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Tobias Langenhan
Torsten Schöneberg *Editors*

Adhesion G Protein-coupled Receptors

Molecular, Physiological and Pharmacological Principles in Health and Disease

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Preface

The very first monograph that was dedicated to a general overview on the adhesion family of G protein-coupled receptors (aGPCRs) was published just in 2010 (Adhesion-GPCRs: Structure to Function. Yona and Stacey, Ed., Landes Bioscience and Springer). It was the earliest attempt by a small group of researchers to cast the scarce information on these enigmatic molecules into a general concept on what they do and how they do it.

The absence of such public face for the biology of aGPCRs was painfully felt by all colleagues who were actively researching aGPCRs in these days. Scepticism was high from many neighbouring fields why aGPCRs rise to such grotesque dimensions with thousands of residues dedicated to their extracellular tails alone. Also how their exotic functions during the development of organs could be accounted for by their peculiar bipartite adhesive/receptive structure was a constant source of doubt (and motivation for further investigation). Not least, whether aGPCRs are 'true' GPCRs and can thus be attacked by the immense technological armoury that has accumulated during the decades of research on other members of the GPCR superfamily was possibly the most pressing question we were confronted with. Next to the simple matter: what do these receptors sense, after all?

While many of these points could not be satisfactorily answered yet back then, the 2010 book project brought them on the map for the first time in a collective effort. Therefore, this venture from a group of adhesion GPCR aficionados was an incontestable sign of a growing community of researchers that had formed to pursue the inherent questions on aGPCRs with seriousness and persistence.

The roster of colleagues that have contributed their expertise, time and dedication to the current monograph bears testimony to that spirit, and we are immensely grateful for their support. We also wish to thank the Editorial Board of the *Handbook of Experimental Pharmacology* for allotting us an entire volume of this eminent book series to document our knowledge on aGPCRs. We are indebted to Susanne Dathe, Wilma McHugh, Rahila Nahid and Sumathy Thanigaivelu from *Springer Nature* for excellent editorial and technical support, and for generous funding from the Deutsche Forschungsgemeinschaft (DFG) to several chapter authors through a Research Unit Grant (FOR 2149), a first award of its kind to a coordinated scientific initiative dedicated to the study of aGPCRs.

The chapters of this volume are authored by renowned experts in the aGPCR field and chart the current state of aGPCR research. Following their contributions, the reader will learn that some of the pressing molecular issues of 2010 have begun to find answers:

- aGPCRs can signal via canonical signaling outlets and a credible mechanism on how they get activated has been recently devised (Liebscher et al., *Tethered agonism: a common activation mechanism of adhesion GPCRs*; Kishore et al., *Versatile signaling activity of adhesion GPCRs*).
- In some cases, the receptors' structural peculiarities have been experimentally matched with highly intriguing biochemical and biological phenomena such as in the case of the GAIN domain and other extracellular protein folds (Araç et al., *Understanding the structural basis of adhesion GPCR functions*).
- One such class of events regards the extensive proteolytic processing of aGPCRs and is discussed by Nieberler et al. (*Control of adhesion GPCR function through proteolytic processing*). Knapp et al. explore the central position of *adhesion GPCRs-related protein networks*, roles that are mainly relayed through their intracellular domains.
- Other vital components of their architecture such as the structure of the heptahelical transmembrane domain of aGPCRs have remained locked to our efforts, but it is clear that in the near future the focus will shift evermore into their direction and offer new vantage points to interfere with their activity. Nijmeijer et al. explored these possibilities in their chapter on *7TM domain structure of adhesion GPCRs*.
- Kovacs et al. review *the relevance of genomic signatures at adhesion GPCR loci* (specifically of human homologs), informing us about their role in phenotypic variation and disease aetiology, an overdue endeavour in the omics era that is aided by the novel harmonised nomenclature and classification system of the aGPCR family introduced by Krishnan et al. (*Classification, nomenclature and structural aspects of adhesion GPCRs*).

The second part of this book is dedicated to physiological and pathological aspects of aGPCRs:

- Scholz et al. describe the emerging concept of *adhesion GPCRs as a putative class of metabotropic mechanosensors*, which distinguishes them from the rest of the GPCR superfamily.
- Several chapters relate to this discovery with specialist focus on its implications in the nervous system (Harty et al., *Adhesion GPCRs as novel actors in neural and glial cell functions: from synaptogenesis to myelination*), in skeletal muscle (White et al., *Control of skeletal muscle cell growth and size through adhesion GPCRs*) and lung physiology (Ludwig et al., *Adhesion GPCR function in pulmonary development and disease*) and in the immune system (Hamann et al., *Adhesion GPCRs as modulators of immune cell function*). Musa et al. describe that *heart development, angiogenesis and blood-brain barrier*

function are modulated by adhesion GPCRs, adding further organ systems that require those receptors for their respective setups and daily operations.
- Finally, Strutt et al. discuss that *adhesion GPCRs govern polarity of epithelia and cell migration*, while the chapter of Aust et al. review the current state of knowledge on *adhesion GPCRs in tumorigenesis.*

We are certain that the research described in this book marks several milestones in the maturation of our understanding on how aGPCRs impact biology. It is to be hoped that the concepts on several aspects of aGPCRs unveiled in the last years are stepping stones to grasp their roles in human disease and therapeutic intervention. We are much looking forward to witness and participate in the exciting developments of this thriving area of biomedical research.

The book will start though with look back at the *History of the adhesion GPCR field* (Hamann and Petrenko), to record the path of our community and remark its scientific course throughout the last 20 years.

Würzburg, Germany — Tobias Langenhan
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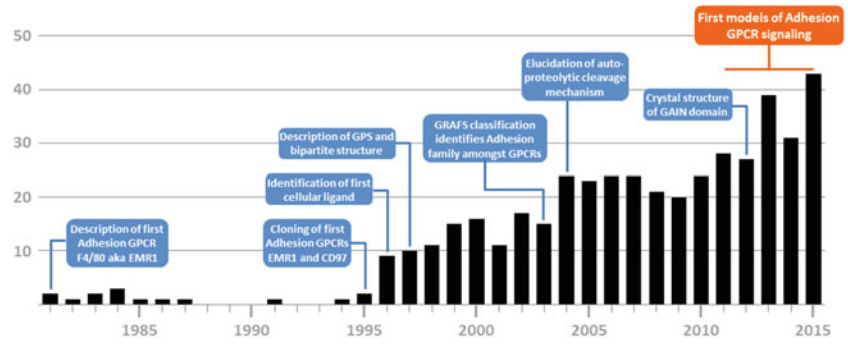
Introduction: History of the Adhesion GPCR Field

Jörg Hamann and Alexander G. Petrenko

Graphical Abstract



Development of the aGPCR scientific field based on PubMed-listed research articles and selected key findings

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Abstract

Since the discovery of adhesion G-protein-coupled receptors (aGPCRs) 20 years ago, reverse genetics approaches have dominated the elucidation of their function and work mechanisms. Seminal findings in this field comprise the description of aGPCRs as seven-transmembrane (7TM) molecules with an extended extracellular region, the identification of matricellular ligands that bind to distinct protein folds at the N-terminus, the clarification of an autoproteolytic cleavage event at a juxtamembranous GPCR proteolysis site (GPS), the elucidation of the crystal structure of the GPCR autoproteolysis-inducing (GAIN) domain that embeds the GPS and connects the receptor fragments, the demonstration that a short N-terminal sequence of the seven-transmembrane (7TM) region can serve as a tethered agonist, and, recently, the notification that aGPCRs can serve as mechanosensors. We here discuss how these discoveries have moved forward aGPCR research and, finally, linked the field to the GPCR field. We argue that crucial questions remain to be addressed before we can fully appreciate the biological nature of these fascinating receptors.

Keywords

Adhesion GPCRs • History • Biology • Structure • Signaling • Pharmacology

1 A Novel Type of Seven-Transmembrane Receptors

After the discovery of hormones as "first messenger" and cyclic adenosine monophosphate (cAMP) as a "second messenger" in the twentieth century, the search for molecules that transduce the "message" through the cell membrane led to the discovery of the first G proteins and then G-protein-coupled receptors (GPCRs). Forward biological approaches, searching for membrane-bound cognate receptors for biocative molecules, subsequently resulted in the identification of many GPCRs and the finding that seven-transmembrane (7TM) receptors possess the largest receptor family in nature. Yet, in contrast to rhodopsin, secretin, glutamate, and Frizzled GPCRs, members of the fifth GPCR family, the adhesion (a) GPCRs, were not discovered via their ligand molecules. Their identification about 20 years ago was the result of genetic approaches that became available through the development of cDNA cloning techniques in the late 1980s (Fig. 1). In 1995, the primary structure of the leukocyte surface molecules CD97 and EMR1 (EGF module-containing, mucin-like hormone receptor 1; in the mouse known as F4/80) was described [1, 2]. The mature proteins were found to comprise a 7TM region, the hallmark of all GPCRs. Most notable was the extended extracellular part, possessing several tandem epidermal growth factor (EGF)-like domains at the N-terminus. With reference to the binary molecule structure, the name EGF-TM7 was coined for these novel receptors [3].

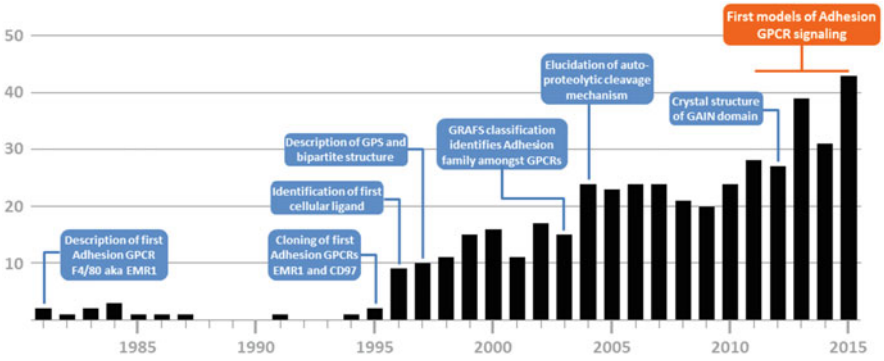

Fig. 1 Development of the aGPCR scientific field. PubMed-listed research articles reporting on aGPCRs indicated per year throughout the last 35 years. Selected key findings are highlighted

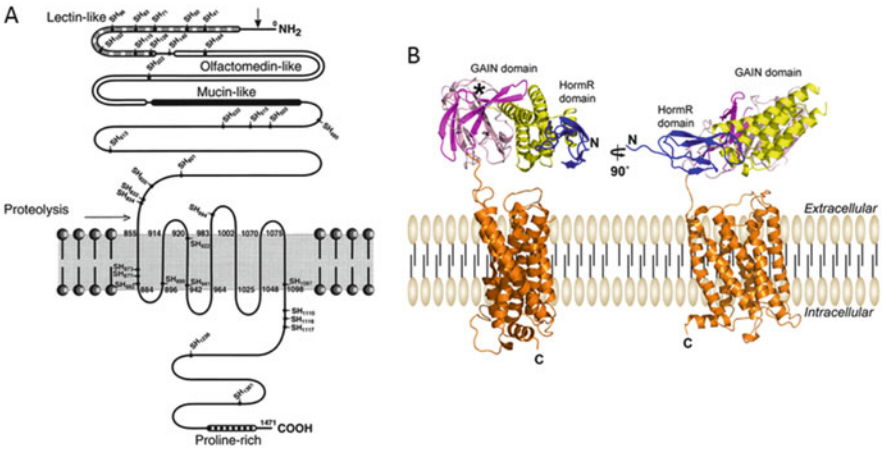

Fig. 2 Progress in the structural understanding of aGPCRs. (**a**) Protein structure of CIRL-1/latrophilin 1 predicted in 1997 from the deciphered amino acid sequence of the mature polypeptide, with indicated the 7TM region, the extended extracellular region with several protein domains, and the juxtamembranous proteolysis site (reproduced from [4]). (**b**) Model of CIRL-1/latrophilin 1 suggested in 2012 based on crystal structures of the GAIN and hormone receptor domain and modeling of the 7TM moiety (reproduced from [6])

Soon after, CIRL-1 (calcium-independent receptor of α-latrotoxin 1)/latrophilin 1, a neuronal receptor for the black widow spider poison α-latrotoxin, was shown to possess a similar structure and strong homology to the 7TM cores of EGF-TM7 receptors [4, 5] (Fig. 2). However, instead of repetitive EGF-like domains, CIRL-1/latrophilin 1 contains singular lectin-like, olfactomedin, and hormone receptor motif domains in its extracellular part. Subsequent description of other homologous 7TM receptors, including latrophilins, EMRs, CELSRs (cadherin EGF LAG seven-pass G-type receptors), BAIs (brain-specific angiogenesis inhibitors), HE6 (human

epididymal 6), and VLGR1 (very large GPCR 1), confirmed the existence of a novel type of GPCR with a large extracellular part, differently composed of structural modules that are typically found in cell adhesion proteins, suggesting their role in coupling cell-to-cell interaction to intracellular signaling.

Right at the beginning, it became clear that these chimeric GPCRs undergo intensive posttranslational modifications and that CD97, CIRL-1/latrophilin 1, and several of their relatives consist of two noncovalently attached fragments that arise from cleavage of the full-length precursor molecules at a juxtamembranous GPCR proteolysis site (GPS) [4, 7, 8]. By pulse-and-chase labeling, it was shown that the cleavage at the GPS site takes place in the endoplasmic reticulum, rendering it fundamentally different from other proteolytic steps, such as furin processing, which occurs in the Golgi apparatus. In 2004, Hsi-Hsien Lin and colleagues showed that the cleavage is an autocatalytic event commonly employed by N-terminal nucleophile hydrolases [9]. The GPS motif appeared to be highly conserved in the aGPCRs family, representing essentially the eighth region of homology within the family. N-terminal to the GPS, larger regions with no sequence homology were found, linking the cell adhesion-like domains. Why the N-terminal protein adhesion-like domains in many aGPCRs are separated from the 7TM part by a large spacer sequence remained unclear for many more years.

Deciphering of the human genome finally disclosed the existence of 33 related receptors that, based on phylogenetic comparison of the 7TM part, assemble a distinct family of GPCRs. The original interest in these proteins was based primarily on their potential of linking cell-to-cell interactions to intracellular signaling. Helgi Schiöth and coworkers thus called them aGPCRs and subdivided them into nine subfamilies [10]. Their unique molecular design clearly sets the aGPCRs apart from other GPCR families, including the secretin GPCRs [11]. It is of note that in spite of their intricate structure, aGPCRs seem to be ancestral to most other GPCR families and have been found even in the most ancient metazoan phyla [12].

As aGPCRs increasingly received attention from a wide spectrum of biomedical fields, the Adhesion GPCR Consortium, together with the International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR), recently proposed a unified nomenclature [13]. The new names carry ADGR as a common dominator, followed by a letter and a number to denote each subfamily and subtype, respectively.

2 Receptor Biology Convenes a New Research Field

In line with the discovery of aGPCRs through genomic approaches, an interest in these molecules developed concurrently in different biomedical areas. In particular, immunologists, neuroscientists, and developmental biologists were among the first who studied these intriguing receptors. Far before any molecular structures were disclosed, Jon Austyn and Siamon Gordon had described in 1981 a monoclonal antibody directed against an antigen on mouse macrophages, called F4/80 [14]. This antigen, currently known as EMR1 (ADGRE1), has become widely

used as a macrophage marker, expressed during development and throughout adult life in a range of inflammatory, infectious, tumor, and other disease models. Other ADGREs (EMRs) are expressed in specific granulocyte populations [15]. More recently, also ADGRBs (BAIs) and ADGRGs have been identified in immune cells, and BAI1 (ADGRB1) attracted interest as a macrophage receptor for danger-associated molecular patterns [16, 17].

A link between aGPCRs and neuronal function was first established by the finding that α-latroxin evokes massive neurotransmitter release and hormone secretion upon binding to CIRL-1/latrophilin 1 (ADGRL1) [5, 18]. More recently, involvement of several ADGRLs (latrophilins) in high-affinity transsynaptic interactions has been reported, suggesting involvement in synaptic functions [19–21]. Another highly intriguing observation was the discovery that defects in the ADGRG subfamily member GPR56 (ADGRG1) cause a cortical malformation, known as bilateral frontoparietal polymicrogyria (BFPP) [22]. GPR56-associated BFPP, also studied in mouse models, has become a prime example for a monogenic disorder arising from aGPCR dysfunction. More recently, additional roles for GPR56 in gyral patterning and in neocortex evolution as well as in oligodendrocyte development have been described [23–25]. Furthermore, elegant studies in zebrafish and mice have linked GPR126 (ADGRG6) and GPR56 on Schwann cells and oligodendrocytes to myelination of peripheral and central nervous axons, respectively [24, 26, 27].

Investigation of invertebrate aGPCRs has helped to understand fundamental developmental processes in health and disease. The Drosophila CELSR (ADGRC) homolog Flamingo/Starry night governs planar cell polarity (PCP) through facilitating the asymmetric distribution of Frizzled and Disheveled [28–30], and chicken CELSR1 (ADGRC1) facilitates core-PCP signaling-mediated closure of the neural tube [31]. In a similar way, the latrophilin homolog LAT-1 organizes cell division planes across the anterior–posterior axis of the *C. elegans* embryo, acting in parallel with noncanonical Wnt/Frizzled signaling [32]. Moreover, CELSR homologs in *C. elegans* and mice regulate axon guidance and neural circuit development [33, 34]. Finally, CELSR1 and VLGR1 (ADGRV1) are required for the development of sensory epithelia; mutations in the latter are associated with the human Usher syndrome, a severe sensory-neuronal disorder that affects vision and hearing [35].

Next to developmental effects in several organ systems, including the reproductive tract, the role of aGPCRs in tumorigenesis evoked interest in the clinical implication of the receptors. Gabriela Aust was the first who showed that expression of CD97 (ADGRE5) correlates with dedifferentiation and invasiveness in various carcinomas [36, 37]. Inversely, GPR56 controls melanoma growth and metastasis [38].

The examples of aGPCR research provided here are far from complete. However, they illustrate a research field that developed in parallel and fairly separated within different biomedical areas, resulting in a steadily growing number of publications (Fig. 1). It was Siamon Gordon who organized in 2002 a 1-day workshop for immunologists and tumor biologists working on EGF-TM7 receptors

Table 1 Biennial adhesion GPCR workshops

Date	Place	Organizers	Talks	Scientific highlights
April 4, 2002	Oxford	Siamon Gordon	16	Identification of cellular ligands
March 19, 2004	Leipzig	Gabriela Aust	19	Autoproteolytic cleavage at the GPS
March 24, 2006	Amsterdam	Jörg Hamann	22	Interaction between receptor fragments
March 29, 2008	Oxford	Martin Stacey	15	Adhesion GPCRs in development
May 1, 2010	Leipzig	Gabriela Aust	19	In vivo models for Adhesion GPCRs
September 6–8, 2012	Würzburg	Tobias Langenhan	23	Crystal structure of the GAIN domain; Autonomous signaling by the CTF; Tethered vs inverse agonist models
June 5–7, 2014	Boston	Xianhua Piao	33	Stachel mechanism of receptor activation; Receptor triggering by mechanosensation
June 2–4, 2016	Leipzig	Torsten Schöneberg; Tobias Langenhan	38	TBD

CTF C-terminal fragment, *GAIN* GPCR autoproteolysis-inducing, *GPCR* G-protein-coupled receptor, *GPS* GPCR proteolysis site, *TBD* to be determined

in Oxford (Table 1). During the following events in Leipzig (2004), Amsterdam (2006), Oxford (2008), and Leipzig (2010), aGPCRs expressed outside the immune system, such as CIRL-1/latrophilin 1, GPR64 (ADGRG2), and VLGR1, slowly entered the stage. The more recent events in Würzburg (2012) and Boston (2014) dealt with all aspects of aGPCR biology and saw a strongly expanding audience [39, 40]. Yet, despite the transformation into 3-day events, the aGPCR Workshops are still informal gatherings, at which novel, unpublished work is presented, and open questions are discussed in an intimate setting. By catalyzing cross talk and collaboration between aGPCR researchers with a different scientific background, the aGPCR Workshops had a tremendous impact on the field. Currently, the community is looking forward to the next event in Leipzig in 2016.

A decisive step in the development of the field was the founding of the aGPCR Consortium (AGC; www.adhesiongpcr.org) in 2012. As an international, open network of academic and nonacademic laboratories interested in aGPCRs, the AGC currently connects more than 60 scientists from 15 countries. The AGC has become a meeting place for everyone interested in aGPCRs and organizes, currently, the biennial workshops. Moreover, the AGC provides information and visibility for the aGPCR community, works on terminology and nomenclature issues, and serves as a starting ground for collaborative research initiatives. The latter has led to the establishment of the Research Unit 2149—*Elucidation of*

Adhesion GPCR signaling—in 2015, which is supported by the Deutsche Forschungsgemeinschaft (www.adhesiongpcr.de).

3 From Molecular Structure to Pharmacology

While work on aGPCRs transcended different biomedical areas, the central question on the mechanism by which these receptors signal remained hard to answer for a long time. The existence of numerous protein domains implied that aGPCRs might engage in cell–cell interactions. A similar function of receptor tyrosine kinases has been very well described, with its importance in cancer biology and development. In 1996, Jörg Hamann demonstrated that CD97 binds decay-accelerating factor/CD55, a molecule associated with regulation of the complement cascade [41]. Since then, interacting partners, often matricellular molecules, have been identified for about ten aGPCRs. However, no comprehensive picture arose that would fit the concept of agonistic ligands as these have identified for other GPCR families [42].

Recently, the juxtamembrane part of the aGPCRs containing the GPS motif has been implicated in the receptor signaling. Demet Araç showed that the GPS is an integral part of a much larger domain that was termed GPCR autoproteolysis-inducing (GAIN) domain [6]. Crystal structures of GAIN domains from CIRL-1/latrophilin 1 and BAI3 (ADGRB3) revealed a conserved, novel fold that fine-tunes the chemical environment at the GPS to catalyze peptide bond hydrolysis (Fig. 2). Another key finding by the groups of Randy Hall and Lei Xu was the observation that the C-terminal fragment (CTF) of some aGPCRs shows intense metabotropic and biological activity, implying that the N-terminal fragment (NTF) controls receptor signaling [43, 44], and that the ectodomain of aGPCRs may act as a tethered ligand for their 7TM domain [45].

Building forth on these studies, the laboratories of Ines Liebscher, Torsten Schöneberg, and Gregory Tall have proposed a tethered agonist mechanism according to which displacement of the NTF exposes a short N-terminal sequence of the 7TM domain, designated *Stachel* (German for stinger), that is hidden within the GAIN domain [46, 47]. Synthetic peptides, comprising these *Stachel* sequences, have been shown to potently trigger various aGPCRs, in vitro and also in vivo. Finally, work from the groups of Bruce Spiegelman, Kelly Monk, and Tobias Langenhan uncovered that mechanical cues trigger the activity of aGPCRs under physiological conditions, adding mechanosensation to the sensory canon of the GPCR superfamily [27, 48, 49].

The ability to activate aGPCRs enabled studies aiming at identifying downstream signaling modes. The demonstration that the receptors can couple to all subclasses of G proteins [46, 50] led to the recognition as bona fide GPCRs [55]. Consequently, established GPCR conferences currently discuss developments in aGPCR research. Yet, uncertainties remain (Table 2). Information concerning the ability of aGPCR binding partners to trigger G proteins is very scarce so far [27, 51], and we do not know whether the functioning of the receptors is confined to

Table 2 Certainties and uncertainties about GPCRs

What do we know	What we are not sure of
• GPCRs are bipartite molecules with a large extracellular region that is connected through a GAIN domain to a 7TM moiety	• A functional link between adhesive capacity and receptor signaling remains to be established
• The majority of aGPCRs undergo autocatalytic processing at a GPS embedded within the GAIN domain	• Lack of cleavage of some aGPCRs suggests that a bipartite structure is not a prerequisite for receptor function
• aGPCRs can be activated through a tethered agonist (*Stachel* sequence)	• Mechanisms allowing exposure of the *Stachel* need to be determined, in particular for solid tissues and non-cleavable receptors
• aGPCRs are widely distributed and cause distinct biological phenotypes	• It is not clear whether aGPCRs display cell type-specific or general cellular functions

7TM seven-transmembrane, aGPCR adhesion GPCR, GAIN GPCR autoproteolysis-inducing, *GPCR* G-protein-coupled receptor, *GPS* GPCR proteolysis site

G-protein signaling. Early studies on PCP in Drosophila showed that the CELSR homolog Flamingo arranges in *trans* and in *cis* with other transmembrane molecules to execute its functions [28–30], possibly presenting another major working mechanism. In addition, CD97 has been shown to heterodimerize with the lysophosphatidic acid (LPA) receptor to amplify LPA-initiated Rho-dependent signaling and invasion in prostate cancer cells [52].

Another interesting possibility exists that the large NTF of aGPCRs may serve autonomously by interacting as a ligand with other receptors. In the original discovery of BAI1, its ectodomain soluble fragment had a role in the inhibition of brain-specific angiogenesis [53]. Also, the presence of a specifically cleaved soluble fragment of CIRL-1/latrophilin 1 was detected in the brain, comprising about 5 % of the total amount of the receptor expressed [54]. Finally, a genetic study uncovered a specific role of the N-terminal fragment of GPR126 in axon sorting [27].

Based on the anecdotal identification of most of its members, the aGPCR cohort is one of the prime examples of genome-sequencing effort-driven identification and definition of an entire molecule class. As a consequence, research on aGPCRs grows out of a "molecule-centered" rather than "biology-centered" history since almost two decades. This situation has recently changed with the advent of molecular models on the signaling paradigm of aGPCRs and their physiological mode of activation. The field has now entered a highly intriguing stage, and we predict that the pharmacological insight and tools that are currently developed will boost novel attempts to understand the biological functions of aGPCRs in health and disease.

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Part I

Molecular and Pharmacological Properties of Adhesion GPCRs

Classification, Nomenclature, and Structural Aspects of Adhesion GPCRs

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Contents

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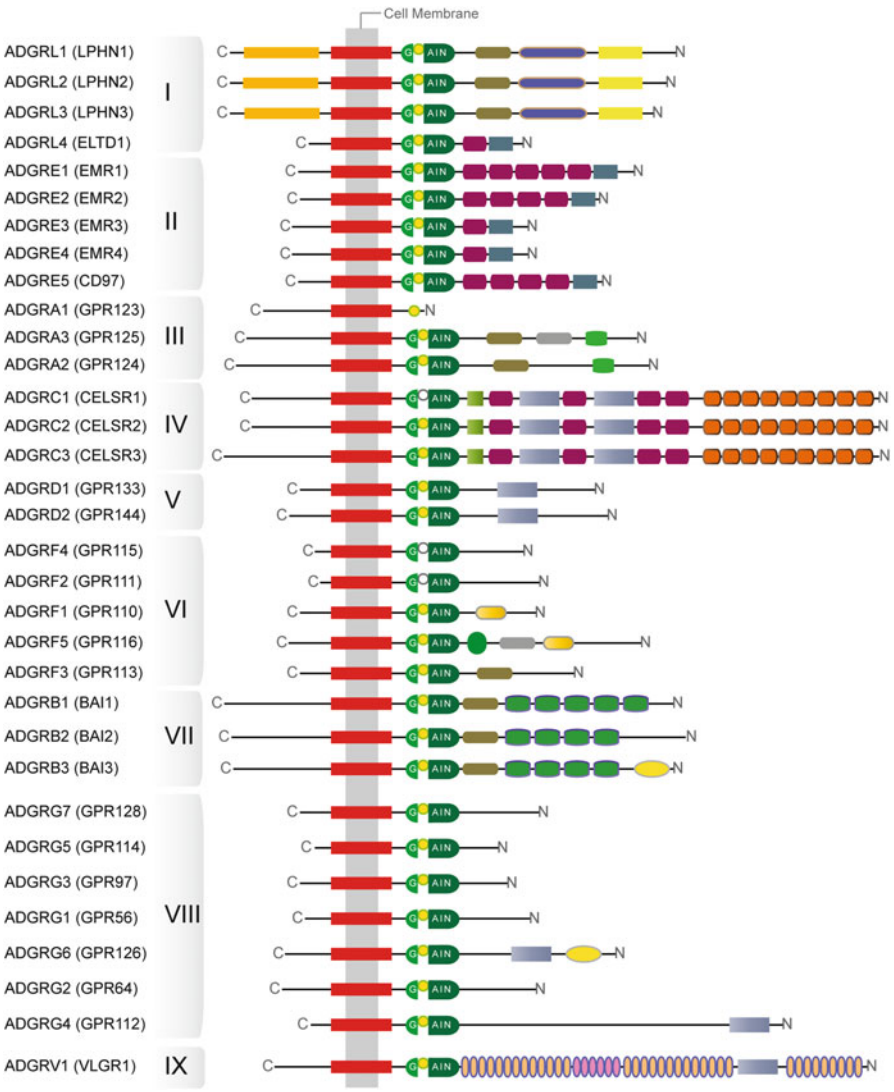
Graphical Abstract



Representation of the nine distinct aGPCR subfamilies and their unique N-terminal domain architecture. The illustration also shows the extracellular structural feature shared by all aGPCRs (except ADGRA1), known as the GPCR autoproteolysis-inducing (GAIN) domain, that mediates autoproteolysis and subsequent attachment of the cleaved NTF and CTF fragments

Abstract

The adhesion family of G protein-coupled receptors (aGPCRs) is unique among all GPCR families with long N-termini and multiple domains that are implicated in cell–cell and cell–matrix interactions. Initially, aGPCRs in the human genome were phylogenetically classified into nine distinct subfamilies based on their 7TM sequence similarity. This phylogenetic grouping of genes into subfamilies was found to be in congruence in closely related mammals and other vertebrates as well. Over the years, aGPCR repertoires have been mapped in many species including model organisms, and, currently, there is a growing interest in exploring the pharmacological aspects of aGPCRs. Nonetheless, the aGPCR nomenclature has been highly diverse because experts in the field have used different names for different family members based on their characteristics (e.g., epidermal growth factor-seven-span transmembrane (EGF-TM7)), but without harmonization with regard to nomenclature efforts. In order to facilitate naming of orthologs and other genetic variants in different species in the future, the Adhesion-GPCR Consortium, together with the International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification, proposed a unified nomenclature for aGPCRs. Here, we review the classification and the most recent/current nomenclature of aGPCRs and as well discuss the structural topology of the extracellular domain (ECD)/N-terminal fragment (NTF) that is comparable with this 7TM subfamily classification. Of note, we systematically describe the structural domains in the ECD of aGPCR subfamilies and highlight their role in aGPCR-protein interactions.

Keywords

Adhesion GPCRs • Nomenclature • Classification • Pharmacology • Drug targets • Homologs • Mammals • Vertebrates • Model organisms • GAIN domain

1 Introduction

The G protein-coupled receptor (GPCR) superfamily is the largest family of cell surface receptors and is grouped into five major families: glutamate, rhodopsin, adhesion, frizzled, and secretin [1, 2]. Among all classes, the adhesion GPCRs (aGPCRs) comprise the second largest family with 33 members in the human genome [1, 2]. Prior to the release of the human genome, aGPCRs were not considered as a separate family of GPCRs. Indeed, at that time, only a few genes were identified that constituted a long extracellular region and a seven transmembrane segment (7TM) characteristic to GPCRs. One of the first aGPCRs cloned was the epidermal growth factor (EGF)-like molecule containing mucin-like hormone receptor 1 and later similar molecules were identified [3–6]. This paved the way for the recognition of these molecules as EGF-TM7-like receptors because of the presence of EGF-like domains in their extracellular region [6]. Similarly, other names that were initially termed for aGPCRs include LN-7TM [7] (for the presence of long N-terminal regions), LNB-7TM [8] (for their similarity to family B secretin-

like GPCRs), or family B2 receptors [9]. Shortly after the release of the human genome, several novel genes were identified, and subsequent gene mining showed that at least 30 GPCR-like sequences exist with a long extracellular region and GPCR proteolysis site (GPS) motif that induce autocatalytic processing [10]. Some of these receptors were often initially denoted or thought as secretin-like GPCRs and were placed in proximity to family B receptors [9]. Nonetheless, the large-scale effort to comprehensively classify the GPCRs in the human genome showed convincing phylogenetic evidence that aGPCRs constitute a separate family of GPCRs [1]. This made clearer that adhesion and secretin families are indeed distinct from each other, although these molecules share vague similarities and are often placed together as family B GPCRs [9]. This view is strengthened as the largest of differences were observed in their extracellular region and in particular aGPCRs are also distinct from secretin GPCRs in molecular function. For example, most aGPCRs contain the GPS motif, which is found in close proximity to the 7TM region (for review see [11]). Moreover, it is currently understood that the GPS motif is a part of a much larger GPCR autoproteolysis-inducing (GAIN) domain, which induces the autocatalytic processing of aGPCRs into an N-terminal fragment (NTF) and a C-terminal fragment (CTF) [12]. In addition, the NTF of aGPCRs contains numerous protein domains implicated in cell and matrix interactions [11, 13], and, thus, the established name of "adhesion" family GPCRs was initially coined to refer this feature. Conversely, the secretin GPCRs do not undergo such autocatalytic processing in their N-termini, however, contains a hormone-binding domain (HBD) to mediate hormonal responses [13, 14]. In this chapter, we review the classification of aGPCRs into nine families in the human genome and briefly discuss the classification and potential homologs of these families in other vertebrate and invertebrate genomes. Of note, we address the recently recommended nomenclature of aGPCRs [15] that aim to provide a coherent and systematic naming system independent of the species and subfamily names. Also, we discuss the similarities between aGPCR subclasses with respect to the organization of their structural topology (e.g., olfactomedin, cadherin, EGF-like and thrombospondin type 1 domain). The analysis nevertheless indicates remarkable differences that demonstrate the structural diversity of aGPCRs, but could also hint at potential interaction partners for orphan aGPCRs or provide information on NTF–CTF interactions. We therefore systematically describe the unique (sub) family-specific structural features and their protein interactions.

2 Classification of aGPCRs

2.1 Human aGPCR Subfamilies

Based on phylogenetic criteria, the human aGPCR repertoire is categorized into nine distinct subfamilies (considering ADGRV1 (VLGR1) as subfamily IX) according to the molecular signature of their 7TM region [1]. The number of genes belonging to the subfamilies I to VIII vary from two genes in "subfamily

V" to seven genes in "subfamily VIII" [1, 16]. However, the subfamily IX contains only a single gene ADGRV1, also known as the very large GPCR1, referring to its unusually large extracellular region of about 6000 amino acids comprising multiple Calx-β repeats [1, 16]. Intriguingly, except for subfamily VI and VIII, each subfamily has a characteristic unique pattern of extracellular domain architecture (see Sect. 4.2) and this molecular signature is used as a marker to distinguish between the subfamilies and as well to categorize novel homologs in other species. Nonetheless, before the classification of human aGPCRs was established in 2003 [1, 10], several important factors were considered to determine the aGPCR classification. One of the major factors strongly taken into account was the strength of the phylogenetic nodes forming distinct groups, as this is central for the classification of subfamilies [1, 10]. This is vital because the tree topology supporting the classification largely depends on the choice of the alignment methods and phylogenetic reconstruction software [17]. Nonetheless, the grouping of aGPCRs into nine distinct families was found reliable when aGPCR repertoires from other mammalian genomes were curated and compared with the human aGPCR repertoire using different phylogenetic algorithms that included maximum parsimony, maximum likelihood, and neighbor-joining analyses [16, 18, 19]. Also, a consensus view of tree topologies obtained from human–mouse, human–rat, and human–dog phylogenetic comparisons showed identical grouping pattern, although the hierarchy within the subfamilies displayed considerable degrees of variation for subfamilies that comprised divergent members (e.g., within subfamily VIII). Overall, these comparative analyses further strengthened the classification, and the human aGPCR classification is now currently utilized in several studies as a reference to further classify aGPCRs in vertebrate and invertebrate genomes.

2.2 aGPCRs in Mammals and Other Vertebrates

Mining of aGPCRs in mammalian and other vertebrate genomes resolved one-to-one orthologous relationships with the human aGPCR members and as well provided insights into the evolution and diversity of aGPCRs. These gene mining studies showed that homologs of most human subfamilies are found in several vertebrate genomes. Nonetheless, the actual gene count of aGPCRs varied from species to species. For example, the mouse genome encodes one-to-one orthologs for 31 of the 33 aGPCRs found in human; however, orthologs of ADGRE2 (EMR2) and ADGRE3 (EMR3) are absent [16, 20, 21]. The same subset of aGPCRs was also observed in the rat genome with no orthologs found for human ADGRE2 and ADGRE3 [18]. Conversely, mining of aGPCRs in other mammalian genomes such as the dog genome showed that all 33 human aGPCRs are found and as well contain additional full-length genes that are homologs of ADGRE2 and ADGRE3 [19]. Additional gene mining studies discovered that the chicken genome contains 21 of 33 aGPCRs, but clearly lack orthologs of other 12 human aGPCRs [22]. Similarly, the fugu genome was surveyed and contains at least 29 aGPCR-like sequences [23]. These robust gene mining studies also suggested that aGPCRs are by no

means a coherent group of equally divergent clusters and they show high degrees of variability in both, between the members of the subfamilies and one-to-one orthologous gene pairs. For example, the overall amino acid similarity within the TM regions of the human aGPCRs belonging to subfamily VII is relatively high similarly (60–70 %), whereas members of subfamily VIII share less percentage sequence identity (20–30 %). Moreover, the percentage of amino acid identity between the orthologous pairs in human–mouse, human–rat, and rat–mouse genomes also shows high degrees of variability from one subfamily to the other. For instance, the human–mouse orthologous pairs of members belonging to subfamily VII (brain-specific angiogenesis inhibitor (BAIs) or ADGRBs) share 98 % identity within their 7TM, while the orthologous pairs of subfamily VI and VIII members share a relatively low percentage identity (50–60 %). Taken together, these differences occasionally make it complicated to classify aGPCRs in distant species and in organisms that constitute local expansions. This was also observed in a recent attempt to classify aGPCRs in the zebra fish genome that constituted local expansions in subfamilies II, VI, and VIII. A few sequences had unstable positioning between the topologies observed using maximum likelihood (ML) and the Bayesian approach [24]. In such cases, aGPCR sequences were classified using a more acute approach of performing separate phylogenetic analyses on each cluster and considering the multiple alignments and extracellular domain architecture [24]. In addition, the exon–intron boundaries with intron phase/positions and the overall conservation of neighboring genes in the genomic scaffolds can serve as additional genetic markers to classify aGPCR orthologs in invertebrates.

2.3 aGPCRs in Invertebrates

In the last decade, robust efforts to sequence the genomes of several invertebrates and some non-bilaterian organisms provided large datasets to further mine and classify aGPCRs across most metazoans (animals). Several earlier studies that sought to determine aGPCRs in these distant species found homologs of a few human aGPCR subfamilies and catalogued many species-specific expansions as well. Genomic survey in closest relatives of vertebrates such as *Ciona* intestinalis and Branchiostoma floridae (amphioxus) exposed the diversity of aGPCRs before the emergence of vertebrates [25, 26]. Amphioxus contained a total of 37 aGPCRs, which is an estimate much similar to that found in the vertebrate genomes. However, amphioxus lacks homologs of human aGPCR subfamilies I, II, IV, VI, and VII and only contains a few aGPCRs that shared some similarities with subfamilies III, V, and VIII. The remaining aGPCRs in amphioxus are most likely species-specific and contained a diverse array of extracellular domains including a few domains that are not commonly found in vertebrate aGPCRs [26]. Similarly, *Ciona* contains 30 aGPCRs, of which only six genes constituted orthologous relationships with the human aGPCR subfamilies (subfamilies I, III, IV, and VIII) [25]. The remaining 24 genes are diverse from the human aGPCR dataset and formed five paralogous groups that are most likely specific to *Ciona* [25]. Mapping

of aGPCRs in *Saccoglossus kowalevskii*, a hemichordate (a deuterostome animal, sister group to echinoderms, and closely related to chordates) found 18 aGPCR-like sequences, which comprise homologs of subfamilies I, III, IV, VII, and VIII, but lack representatives of other aGPCR subfamilies [27]. In addition, previous studies have also estimated the number of aGPCRs in other metazoans including, echinoderms (sea urchin) [28, 29], nematodes (*Caenorhabditis elegans*) [30], arthropods (*Drosophila melanogaster*) [31], cnidarians (*Nematostella vectensis*) [32], placozoans (*Trichoplax adhaerens*) [33], and sponges (*Amphimedon queenslandica*) [34, 35]. These ancient and primitive animals constituted several species-specific aGPCRs, but also contain a few homologs of some human aGPCR subfamilies. For instance, *N. vectensis* contains homologs of subfamilies III, IV, and IX, while *T. adhaerens* has a homolog of subfamily IV [32, 35]. Nonetheless, it must be mentioned here that these phylogenetically basal species contained highly diverse aGPCR datasets and earlier attempts to phylogenetically resolve their relationships with human and other vertebrate aGPCRs) have resulted in either unresolved topologies or lack of sufficient support to categorize them into the known subfamilies. This scenario is also observed in our recent attempt where we classified sponge aGPCRs and resolved their relationships with aGPCR datasets from other non-bilaterian species [34]. Taken together, these studies elucidate the difficulties in classifying aGPCRs in distant species. Moreover, the complexity is also aggravated in naming of these homologs and other genetic variants in different species, due to the lack of harmonized nomenclature for aGPCRs. Nonetheless, the current nomenclature (see below) will provide a better platform to classify these ancient GPCRs and to further examine the aGPCR diversity.

3 Recommended Nomenclature of aGPCRs

As described in previous sections, aGPCRs are highly diverse and are found in a wide range of species, where they perform important and diverse biological functions. However, aGPCRs lack a harmonized nomenclature and over the years diverse names have been used for the genes belongings to different subfamilies. Names such as cadherin EGF LAG seven-pass G-type receptor (CELSR), EGF-TM7 (epidermal growth factor-seven-span transmembrane), brain-specific angiogenesis inhibitor (BAI), and very large GPCR (VLGR) were initially created by pioneers of this research field much before the time point where aGPCRs were not considered as a separate family of GPCRs. Later after the release of the human genome, many new aGPCRs genes were identified and GPR# names were assigned in collaboration with the Human Genome Organization (HUGO) Gene Nomenclature Committee (HGNC). Nonetheless, these GPR# names were also considered as temporary identifiers until additional information about the protein function was elucidated. Currently, the aGPCRs are considered to assemble a separate family of GPCRs. Over the years the research field has expanded widely and more genome-wide studies including genomics/genetics, expression, and epigenetics are consistently been carried out. This permanent growth of the aGPCR research field raised

the requirement for a harmonized naming system that would clearly illustrate the relationship between these proteins/genes for diverse research groups. The highly diverse aGPCR naming system was discussed in IUPHAR meetings and this has been taken into consideration by the IUPHAR Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR). The Adhesion-GPCR Consortium with assistance from HGNC worked on a coherent naming system, sought to provide aGPCRs a prefix that identifies any adhesion GPCR homolog, independent of species or subfamily [15]. Providing a unique and most appropriate prefix for all aGPCR families was considered highly important because any such naming system will help to name orthologs and other genetic variants in different species in the future [15].

The most appropriate and unique prefix found was ADGR, which stands for *ad*hesion *G* protein-coupled *r*eceptor. Moreover for easy recognition, each subfamily was then assigned a letter to relate to their previous names. For example, L for the latrophilins, E for the EGF-TM7 receptors, C for the CELSRs, B for the BAIs, and V for VLGR, while the subfamilies with GPR# names have been given a letter in alphabetic order (A, D, F, G). As stated above the members within each subfamily (I to IX) were assigned a sequential number based on the phylogenetic clustering pattern according to the molecular signature of the 7TM regions. For example, the members of the subfamily I, the latrophilins, LPHN1, LPHN2, LPHN3, and ELTD1, were named as ADGR*L* for latrophilins, and the members were assigned numbers as such where LPHN1 is now ADGRL1, LPHN2 is ADGRL2, LPHN3 is ADGRL3, and ELTD1 is ADGRL4 [15]. Likewise members of the other subfamilies contain the prefix ADGR, the associated letter to relate to previous names, followed by numbers for the paralogs. This nomenclature (Table 1) has been accepted by both HGNC and NC-IUPHAR, and both organizations encourage the use of this nomenclature in all literature and databases [15]. Upon recommendation, the new nomenclature is currently being used together with the old names, for example, "ADGRE5 (CD97)" or "ADGRL1 (LPHN1)," until the new names are fully established.

4 Structural Aspects of aGPCRs

The 33 human aGPCRs can be divided into nine subfamilies based on their 7TM sequence similarity. Interestingly, the 7TM-based classification reflects similarities within aGPCR subfamilies with respect to the organization of their extracellular domains. Class-specific features, such as the GAIN domain and often-occurring sequences like the HBD and EGF-like domains are present in several aGPCR subfamilies. In contrast, cadherin and thrombospondin type 1 repeats are subfamily specific. A subfamily ordered description of all domain structures and their identified interaction partners gives insight in the structural diversity of aGPCR and also highlights the dual function of both the NTF and the CTF.

Table 1 Currently used nomenclature of adhesion GPCRs

Subfamilies	Previous/old gene names	Proposed new names for subfamilies	Currently accepted nomenclature/ used new gene names
I	LPHN1	L (Latrophilin)	ADGRL1
	LPHN2		ADGRL2
	LPHN3		ADGRL3
	ELTD1		ADGRL4
II	EMR1	E (EGF-TM7)	ADGRE1
	EMR2		ADGRE2
	EMR3		ADGRE3
	EMR4		ADGRE4
	CD97		ADGRE5
III	GPR123	A	ADGRA1
	GPR124		ADGRA2
	GPR125		ADGRA3
IV	CELSR1	C (CELSR)	ADGRC1
	CELSR2		ADGRC2
	CELSR3		ADGRC3
V	GPR133	D	ADGRD1
	GPR144		ADGRD2
VI	GPR110	F	ADGRF1
	GPR111		ADGRF2
	GPR113		ADGRF3
	GPR115		ADGRF4
	GPR116		ADGRF5
VII	BAI1	B (BAI)	ADGRB1
	BAI2		ADGRB2
	BAI3		ADGRB3
VIII	GPR56	G	ADGRG1
	GPR64		ADGRG2
	GPR97		ADGRG3
	GPR112		ADGRG4
	GPR114		ADGRG5
	GPR126		ADGRG6
	GPR128		ADGRG7
IX	GPR98	V	ADGRV1

BAI brain-specific angiogenesis inhibitor, *CD* cluster of differentiation, *CELSR* cadherin EGF LAG seven-pass G-type receptor, *EGF-TM7* epidermal growth factor-seven-span transmembrane, *ELTD* EGF, latrophilin, and seven-transmembrane domain-containing protein, *EMR* EGF-like molecule-containing mucin-like hormone receptor

4.1 General Structural Features of aGPCRs

4.1.1 7TM

All aGPCRs possess the classical 7TM architecture. Based on sequence conservation and gene splicing sites localization [32], aGPCRs are more closely related to family B secretin receptors than to family A rhodopsin GPCRs (see Chapter IV for a complete aGPCR/secretin family B structural comparison). Seven TM sequence homology between aGPCR subfamilies classifies them in nine distinct subfamilies [16, 36]. Intriguingly, this classification can be extended to the extracellular domain (ECD), suggesting an evolutionary conserved relationship between the extracellular domains and 7TM functioning. Indeed, for some aGPCRs it has been described that protein binding to the extracellular domain results in 7TM-dependent signaling [11, 15]. Recently, an activation mechanism has been proposed in which the 7TM N-terminus (Stachel; see below) is exposed upon removal of the non-covalently attached N-terminal fragment (NTF). This tethered sequence can subsequently interact with the 7TM structure and induce receptor activation [37–40]. This activation mechanism seems applicable for the whole aGPCR family, since the majority of aGPCRs possess this conserved sequence motif (see [41]).

4.1.2 ECD

Each aGPCR has a typically large N-terminal extracellular domain (ECD) that encompasses both aGPCR subfamily-dependent structural features, e.g., adhesion domains (see Sect. 4.2 and [42]), and aGPCR-specific features like the GAIN domain. Here, we will briefly address all features and, moreover, give a tabular overview of the different aGPCR subfamilies with their characteristic domains (Table 2).

4.1.3 GAIN

The GAIN domain, which is conserved in 32 out of 33 human aGPCR (absent in ADGRA1/GPR123), can be found directly N-terminally of the first transmembrane helix (TM1). In 2012 the crystal structures of the GAIN domain in ADGRL1 (latrophilin-1) and ADGRB3 (BAI3) were published [12, 44] (see also [42]). The GAIN domain consists of domain A that contains 6 alpha-helices and domain B that has a twisted beta-sandwich with 13 beta-strands and 2 alpha-helices [12, 44]. Within in the GAIN domain lays a conserved GPCR proteolytic site (GPS) where aGPCRs are autoproteolytically cleaved into an N-terminal fragment (NTF) and C-terminal fragment (CTF) during protein maturation (Table 2). GPS cleavage takes place between the last two (12–13) beta-strands. Importantly, it was shown that the GPS of ADGRB3 (BAI3) is situated in a sharply kinked loop and is therefore non-cleavable [12]. Indeed, not all aGPCRs are actually cleaved at their GPS: ADGRF2 (GPR111), ADGRG5 (GPR114) [40], ADGRF4 (GPR115) [45], and ADGRC1 (CELSR1) [46] have been shown to be cleavage deficient, whereas ADGRE1 (EMR1), ADGRA1, ADGRA2 (GPR124), ADGRA3 (GPR125), ADGRC3 (CELSR3), and ADGRB3 (BAI3) [12] are expected to be non-cleavable due to a non-consensus sequence (i.e., the general base H is absent

Table 2 Overview of aGPCR structural NTF features

Structural Feature -> / aGPCR	GPS (aa position)	GAIN	Hormone binding domain	Olfactomedin	Lectin	STP rich region	EGF Ca2+	EGF	RGD motif	Immunoglobulin	Leucine rich repeats	Laminin-EGF	Laminin G	Cadherin	Pentraxin	SEA	Thrompospondin type 1 repeat	CUB	Calx-beta	EPTP	Additional cleavage sites	Binding partners identified	Stachel-induced activation
ADGRL1 (LPHN1)	838-839	1	1	1	1	1																	
ADGRL2 (LPHN2)	824-825	1	1	1	1	1																	
ADGRL3 (LPHN3)	841-842	1	1	1	1	1																	
ADGRL4 (ELTD1)	406-407	1					1	1															
ADGRE1 (EMR1)	584-585*	1					5	1															
ADGRE2 (EMR2)	517-518	1					4	1															
ADGRE3 (EMR3)	338-339	1					1	1															
ADGRE4 (EMR4)	173-174	1					1	1															
ADGRE5 (CD97)	530-531	1					4	1	1														
ADGRA1 (GPR123)																							
ADGRA2 (GPR124)	746-747*	1	1						1		1												
ADGRA3 (GPR125)	737-738	1	1							1	1												
ADGRC1 (CELSR1)	2448-2449*	1	1				4					1	2	9									
ADGRC2 (CELSR2)	2356-2357*	1	1				4					1	2	9									
ADGRC3 (CELSR3)	2517-2518*	1	1				4					2	2	9									
ADGRD1 (GPR133)	544-545	1																					
ADGRD2 (GPR144)	636-637	1													1								
ADGRF1 (GPR110)	566-567	1														1							
ADGRF2 (GPR111)	426-427*	1																					
ADGRF3 (GPR113)	752-753	1	1					1															
ADGRF4 (GPR115)	381-382*	1																					
ADGRF5 (GPR116)	990-991	1								1						1							
ADGRB1 (BAI1)	926-927	1	1						1								5						
ADGRB2 (BAI2)	911-912	1	1														4						
ADGRB3 (BAI3)	856-857	1	1														4	1					
ADGRG1 (GPR56)	282-283	1																					
ADGRG2 (GPR64)	606-607	1																					
ADGRG3 (GPR97)	249-250	1																					
ADGRG4 (GPR112)	3078-3080	1							1						1								
ADGRG5 (GPR114)	226-227	1																					
ADGRG6 (GPR126)	840-841	1	1										**		1			1					
ADGRG7 (GPR128)	415-416	1																					
ADGRV1 (VLGR1)	5890-5891	1							1						1				35	6			

Structural features present in extracellular domains of aGPCR family members. Numbers indicate the amount of repeats. In *blue*, receptors that have additional cleavage sites identified in NTF, identified binding partners, and/or Stachel-induced receptor activation are reported. *Red box*: Non-cleavage (experimental evidence). *Orange box*: theoretically non-cleavable. *According to GPS sequence alignment Lin et al. [43], **Novel laminin domain in ADGRG6

or replaced by an interfering residue) at their GPS [15]. Except for the ADGRAs, this cleavage deficiency seems random and subfamily independent (Table 2 and [47]).

4.1.4 HBD

Directly N-terminal of the GAIN domain is a so-called hormone-binding domain (HBD; ~70 aa) in 12 of the 33 aGPCRs [12] (Table 2 and [42]). This domain consists of two long and two short antiparallel beta-strands that are connected with random loop structures. Interestingly, such an HBD is also found in family B secretin receptors, the most closely related GPCRs [32]. This domain has been considered as potential ligand-binding domain [12, 48]. However, the crystal structure shows a conformation of the HBD that does not allow hormone binding due to steric hindrance of the GAIN domain [12]. It should be noted however that this conformation is just a snapshot and may not represent a dynamic real-life

situation. In contrast to family B secretin receptors, the aGPCR HBDs lack an N-terminal alpha-helix, which has proven important, but not essential for peptide and hormone interactions in family B secretin receptors (e.g., GLP-1 receptor [49]).

4.1.5 EGF-Like Domain

The EGF-like domain (with or without calcium binding) is another domain that is present in several aGPCRs (i.e., ADGRL4, ADGREs, and ADGRCs). EGF-like domains consist of a large N- and short C-terminal-located two-stranded beta-sheet, which are connected by a loop structure [50]. Multiple EGF-like domains fold together to form a functional solenoid architecture. The structural organization of these domains is facilitated by domain–domain contacts that can be stabilized by calcium ion binding (see also [42]).

4.2 aGPCR Subfamily-Specific Structural Features and Protein Interactions of NTF

4.2.1 ADGRL

The NTF of the ADGRL (I) subfamily has a common structure for ADGRL1–3, namely, a cysteine-rich region homologous to a galactose-binding lectin (GBL or lectin) domain (108 aa) [51, 52]; olfactomedin domain (260 aa) (a glycoprotein of the extracellular matrix of the olfactory neuroepithelium) [53]; serine, threonine, and proline (STP)-rich region (79 aa); and an HBD [32, 54].

The structure of the lectin domain was resolved by NMR, showing a beta-sandwich including two antiparallel sheets, surrounded by a 10-residue alpha-helix and two extended loops [55]. L-Rhamnose can bind with low affinity to one of the loops [55, 56]; however, no data on ADGRL function has been tested. The lectin domain is conserved in most ADGRL1–3 orthologs, except coelenterates which could hint at an important role in receptor functioning [57]. Crystallographic data for the lectin and olf domains of the mouse ADGRL3 showed that olf-b has a propeller fold and a conserved calcium-binding site [58]. Note that the olfactomedin domain is only present in vertebrate orthologs aGPCRs, which could indicate that this domain is acquired during early vertebrate evolution [59]. The STP domain is present in vertebrate and insect orthologs, however, is absent in *C. elegans* [59]. In contrast, the fourth member of the ADGRL class, ADRGL4 (ELTD1), has a different structural composition of its NTF (i.e., EGF-like domain and Ca^{2+}-binding EGF-like domain) [60, 61] (see Sect. 4.1). The ADGRL receptors were discovered due to binding of an exogenous binding partner to ADGRL1, α-latrotoxin—a black widow spider venom [62]. This venom binds to the HBD and GAIN domains in the NTF of ADGRL1 [63]. In later years three endogenous binding partners have been found to interact with ADGRL1–3. All three belong to single-span transmembrane protein families: teneurins, fibronectin leucine-rich transmembranes (FLRTs), and neurexins.

The large postsynaptic glycoprotein teneurin-2 [also named Lasso (latrophilin-associated synaptic surface organizer)] binds to the lectin domain of presynaptic

ADGRL1 "in trans" ($K_d < 2$ nM) [59, 64]. Interestingly, while ADGRL1-3 have similar domains in their NTF, teneurin-2 only binds ADGRL1 and does only bind weakly (ADGRL2) or does not bind at all (ADGRL3) to the other subfamily members [64]. In addition, ADGRL3 binds to teneurin-3 [65] and teneurin-4 binds to ADGRL1 [66]. This indicates that, although the structural similarity is present, there is room for receptor-specific targeting.

Very recently, it was shown that a short amino acid sequence on the distal extracellular tip of each teneurin named teneurin C-terminal-associated peptide (TCAP) exhibits an effect on ADGLRs, independent of the teneurin protein. Interestingly, TCAP has remarkable structural homology with peptide ligands that bind to the secretin GPCR subfamily (i.e., CRF peptide family). TCAP binds to the lectin domain of ADGRL1–3 [67].

Cell adhesion proteins fibronectin leucine-rich transmembranes (FLRTs) were initially thought to bind "in trans" to the lectin domain of ADGRL1–3 [65]. However, recent elucidation of the propeller-like olf domain showed a major role for this domain in FLRT interaction [58, 68, 69]. ADGRL1 and ADGRL3 bind both FLRT1 and FLRT3, but ADGRL2 only interacts with FLRT3. Their extracellular regions interact with ADGRLs and with uncoordinated-5 (UNC5/netrin) receptors [68].

ADGRL1 also interacts "in trans" with a family of highly variable presynaptically localized neuronal cell surface receptors (i.e., neurexins) [70, 71]. Neurexin-1a, neurexin-1b, neurexin-2a, neurexin-2b, and neurexin-3b proteins bind ADGRLs via the receptor its olf domain. This results in a transsynaptic adhesion complex [66].

Of interest, the ADGRL family has been extensively studied in *C. elegans*. The ADGRL1 homolog LAT-1 has been shown to exhibit an NTF- and CTF-independent function. The NTF is anchored to the membrane and mediates fertility, independently from the CTF [45]. Likewise, the role of the CTF part in tissue polarity is independent of the NTF. Note that these effects are GPS cleavage independent, since a non-cleavable mutant did still possess the two independent activities [45]. ADGRL1–3 have the ability to reassociate upon protein binding to their NTF. Interestingly, this reassociation does not require a CTF from the original ADGRL subfamily member, but the protein-bound NTF can bind to a CTF from another ADGRL subfamily member [57]. Since the GAIN domain has high structural homology within the aGPCR class, even functional interactions with different subfamily members have been observed (named crisscross associations) [57].

No interaction data is known to date regarding the NTF of ADGRL4. The question arises whether this aGPCR truly belongs in this aGPCR subfamily. Structure-wise ADGRL4 is able to form homodimers at the cell surface consisting of two full-length ADGRL4 proteins [61].

4.2.2 ADGRE

Members of the ADGRE subfamily have a very similar NTF consisting of 2–5 tandemly arranged EGF-like domains (see Sect. 4.1). The 1–4 EGF-like domains that are located C-terminal of the first EGF domain have a calcium-binding site and belong to the fibrillin-like family I type [6]. The NTF similarity in ADGREs is

mainly caused by their evolutionary history of gene duplication and exon shuffling [6]. ADGRE4 is most likely a pseudo-gene evolved after separation from the great apes [72]. ADGRE2 has been present from early placental mammals. Both ADGRE2 and ADGRE3 have no homologs in Murinae [73]. The 7TM of ADGRE2 have high amino acid similarity to ADGRE3, but the NTF part is most similar to ADGRE5 (6 aa difference) [74]. Of interest is the fact that ADGREs (i.e., 1, 2, 5, but also EGF-like domain-containing ADGRL4) have multiple isoforms. These isoforms vary in their EGF-like domain numbers and thus the structural formation of their NTF.

Based on the NTF structural features, ADGRL4 was previously believed to be part of the ADGRE subfamily [6]. However, based on chromosome localization (all genes except of ADGRL4 are located on chromosome 19), ADGRL4 does not fit in this family [6]. In addition, an ADGRL4-like gene in zebra fish (where ADGRE members are restricted to mammals) and the expression pattern of ADGRL4 also exclude ADGRL4 from the ADGRE subfamily [6].

CD55 interacts with the first two EGF-like domains in ADGRE5, whereas the third EGF-like domain is needed for structural integrity of the binding interface [75, 76]. Strikingly, ADGRE2 which differs only three amino acids in its EGF-like domains does not bind CD55 [74], demonstrating the high specificity in CD55 binding interaction to ADGRE5 (CD97). Different isoforms (i.e., different numbers of EGF-like domains) have different affinity for CD55 [76, 77]. Also, glycosaminoglycan side chain chondroitin sulfate B [78], α5β1 and αvβ3 integrins [79], as well as CD90 [80] interact with ADGRE5. Chondroitin sulfate binds to the fourth EGF-like domain of ADGRE5 and ADGRE2, which is only present in the largest isoform of this receptor. Integrins bind to the RGD motif and CD90 binds to the GAIN domain. It was also shown that NTF of ADGRE5 can simultaneously bind CD55 and chondroitin sulfate B [81], although it has been suggested that this dual interaction is not likely to occur in vivo due to affinity for different isoforms [76, 77]. ADGRE5 also interacts "in *cis*" with lysophosphatidic acid receptor 1 (LPA) [82, 83], although structural requirements for this interaction are not yet known. Exogenous ligands have been developed for all members of this aGPCR subfamily by means of NTF-targeting antibodies [29]. ADGRE5-directed antibodies bind to different EGF domains [84].

4.2.3 ADGRA

The ADGRA subfamily has three members and all three have quite different NTF compositions. ADGRA1 is the only aGPCR that does not include the characteristic GAIN domain. There are also no known functional domains in the short NTF region of ADGRA1 [85]. ADGRA2 and ADGRA3 both have very similar leucine-rich repeats (LRRs), an immunoglobulin (Ig) domain, and an HBD [85]. In addition, ADGRA2 also contains an Arg-Gly-Asp (RGD) motif [86]. The LRRs (~25 aa) form a characteristic alpha/beta horseshoe fold with a beta-sheet inside and helices pointing outside [87]. Ig domains in ADGRA2 and ADGRA3 have some similarity to this domain in ADGRF5 (GPR116) [85]. The HBD of ADGRA2 and ADGRA3 is most closely related to the HBD of ADGRB3 (BAI3) receptor [85].

ADGRA2 has a GPS site, but is predicted to be non-cleavable. Interestingly, shed NTF of this receptor could be detected in cell culture medium. This is caused by a cleavage site in the NTF that is an interaction point for thrombin-induced shedding promoted by cell surface protein disulfide isomerase [88]. The RGD motif in ADGRA2 can bind αvβ3 integrin [86].

4.2.4 ADGRC

ADGRC (CELSR) subfamily members have several protein domains in their NTF. Their former name (i.e., CELSR) is actually an abbreviation of these different domains: cadherin, EGF LAG, and seven-pass G-type receptor [89]. ADGRCs are among the oldest aGPCRs. ADGRC consists of nine cadherin repeats (110 aa), followed by six EGF-like domains (see Sect. 4.1), two LAG domains, and an HBD (see Sect. 4.1). The cadherin repeats play a role in extracellular calcium binding [90]. The 3D structure of several cadherin domains in other proteins has been solved lately. These cadherin domains are formed by seven beta-strands and show similarity to immunoglobulin constant domains [91, 92]. Comparable with EGF-like repeats, the quaternary structural composition of several repeats is mediated by calcium binding [93]. Five calcium-binding EGF-like domains (40 aa) interrupted by two laminin G-like (LAG) domains follow the cadherin repeats. The LAG domains have a jelly roll fold structure and could also bind calcium [94]. EGF-Lam domains (60 aa) are situated between the EGF-like and LAG domain structures and the HBD (see Sect. 4.1). ADGRC1 and ADGRC2 contain one EGF-Lam domain and ADGRC3 has two EGF-Lam domains. EGF-Lam domains contain eight conserved cysteine residues [89].

Although the large NTF of ADGRC subfamily members has multiple protein domains and therefore potential interaction interfaces, no endogenous protein-binding partners have been found to date. Activation is however modulated by calcium-induced *trans*-homodimer formation by cadherin–cadherin interactions [95, 96]. Also *cis*-homodimers have been reported, and these can also be formed in absence of calcium. We could therefore speculate that one ADGRC is the "ligand" for another ADGRC and vice versa. Unfortunately, no information is yet known about the exact interaction points and/or mechanisms by which ADGRC form *trans*- and/or *cis*-dimers.

4.2.5 ADGRD

ADGRD have quite short NTFs compared to other aGPCR subfamilies. ADGRD1 does not have any known additional structural features in its NTF, but ADGRD2 has a pentraxin (PTX) domain [16]. This PTX domain has similarities (33 %) with the PTX domain in ADGRG4 (GPR112) [16]. Pentraxins consist of up to five non-covalently bound identical subunits that form a flat pentameric disk [97]. An interesting observation is that the ADGRD subgroup is the most closely related to the family B secretin receptors [32].

No binding partners have been identified for ADGRDs. However, deletion of the NTF activates ADGRD1 by exposing the tethered agonist to the 7TM [37].

4.2.6 ADGRF

ADGRF1 (GPR110) has an additional cleavage site in its NTF, which is named sperm protein/enterokinase/agrin (SEA) domain (~80 aa) [98]. This motif might have a successive beta-strand structure that is interrupted by one alpha-helix [99]. ADGRF2 and ADGRF4 show high sequence similarity and both have no functional domains in their NTF [85]. ADGRF3 (GPR113) [100] has one EGF domain and an HBD (see Sect. 4.1). There is some structural similarity between ADGRF3 and the ADGRE subfamily, as well as with ADGRL4 [85]. The HBD is mostly related to ADGRL subfamily, especially ADGRL3 [85]. ADGRF5 has a SEA box and contains two immunoglobulin-like repeats [85, 101]. ADGRF5 also has an additional cleavage site in its NTF that is sensitive for furin [102]. Moreover, ADGRF5 can form homodimers on the cell surface in *cis* [103] and has been suggested to interact with the surfactant component: surfactant protein D [104].

4.2.7 ADGRB

The NTF of the ADGRB subfamily consists of four or five thrombospondin type 1 repeats (TSRs) and an HBD (see Sect. 4.1). In addition, ADGRB1 (BAI1) contains an RGD motif (231–233 aa) (see ADGRA2) in its NTF [105]. ADGRB3 has an additional Cs1 and Csr/Uegf/BMP-1 (CUB) domain (110 aa) at the N-terminus [106].

CUB domains are made up of a beta-sandwich with a jelly roll fold. They furthermore contain four conserved cysteines that are thought to make disulfide bonds [107].

Thrombospondin type 1 repeats (60 aa) are conserved domains that are comprised of an elongated, three stranded beta-sheet [108]. One site contains a helical groove that contains a high amount of positively charged residues. Variations in the positive charge (distribution or density) is often observed within different TSRs and also within the ADGRBs and is believed to contribute to protein-binding specificity [109].

An interesting feature of the NTF of ADGRB members is their ability to be cleaved, in addition to GPS autoproteolysis [109]. Cleavage results in two fragments of 120 kDa (vasculostatin) [105, 110] and 40 kDa (vasculostatin-40). Vasculostatin-40 contains one TSR [111]. Phosphatidylserine is an endogenous ligand for ADGRB1 (BAI1) and binds to thrombospondin type 1 repeats [112, 113]. ADGRB1 can also bind the exogenous lipopolysaccharide (LPS) of Gram-negative bacteria in macrophages [109]. ADGRB1 and ADGRB2 both showed increased constitutive activity upon deletion of their NTF domains, compared to full-length receptors. This indicates that the NTF has an inhibiting effect on CTF functioning [114]. The RGD motif from ADGRB1 (BAI1) can interact with integrins [105, 115]. The shed NTF fragments vasculostatin and vasculostatin-40 interact with αvβ5 integrins [105, 110, 111]. Vasculostatin binds to CD36 or histidine-rich glycoprotein (HRGP) [116]. ADGRB3 (BAI3) binds secreted C1q-like proteins with its thrombospondin type 1 repeats and CUB domain [106, 117].

4.2.8 ADGRG

The majority of ADGRG subfamily members are characterized by their lack of known functional domains [i.e., ADGRG1 (GPR56), ADGRG3 (GPR97), ADGRG5, and ADGRG7 (GPR128)] [10]. Only ADGRG6 (GPR126), which contains a CUB domain, PTX domain, and HBD, and ADGRG4 that possesses a PTX domain and RGD motif have additional features besides the GAIN domain. Interestingly, ADGRG6 NTF is additionally cleaved by furin during intracellular processing steps, which leads to the release of sub-NTF fragments [118]. Recently, ADGRG1, ADGRG2 (GPR64), ADGRG5, and ADGRG6 were shown to be activated by a tethered peptide agonist upon NTF removal [37–39, 119]. Curiously, ADGRG2 full-length proteins activate different signaling pathways than truncated ADGRG2 in which the NTF is removed [120].

Collagen III and major cross-linking enzyme transglutaminase 2 (TG2) have been identified as binding partners for ADGRG1 [121, 122]. Collagen III binds in the NTF region range 27–160 aa, and, noteworthy, glycosylation is not a prerequisite for interaction to take place [123]. TG2 binds between 108 and 177 aa in the NTF of ADGRG1 [121, 124]. It is not clear whether collagen III and TG2 can bind simultaneously to the NTF.

ADGRG3 is the only aGPCR for which an interaction with a drug (i.e., corticosteroid beclomethasone dipropionate) has been reported [125].

ADGRG6 interacts with collagen IV and laminin-211. Collagen IV binds to the CUB and PTX domains [126]. Laminin-211 has been shown to interact with a novel laminin-binding domain (446–807 aa) in the ADGRG6 NTF and thereby prevents Stachel-induced receptor activation [127].

4.2.9 ADGRV

ADGRV is the only receptor in this subfamily and has the largest NTF of all aGPCRs. The NTF consists of 35 Calx-beta motifs (45 aa) that are interrupted by a pentraxin (PTX) domain (200 aa) (between the ninth and tenth Calx-beta motif) and an epilepsy-associated repeat (EAR) domain between the 22nd and 23rd Calx-beta motifs [128]. The Calx-beta motif has most likely an alpha, beta, beta, alpha, beta topology. The EAR domain has a predicted structure that consists of seven-bladed beta propellers [129].

The Calx-beta motifs 32–35 of ADGRV can bind calcium [130]. Other ligands or binding partners have not yet been identified. The beta propeller structures of the EAR, PTX, and Calx-beta domains and motif are potential binding interfaces for peptides and proteins. In addition, this has been suggested that ADGRV NTF interacts with ADGRV NTF via its Calx-repeats on other cells via NTF homodimerization [131].

5 Summary

aGPCRs are an object of a currently growing research field, and exploration of the pharmacological potential of these important signaling molecules is developing. This family of GPCRs was phylogenetically shown to form a separate cluster from other GPCR families and was categorized into nine distinct subfamilies. Repertoires of aGPCRs have been curated in several species including some model organisms. However, naming these homologs in a harmonized manner was found to be difficult, as a coherent nomenclature system of aGPCRs is not available. Moreover, the complexity of aGPCRs such as sharing low sequence similarity within some subfamily members and their diverse long N-termini with multiple functional domains adds on to the difficulty in naming and classification of homologs in distant species. This is certainly important as proper classification and curation of homologs in important established model organisms and other emerging model organisms (distant metazoan species) is essential in exploring the aGPCR biology. Recently, the Adhesion-GPCR Consortium with assistance from HGNC worked on a coherent naming system and provided aGPCRs a prefix that identifies any adhesion GPCR homolog, independent of species or subfamily [15]. In such context, ADGR was found to be the most appropriate and unique prefix, standing for adhesion G protein-coupled receptor. And each subfamily was assigned a single-letter acronym based on their previous subfamily names (e.g., E for the EGF-TM7 receptors, C for the CELSRs). This nomenclature is currently used and will be essential for easy naming of orthologs and other genetic variants in different species, including those that encode large expansions of aGPCRs. With the current nomenclature system, future comparisons of aGPCRs repertoires will help to better classify and define novel aGPCRs in important emerging model organisms, and this would provide a key foundation for elucidating aGPCR functions and pursuing aGPCRs as therapeutic targets. The current aGPCR classification into nine distinct subfamilies is based on their 7TM sequence similarity. Moreover, aGPCRs have family-specific features, like the GAIN domain, but also possess subfamily-specific domains in their NTF. Interestingly, overlap is observed with respect to the organization of aGPCR structural NTF features and the 7TM-based classification. A systematic description of the structural topology in aGPCR NTF regions provides information on protein-binding domains and NTF–CTF interactions and could ultimately help to get insight in (novel) aGPCR interaction partners for orphan aGPCRs.

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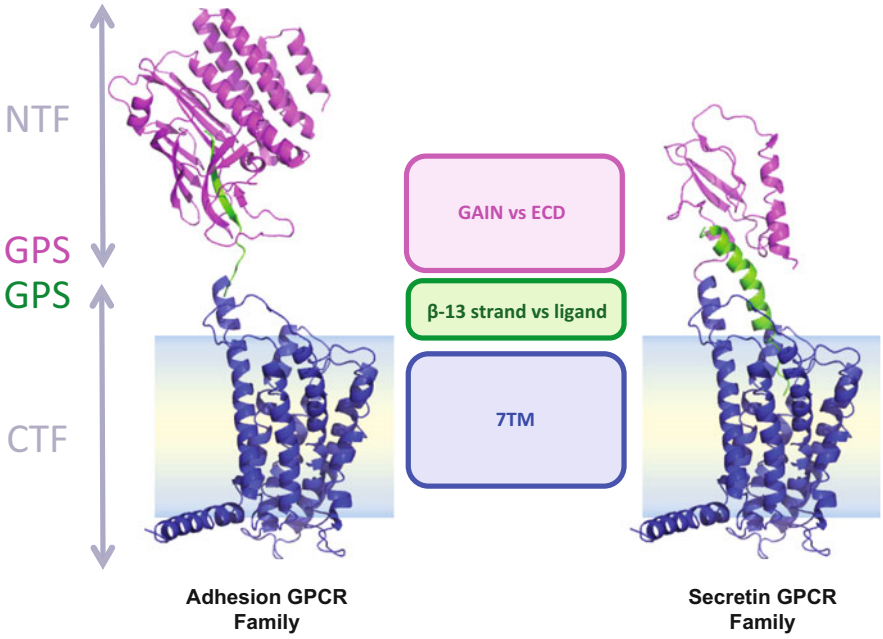
7TM Domain Structure of Adhesion GPCRs

Chris de Graaf, Saskia Nijmeijer, Steffen Wolf, and Oliver P. Ernst

Graphical Abstract



Schematic presentation of the overall adhesion G Protein-Coupled Receptor (aGPCR) structure and functional domains, covering an extracellular N-terminal fragment (NTF), a membrane-spanning C-terminal fragment (CTF) and a GPCR proteolysis site (GPS). (*Left side*) aGPCR model constructed based on the seven-transmembrane (7TM) structure (*blue*) of secretin family glucagon receptor (GCGR) (PDB, 4L6R) [11] and the GPCR autoproteolysis inducing (GAIN) domain (*magenta*) structure of latrophilin 1 (PDB, 4DLQ) [9]. The β-13 strand residues are depicted in *green*. (*Right side*) The experimentally validated full-length secretin family GCGR

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structure combining structural and experimental information from the GCGR 7TM crystal structure (PDB, 4L6R) (*blue*), the GCGR extracellular domain (ECD) structure (PDB, 4ERS) (*magenta*) and the ECD structure of glucagon-like peptide-1 (GLP-1)-bound glucagon-like peptide-1 receptor (GLP-1R) (PDB, 3IOL) (*green*), complemented by site-directed mutagenesis, electron microscopy (EM), hydrogen–deuterium exchange (HDX) and cross-linking studies [11–13])

Contents

Abstract

Despite the recent breakthroughs in the elucidation of the three-dimensional structures of the seven transmembrane (7TM) domain of the G protein-coupled receptor (GPCR) superfamily, a corresponding structure of a member of the adhesion GPCR (aGPCR) family has not yet been solved. In this chapter, we give an overview of the current knowledge of the 7TM domain of aGPCRs by comparative structure-based sequence similarity analyses between aGPCRs and GPCRs with known crystal structure. Of the GPCR superfamily, only the

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secretin family shares some sequence similarity with aGPCRs. This chapter will therefore emphasize on the comparison of these two GPCR families. Two 7TM domain structures of secretin family GPCRs are known that provide insight into the structure-function relationships of conserved sequence motifs that play important roles and are also present in most aGPCRs. This suggests that the 7TM domains of aGPCRs and secretin family GPCRs share a similar structural fold and that the conserved residues in both families may be involved in similar intermolecular interaction networks and facilitate similar conformational changes. Comparison of the residues that line the large peptide hormone binding pocket in the 7TM domain of secretin family GPCRs with corresponding residues in aGPCRs indicates that in the latter, the corresponding pocket in the 7TM domain is relatively hydrophobic and may be even larger. Improved knowledge on these conserved sequence motifs will help to understand the interactions of the aGPCR 7TM domain with ligands and gain insight into the activation mechanism of aGPCRs.

Keywords
GPCR sequence alignment • Adhesion GPCR residue nomenclature • Adhesion GPCR sequence-structure relationship • Druggability adhesion GPCRs

1 Introduction

1.1 Structural Topology of Adhesion GPCRs

aGPCRs are cell-surface proteins that belong to the large GPCR superfamily comprising the five main families, named according to the GRAFS classification system glutamate, rhodopsin, adhesion, frizzled/taste2, and secretin [1, 2]. Alternatively, GPCRs can be classified into families A, B, C and F (Table 1) [3]. A phylogenetic analysis of the seven-transmembrane (7TM) α helices of the human GPCR superfamily classified aGPCRs as a separate family [4] (the 'A' in the GRAFS or B2 in A–F classification). The GRAFS classification system clearly distinguishes aGPCRs from secretin-like GPCRs, and therefore this nomenclature

Table 1 Overview of GPCR family classifications, which allows easy comparison between the GRAFS and A–F classifications and unique receptors (unique orthologues) for which a crystal structure is available in the Protein Data Bank

Classification of GPCR superfamily			
General family name	GRAFS	A–F families	Unique receptors for which a crystal structure is available[a]
Glutamate-like GPCRs	G	C	2
Rhodopsin-like GPCRs	R	A	27
Adhesion GPCRs	A	B2	0
Frizzled/taste2 GPCRs	F	F	1
Secretin-like GPCRs	S	B1	2

[a]accessed 17 Jan 2016

will be used throughout this chapter. Note that at least one GPCR crystal structure is available for all subfamilies, except for the aGPCR and Taste2 receptor family [5].

The aGPCR family can be further divided into nine distinct subfamilies that all share typical aGPCR features [6]. aGPCRs have a twofold protein structure consisting of a large extracellular domain (ECD, ~320–5878 residues) and a membrane-spanning 7TM domain followed by an intracellular domain (ICD) (~300–600 residues) [6]. A unique feature of the aGPCR family is their autoproteolytical cleavage at the GPCR proteolysis site (GPS) [7, 8], which is embedded in the conserved GPCR autoproteolysis inducing (GAIN) domain [9, 10] that forms an important part of the ECD. This cleavage results in a split aGPCR in which an extracellular N-terminal fragment (NTF) and a membrane-spanning C-terminal fragment (CTF) that includes the ICD are non-covalently associated (Fig. 1).

It is still under debate whether NTF and CTF complement each others' function by working as a single entity and/or that both fragments possess separate functions

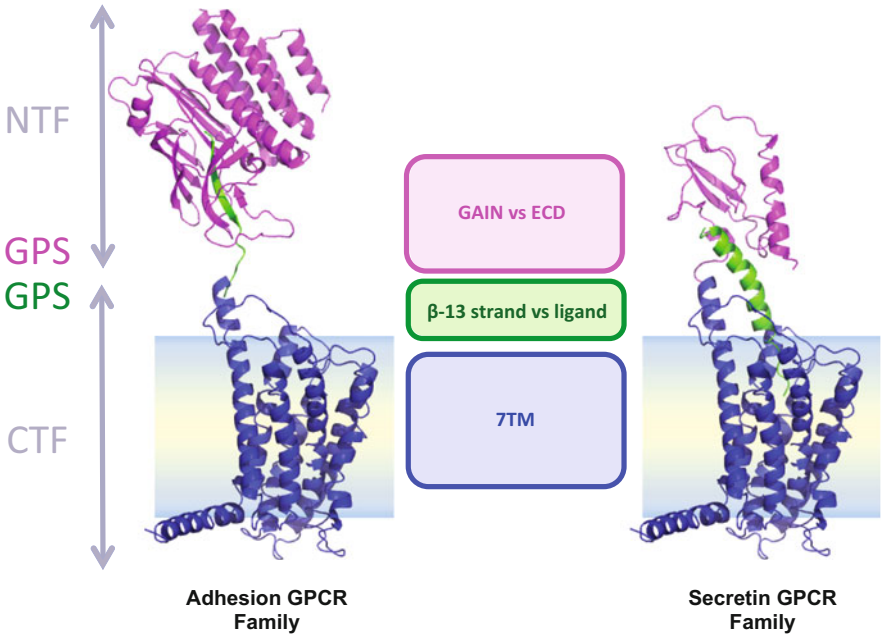

Fig. 1 Schematic presentation of the overall aGPCR structure and functional domains, covering an extracellular N-terminal fragment (NTF), a membrane-spanning C-terminal fragment (CTF) and a GPCR proteolysis site (GPS). (*Left side*) aGPCR model constructed based on the seven-transmembrane (7TM) structure (*blue*) of secretin family glucagon receptor (GCGR) (PDB, 4L6R) [11] and GPCR autoproteolysis inducing (GAIN) domain (*magenta*) structure of latrophilin 1 (PDB, 4DLQ) [9]. The β-13 strand residues are depicted in *green*. (*Right side*) The experimentally validated full-length secretin family GCGR structure combining structural and experimental information from the GCGR 7TM crystal structure (PDB, 4L6R) (*blue*), the GCGR extracellular domain (ECD) structure (PDB, 4ERS) (*magenta*) and the ECD structure of glucagon-like peptide-1 (GLP-1)-bound glucagon-like peptide-1 receptor (GLP-1R) (PDB, 3IOL) (*green*), complemented by site-directed mutagenesis, electron microscopy (EM), hydrogen–deuterium exchange (HDX) and cross-linking studies [11–13])

[6]. The NTF contains functional domains that often mediate cell-cell or cell-matrix adhesion [2] by binding to endogenous extracellular interaction partners. These interacting proteins can be part of the extracellular matrix or situated on the cell outer membrane of opposing cells (trans) as well as the same cell (cis) [6, 14]. Although binding interfaces have been investigated with purified NTF proteins, a detailed view of the interactions between NTF and its binding partners is often absent (see [15]). Only recently the binding site for a cell-adhesion protein, fibronectin leucine-rich repeat transmembrane protein 3 (FLRT3), to the latrophilin 3 aGPCR (LPHN3, ADGRL3) was described in detail where the interaction hotspot was located at the second and third blades of the olfactomedin domain propeller in the LPHN3 NTF [16]. Importantly, not all binding events at the NTF result in aGPCR signalling, and it remains to be determined whether ligand binding to the NTF is a prerequisite for aGPCR activation.

The only extracellular domain that is shared by almost all aGPCRs [excl. ADGRA1 (GPR123)] is the ~320 residues large GAIN domain, which is located immediately N-terminal of TM1 (see [15]). The importance of the GAIN domain structure in the autoproteolysis of aGPCRs has recently been highlighted for the latrophilin 1 receptor (ADGRL1) and ADGRB3 (BAI3) [9, 10]. Noteworthy, cleavage takes place at the L↓T/S/C site [17] within the GPS motif that encompasses the last five beta strands of the GAIN domain, leaving the last beta strand (β-13) of the GAIN domain still attached to the CTF [9, 18–20].

The CTF of aGPCRs resembles the classical 7TM structure of the rhodopsin- or secretin-like GPCR families [2, 17]. Increasing evidence shows that the CTF possesses signalling properties such as G-protein coupling [21–24], β-arrestin interaction [24–26] and receptor internalization [27] that clearly hints at conformational changes general to the GPCR superfamily. A more detailed look at the CTF shows that it can be divided into four parts: (1) the β-13 strand (also called the Stachel sequence), (2) the 7TM helices that cover the predominant part of the CTF, (3) the extracellular loops (ECLs) and (4) the intracellular loops (ICLs) and C-tail.

The protruding and hydrophobic β-13 strand/Stachel, which is the remaining part of the GAIN domain after cleavage, has been recently highlighted as tethered agonist sequence capable to activate aGPCRs (i.e. GPR56, GPR110, GPR126, GPR133, GPR114, GPR64) [18, 20, 28, 29] (see [30]). While this very recent work on aGPCR activation has been rapidly progressing, it remains to be revealed in what way the β-13 strand/Stachel interacts with amino acids within the 7TM helices and/or ECLs and how this leads to an active aGPCR conformation.

To date, no evidence is available on the role of ECLs and ICLs on aGPCR functioning. This is understandable, considering the limited information on aGPCR signal transduction for the majority of aGPCRs and hence very rare site-directed mutagenesis data. Potential protein-protein interaction sites/motifs have been predicted in aGPCR C-tail sequences, which led to the identification of non-canonical signalling partners (i.e. ELMO/Dock180 or dishevelled) for ADGRB1 (BAI1) [31, 32] and ADGRA3 (GPR125), respectively [33]. The 7TM region has been investigated in comparative sequence alignment studies, but experimental evidence further supporting the importance of specific residues and/or motifs is absent.

1.2 Sequence Similarity Between the 7TM Domains of aGPCR and Other GPCR Subfamilies

In earlier amino acid sequence alignment analyses of aGPCRs, a high sequence similarity in the TM helices (25–70 % amino acid identity) between members of the family was observed [17]. Previously reported aGPCR sequence alignments with secretin-like GPCRs showed a 25–30 % amino acid identity with aGPCRs [17], and sharing of several conserved residues [2, 17]. This is in line with earlier studies that not only showed sequence similarities but also a similar splice site setup [34]. Indeed, it was even suggested that secretin-like GPCRs are descendants from aGPCRs [34]. In particular aGPCRs ADGRG5 (GPR114) and ADGRG7 (GPR128) share the closest sequence similarity with the secretin family GPCRs glucagon receptor (GCGR or GLR) and corticotropin-releasing factor receptor 1 (CRFR1).

Figure 2 shows a NeighborNet analysis [35] of a MUSCLE [36] alignment of aGPCR sequences and 7TM domain sequences from crystallized GPCRs following the method of Wolf and Grünewald [37]. The NeighborNet-based network, a superposition of multiple possible phylogenetic trees, shows that aGPCRs indeed are most related to the GCGR and CRFR1 receptors. The recently solved crystal structures of the 7TM domains of GCGR [11, 40] and CRFR1 [41] therefore offer useful structural templates for the 7TM regions of aGPCRs as will be further demonstrated in this chapter. In contrast, the sequence similarities between aGPCRs and rhodopsin, glutamate and frizzled families, for which 7TM crystal structures have been solved [42–44], are relatively low (Fig. 2), although few structural features may also be conserved between aGPCRs and these GPCR families (Fig. 3).

To allow comparison between the residues at different positions in the TM helices of different GPCRs within and between different families, residues are numbered according to the *Ballesteros-Weinstein* numbering scheme [50] where the single most conserved residue in each TM helix is designated X.50 (Table 2). X is the TM helix number, and all other residues in that helix are numbered relative to this conserved position [45]. The *Ballesteros-Weinstein* numbering scheme has been initially defined for family A/rhodopsin family members (X.50a [50]), and similar residue numbering schemes are used to allow comparison between B1/secretin family (*Wootten*, defining reference position X.50b [51]), family C/glutamate family (*Pin*, X.50c [50]) or family F/frizzled family (*Wang*, X.50f [44]) GPCRs. Residues located in extracellular loop 2 are numbered based on their relative position to a conserved cysteine ($C^{45.50}$) that forms a disulphide bond with a cysteine in TM3 ($C^{3.25}$/$C^{3.29}$) in all GPCR families [51]. The *structural* alignment of the TM helices of the crystal structures of rhodopsin, secretin, glutamate and frizzled receptors allows the definition of a *structure-based* sequence alignment of these GPCR families (Table 2) that can be extended to aGPCRs based on the sequence alignment between the aGPCRs and homologous secretin-like receptors (Fig. 3).

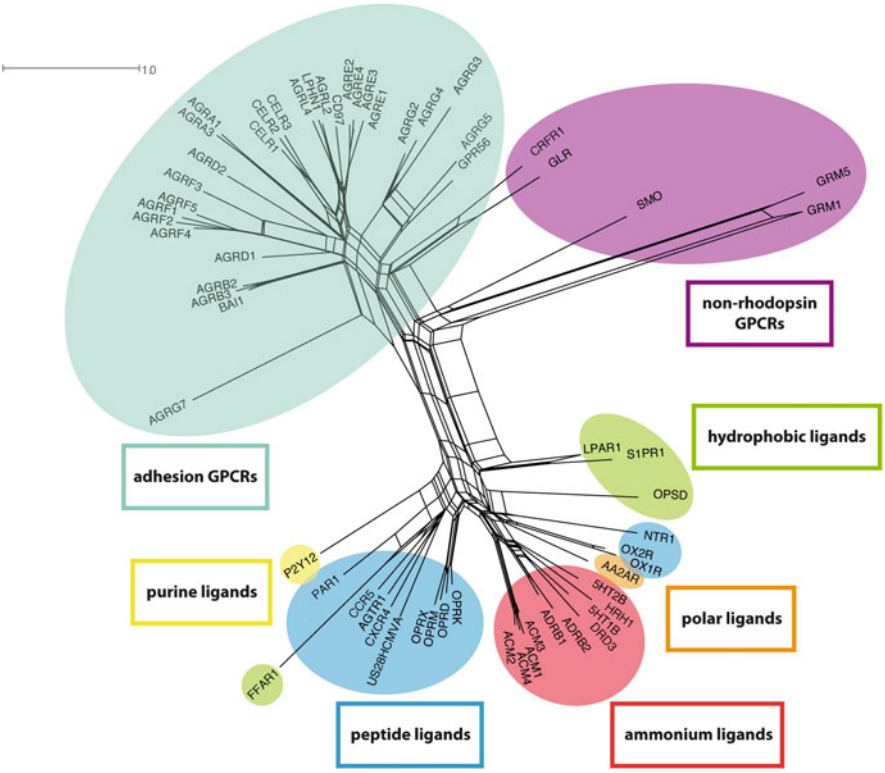

Fig. 2 NeighborNet analysis [35] of a MUSCLE [36] alignment of adhesion GPCR sequences and 7TM domain sequences from crystallized GPCRs following the method of Wolf and Grünewald [37]. The displayed network was calculated in SplitsTree [38] using maximum-likelihood distances [39]. Receptor abbrevations are given according to their Uniprot entry names. Full GPCR sequences were used to obtain the alignment, but only the 7TM domain sequences according to crystal structures were used for the analysis

Table 2 shows that the reference residues of the Wootten numbering scheme for family B1/secretin-like GPCRs are also the most conserved for five of the family B2/aGPCR TM helices ($S^{1.50b}$, $H^{2.50b}$, $N^{5.50b}$, $G^{6.50b}$, $G^{7.50b}$), and the remaining two residues in TM3–4 still display a high conservation within the aGPCR family ($E^{3.50b}$ 67 % and $W^{4.50b}$ 48 % within human aGPCRs). For example, for the glucagon receptor with its known crystal structure, the residues that are most conserved in secretin family GPCRs ($S152^{1.50b}$, $H177^{2.50b}$, $E244^{3.50b}$, $W271^{4.50b}$, $N317^{5.50b}$, $G359^{6.50b}$, $G391^{7.50b}$) are also present in most aGPCRs, for example, CD97 (ADGRE5: $S558^{1.50b}$, $H583^{2.50b}$, $E633^{3.50b}$, $Y666^{4.50b}$, $N707^{5.50b}$, $G750^{6.50b}$, $G779^{7.50b}$) or GPR56 ($S414^{1.50b}$, $H446^{2.50b}$, $E496^{3.50b}$, $W524^{4.50b}$, $N585^{5.50b}$, $G620^{6.50b}$, $G651^{7.50b}$). The highly conserved tryptophan residue in TM3 of aGPCRs (e.g. $W629^{3.46b}$ in CD97) and secretin-like receptors ($W^{3.46b}$) is the most conserved residue in TM3 of frizzled receptors ($W^{3.50f}$), while the conserved tryptophan residue in TM4 of aGPCRs (e.g. W623 in GPR56) and secretin-like

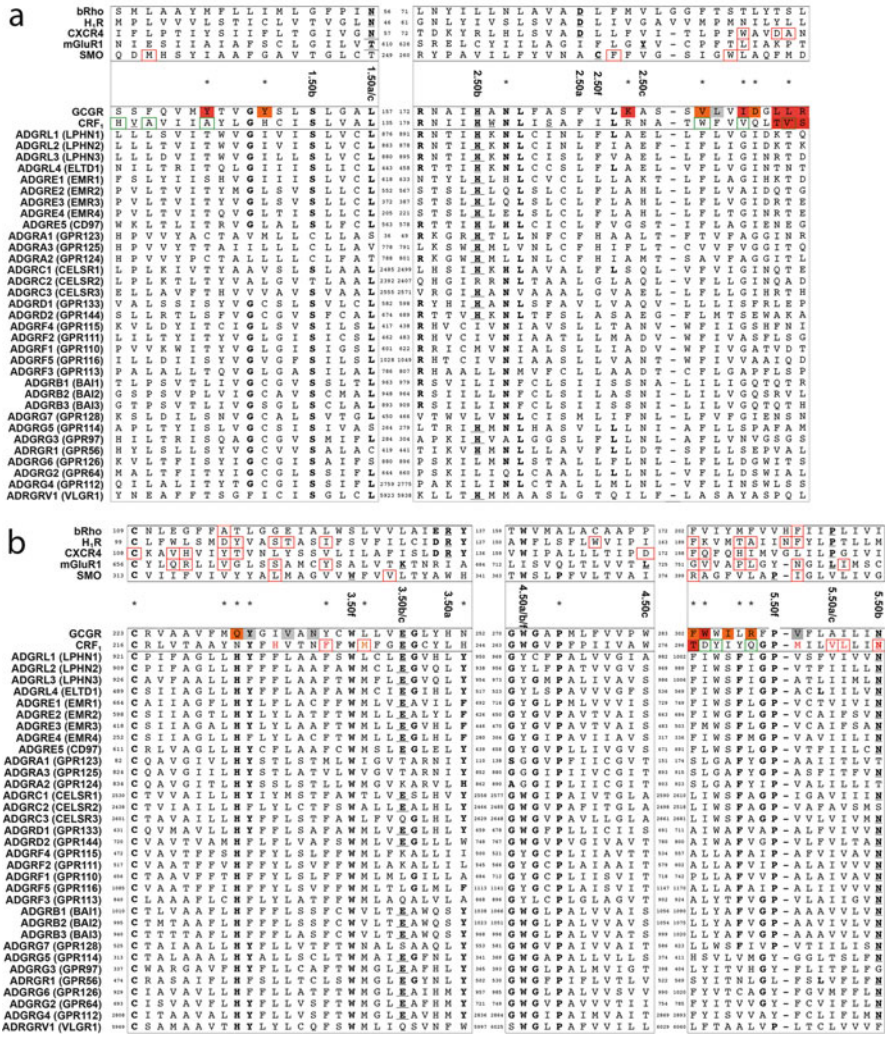

Fig. 3 Sequence alignment of the 7TM domains of all 33 human aGPCRs and representative rhodopsin, glutamate, frizzled and secretin family GPCRs for which crystal structures are available. Effects of mutation studies in GCGR and corticotropin-releasing factor receptor 1 (CRFR1) receptors [13, 45] are mapped on a structure-based sequence alignment between representative rhodopsin family (bovine rhodopsin (bRho; PDB, 1F88) [46], histamine H_1 receptor (H_1R; PDB, 3RZE) [47] and CXC-chemokine receptor 4 (CXCR4; PDB, 3ODU, 3OE0) [48]), glutamate family (metabotropic glutamate receptor 1 (mGluR1; PDB, 4OR2) [43]), frizzled family (smoothened receptor (SMO; PDB, 4JKV) [44]) and secretin family [GCGR (PDB, 4L6R [11], 5EE7 [40])] CRFR1 (PDB, 4K5Y) [41] GPCRs. Only specific parts of TM1–2 (panel a), TM3-5 (panel b), and TM6–7 (panel c) are shown, separated by *grey dashed lines*, and residue numbers of defining missing regions in the alignment are indicated. The alignment considers helix bulges and constrictions as described in [5]. Mutated residues that show four- to tenfold (*orange*) and >tenfold (*red*) changes of K_i/IC_{50} values for ligand binding (or ligand potency/EC_{50} value if no K_i/IC_{50} value has been reported) are marked as *full coloured box* (peptide ligands) and *open*

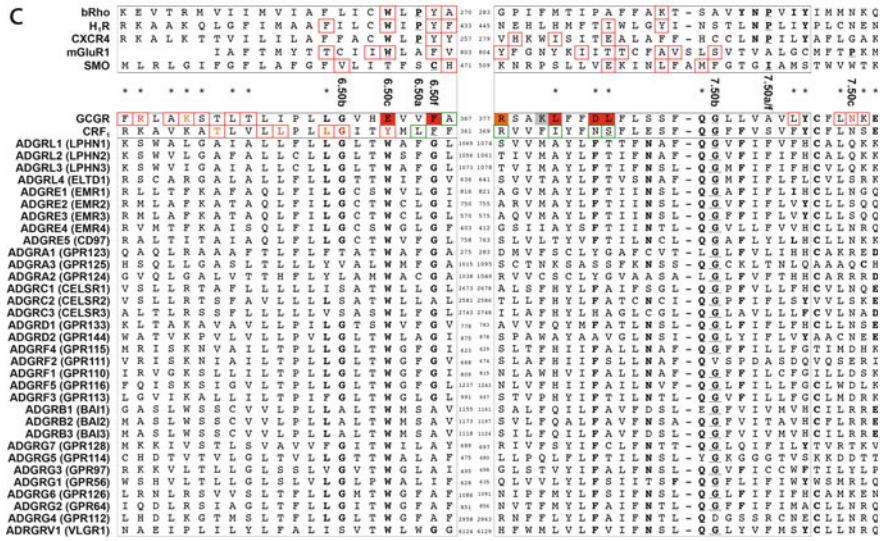

Fig. 3 (continued) coloured box (non-peptide ligands). Mutants that show receptor expression <30 % of wild type are marked *grey*. Receptor residues that covalently bind peptide ligands in photo-cross-linking or cysteine-trapping studies [13, 45, 49] are boxed *green*. Ligand contact residues in GCGR, CRFR1, bRho, H_1R, CXCR4, mGluR1 and SMO crystal structures are boxed *red*. The most conserved residues in TM1–7 of family A (X.50), family B (X.50b), family C (X.50c) and family F (X.50f) GPCRs are *underlined* and shown in *bold*. The positions of other residues described in the text are indicated with an *asterisk*

receptors ($W^{4.50b}$) is also defined as most conserved reference residue in family A/rhodopsin ($W^{4.50a}$) and family F/frizzled ($W^{4.50f}$) GPCRs. The most conserved proline residue in TM5 of frizzled ($P^{5.50f}$) is also present in most aGPCRs (e.g. $P699^{6.42b}$ in CD97) and secretin receptors ($P^{5.42b}$), while the most conserved tryptophan residue in TM6 of glutamate family GPCRs ($W^{6.50b}$) is also conserved in rhodopsin family GPCRs ($W^{6.48b}$) and aGPCRs (e.g. $W753^{6.53b}$ in CD97). The number of shared conserved residues between aGPCRs and other GPCR families reflects the network analysis in Fig. 2 and indicates that aGPCRs are most homologous to secretin-like receptors, but that also frizzled, rhodopsin and glutamate GPCRs share some local sequence similarity in specific TM helices.

Based on (1) the fact that aGPCRs share the highest overall sequence similarity to secretin-like receptors (Fig. 2) (2) the fact that aGPCRs and secretin-like receptors share almost all highly conserved residues defined as reference positions in secretin-like receptors (or other GPCR families, Table 2) and (3) the fact that aGPCRs and secretin-like receptors have historically been defined as the same GPCR family B, we propose to consistently use the family B/secretin-like numbering scheme [51, 53] for aGPCRs. Therefore, to allow systematic comparison of the residues at different positions in the TM helices of different GPCRs within and between different families, receptor residue numbers are annotated throughout this

Table 2 Comparison of Ballesteros-Weinstein reference positions X.50 [50] for representative family A/rhodopsin-like [bovine rhodopsin (bRho)], family B1/secretin-like [glucagon receptor (GCGR)], family C/glutamate [metabotropic glutamate receptor 1 (mGluR1)] and family F/frizzled [smoothened (SMO)] to representative family B2/aGPCRs CD97 and GPR56

Helix	Family B2 adhesion (CD97)	Family B2 adhesion (GPR56)	Family B1 secretin (GCGR)	Family A Rhodopsin (bRho)	Family C Glutamate (mGluR1)	Family F Frizzled (SMO)
TM1	S558$^{1.50b}$	S414$^{1.50b}$	S152$^{1.50b}$	G51$^{1.46a}$	G603$^{1.50c}$	T241$^{1.43f}$
	L562$^{1.54b}$	C418$^{1.54b}$	L156$^{1.54b}$	N55$^{1.50a}$	T607$^{1.54c}$	T245$^{1.47f}$
	C565$^{1.57b}$	T421$^{1.57b}$	A159$^{1.57b}$	T58$^{1.53a}$	V610$^{1.57b}$	T248$^{1.50f}$
TM2	H583$^{2.50b}$	H446$^{2.50b}$	H177$^{2.50b}$	L76$^{2.43a}$	C631$^{1.39c}$	L267$^{2.42f}$
	L590$^{2.57b}$	V453$^{2.57b}$	F184$^{2.57b}$	D83$^{2.50a}$	I638$^{1.46c}$	A272$^{2.49f}$
	F591$^{2.58b}$	F454$^{2.58b}$	V185$^{2.58b}$	L84$^{2.51a}$	F639$^{1.47c}$	C273$^{2.50f}$
	T595$^{2.62b}$	F454$^{2.58b}$	S189$^{2.62b}$	F88$^{2.55a}$	Y642$^{2.50c}$	V276$^{2.53f}$
TM3	C612$^{3.29b}$	C475$^{3.29b}$	C224$^{3.29b}$	C110$^{3.25a}$	C657$^{3.29c}$	C314$^{3.25f}$
	W629$^{3.46b}$	W492$^{3.46b}$	W241$^{3.46b}$	S127$^{3.42a}$	A674$^{3.46c}$	W323$^{3.50f}$
	E633$^{3.50b}$	E496$^{3.50b}$	E245$^{3.50b}$	L131$^{3.46a}$	K678$^{3.50c}$	A327$^{3.54f}$
	L637$^{3.54b}$	L500$^{3.54b}$	L249$^{3.54b}$	R135$^{3.50a}$	I354$^{3.54c}$	W331$^{3.58f}$
TM4	Y666$^{4.50b}$	W524$^{4.50b}$	W272$^{4.50b}$	W161$^{4.50a}$	I714$^{4.40c}$	W365$^{4.50f}$
	S670$^{4.60b}$	V534$^{4.60b}$	W282$^{4.60b}$	P171$^{4.60a}$	L724$^{4.50c}$	I375$^{4.60f}$
EL2	C684$^{45.50}$	C562$^{45.50}$	C294$^{45.50}$	C187$^{45.50}$	C746$^{45.50}$	C390$^{45.50}$
TM5	P699$^{5.42b}$	L577$^{5.42b}$	P310$^{5.42b}$	V211$^{5.45x46a}$	Y759$^{5.46c}$	P407$^{5.50f}$
	I703$^{5.46b}$	V581$^{5.46b}$	A314$^{5.46b}$	P215$^{5.50a}$	L763$^{5.50c}$	V411$^{5.54f}$
	N707$^{5.50b}$	N585$^{5.50b}$	N318$^{5.50b}$	I219$^{5.54a}$	C767$^{5.54c}$	G415$^{5.58f}$
TM6	G750$^{6.50b}$	G620$^{6.50b}$	G359$^{6.50b}$	L262$^{6.45a}$	C795$^{6.47c}$	V463$^{6.47f}$
	W753$^{6.53b}$	W623$^{6.53b}$	E362$^{6.53b}$	W265$^{6.48a}$	W798$^{6.50c}$	T466$^{6.47f}$
	F755$^{6.55b}$	L625$^{6.55b}$	V364$^{6.55b}$	P267$^{6.50a}$	A800$^{6.52c}$	S468$^{6.49f}$
	G756$^{6.56b}$	I626$^{6.56b}$	F365$^{6.56b}$	Y268$^{6.51a}$	F801$^{6.53c}$	C469$^{6.50f}$
TM7	G779$^{7.50b}$	G651$^{7.50b}$	G393$^{7.50b}$	A299$^{7.46a}$	V823$^{7.40c}$	F526$^{7.46f}$
	Y783$^{7.54b}$	F655$^{7.54b}$	A397$^{7.54b}$	P303$^{7.50a}$	L827$^{7.44c}$	I530$^{7.50f}$
	H786$^{7.57b}$	Y658$^{7.57b}$	Y400$^{7.57b}$	Y306$^{7.53a}$	C829$^{7.46x47c}$	S533$^{7.53f}$
	L789$^{7.60b}$	M661$^{7.60b}$	L403$^{7.60b}$	M309$^{7.56a}$	P833$^{7.50c}$	V536$^{7.56f}$

Receptor-specific Uniprot numbers are coloured *grey*, and their Ballesteros-Weinstein residue numbers appended by the corresponding GPCR family as small letter are indicated as superscript (e.g. S558$^{1.50b}$, serine residue 558 of CD97 located at Ballesteros-Weinstein position 1.50 in family B GPCRs; N55$^{1.50a}$, asparagine residue 55 in bovine rhodopsin at position 1.50 in family A GPCRs). In addition to Ballesteros-Weinstein reference positions, conserved residues C$^{3.25a/f}$/C$^{3.29b/c}$, C$^{45.50}$ and Y$^{7.53a/7.57b}$ are indicated. Conserved residues (>45 %) between GPCR families are marked *orange*, while conserved reference residues for a specific family that is not conserved in other families are marked *cyan*

chapter by their Uniprot numbers (for specific receptors) as well as their Ballesteros-Weinstein/Wootten residue number and secondary structure motif (as superscript), according to IUPHAR guidelines [55] and secretin family GPCR residue numbering [51, 54] guidelines, respectively.

1.3 Aims of This Chapter

Assuming that the tethered agonist sequence directly influences the 7TM structure, no other endogenous ligands have been described that bind to the 7TM domain of aGPCRs. This absence of suitable 7TM ligands affects the progress of aGPCR crystallization efforts, and hence we are still waiting for the first crystal structure of aGPCR proteins. Until then, the aGPCR field relies on structure-based homology models based on different GPCR superfamily members to give insight in the presence and accessibility of potential aGPCR 7TM binding cavities.

As indicated in Sect. 1.2 and described in more detail in the following paragraph, aGPCRs share sequence similarity with the secretin GPCR family, which justifies the analysis and extrapolation of sequence-structure relationships from the crystal structures for this subfamily [45], CRFR1 [41] and GCGR [11, 40], as well as the wealth of mutation data available for the 7TM domain of secretin family receptors, in particular the regions that share sequence similarity with aGPCRs.

We therefore aim to make aGPCR structure-based sequence alignments, based on our expertise in both rhodopsin and secretin family GPCR structures. Moreover, based on the similarity with secretin family GPCRs, we will identify important/conserved amino acids (motifs) in the 7TM domain of aGPCRs. In addition, the well-established Ballesteros-Weinstein numbering scheme [50] for amino acid residues within GPCR TM helices will be introduced for aGPCR family members.

2 Conserved Structure-Sequence Relationships Between Adhesion, Secretin and Rhodopsin Family GPCRs

The following section provides a detailed description of conserved structural motifs in the 7TM domains of aGPCRs and secretin family GPCRs and comparison to functionally and structurally important sequence motifs in similar regions in the 7TM domains of rhodopsin, glutamate and frizzled family GPCRs, based on structure-based sequence alignments of the crystal structures of secretin (GCGR and CRFR1), rhodopsin, glutamate and frizzled family GPCRs [5] (Fig. 3). This GPCR crystal structure-based aGPCR to secretin family alignment is in line with the previous sequence alignment studies between a selection of aGPCRs (CTFs) and the secretin-like human calcitonin receptor sequence, leading to the identification of potentially conserved residues/motifs: TM3 (WMLxE$^{3.50b}$G), TM4 (GW$^{4.50b}$GxP), ECL2 (C$^{45.50b}$WL), TM5 (GPVxxN$^{5.50b}$), TM6 (LLG$^{6.50b}$) and TM7 (QG$^{7.50b}$xF, C$^{7.54b}$, V$^{7.64b}$) [17]. In a more extensive amino acid sequence alignment of aGPCR 7TM regions, involving all 33 human aGPCRs, three residues were conserved in all aGPCRs (i.e. H$^{3.37b}$ and W$^{3.46b}$) in TM3 and P$^{4.42b}$ in TM4 (Table 2, Fig. 3) [2].

2.1 Transmission Switch: TM3, TM5, TM6 and TM7

$W^{6.53b}$ in TM6 is conserved in aGPCRs and structurally aligned with the highly conserved $W^{6.48b}$ in the rhodopsin family and $W^{6.50c}$ in glutamate family GPCRs (Table 2, Fig. 3). In rhodopsin family GPCRs, $W^{6.48b}$ acts as a rotameric toggle switch [56] that controls the structural transduction of ligand-induced receptor signalling between the extracellular 7TM ligand binding site and the intracellular G-protein recognition site. The GCGR crystal structure shows that the TM3–TM6 interface in secretin family GPCRs (one helical turn below $W^{6.48b}$) contains large hydrophobic residues $Y/F^{3.44b}$, $M/L^{3.47b}$ and $L/F^{6.49b}$ that make similar hydrophobic interactions as structurally aligned $I/V/L^{3.40a}$, $L/I^{3.43a}$ and $F^{6.44a}$ residues present in most rhodopsin family GPCRs (Fig. 4b). aGPCRs contain similar large apolar residues ($F/L^{3.44b}$, $M/L^{3.47b}$, $L/F^{6.49b}$) at these positions. The GCGR crystal structures show that this interface (defined as receptor signal transmission switch in rhodopsin family GPCRs) [42, 57] is further stabilized in secretin family GPCRs by close contact between the conserved $Y^{3.44b}$ and $G^{6.50b}$, as well as a secretin family-specific inter-helical hydrogen bond between the conserved $N^{5.50b}$ and the backbone of $L^{3.47b}$ at the TM3–TM5 interface (Fig. 4b). The same or homologous

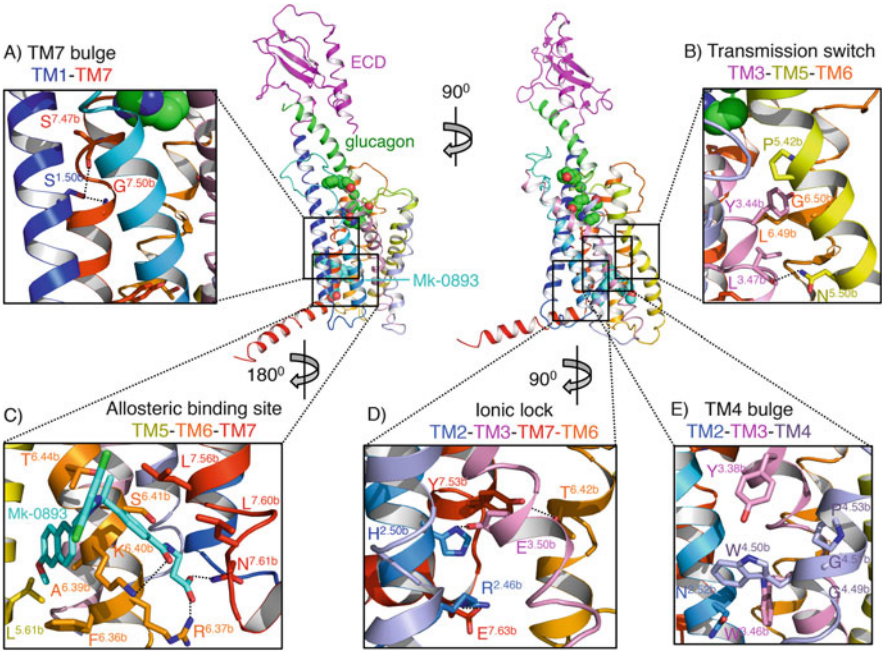

Fig. 4 Conserved structure-sequence features in secretin and adhesion family GPCRs, described in Sects. (**a**) 2.2, (**b**) 2.1, (**c**) 2.3 and (**d**) 2.4 and shown in the full-length glucagon-bound GCGR structure described and presented in Fig. 1 (**a**–**c** and **e**) [11–13] and the recently published small allosteric antagonist (Mk-0893)-bound GCGR structure [40] (**d**). Family B Ballesteros-Weinstein numbers and sequence alignments are defined in Fig. 3

residues involved in these contacts are also present in aGPCRs (e.g. F625$^{3.44b}$, M628$^{3.47b}$, N707$^{5.50b}$ and G750$^{6.50b}$ in CD97), suggesting that similar interaction networks are present in the 7TM of aGPCRs (Fig. 3). Moreover, the conserved P$^{5.42b}$ in secretin and adhesion family members (position 5.46a in rhodopsin family) and P$^{5.50a}$ in rhodopsin-like GPCRs (position 5.46b in secretin family) stabilize a similar bulge in TM5 that can mediate this conformational switch.

2.2 TM7 Bulge: TM1 and TM7

A serine residue (S$^{1.50b}$) in TM helix 1 (TM1) is conserved in most aGPCRs, which is also conserved in secretin family GPCRs (e.g. S558$^{1.50b}$ in CD97, Table 2, Fig. 3). In the crystal structures of GCGR and CRFR1, S$^{1.50b}$ interacts with the backbone of TM7 at S$^{7.47b}$, G$^{7.50b}$ and F/L$^{7.51b}$, thereby stabilizing the bulge in the TM7 helix of secretin family GPCRs [11] (Fig. 4a). In rhodopsin family GPCRs, the kink in TM7 is located one helical turn lower by a conserved P$^{7.50a}$ residue (aligned with position 7.54b in aGPCRs). The importance of S$^{1.50b}$ and S$^{7.47b}$ is consistent with mutation studies of secretin-like GPCRs showing that mutation of these residues alters receptor signalling [51]. S$^{1.50b}$ likely plays a similar structural role in aGPCRs by interacting in a similar fashion with the backbone of the small polar residue at position 7.47b (A/S/T) and the conserved G$^{7.50b}$ (e.g. G779$^{7.50b}$ in CD97). Most aGPCRs furthermore contain a medium-sized polar residue (N/E/D/H/Q) at position 2.61b (aligned with position 2.54 in rhodopsin family GPCRs) that may participate in this polar interaction network between TM1 and TM7 (Fig. 3). The only four aGPCRs that do not contain a serine residue at position 1.50b contain alternative small polar residues that can mediate similar H-bond networks, namely, C$^{1.50b}$ [GPR123 (ADGRA1), GPR124 (ADGRA2), GPR125 (ADGRA3)] and T$^{1.50b}$ [CELSR2 (ADGRC2)]. Position 1.50b is structurally aligned to position 1.46, which is a Gly residue in most family A GPCRs [58]. G$^{1.46a}$ is located one helical turn before the conserved residue N$^{1.50a}$ that controls rhodopsin family GPCR signalling by forming an H-bond network with N$^{7.49a}$ in TM7. N$^{7.49a}$ is structurally aligned with position 7.53b in aGPCRs (Fig. 4c) located one helical turn lower than the conserved G$^{7.50b}$ residue.

2.3 Ionic Lock: TM2, TM3, TM6, TM7 and Helix 8

At the intracellular side of the 7TM domain, H$^{2.50b}$ and E$^{3.50b}$ are conserved in secretin family GPCRs and present in most aGPCRs (e.g. H583$^{2.50b}$ and E633$^{3.50b}$ and CD97, Table 2, Fig. 3). These residues form an H-bond network within GCGR and CRFR1 crystal structures [11, 41] (Fig. 4c). Similar polar interaction networks are present in distinct, but closely located regions in rhodopsin family GPCRs, including (1) a water-mediated, sodium-stabilized H-bond interaction between D$^{2.50a}$ and N$^{7.49a}$ [59], structurally aligned with hydrophobic residues L/F$^{2.57b}$ and I/V$^{7.53b}$ that form a hydrophobic interface between TM2 and TM7 in

secretin family GPCRs and (2) an ionic H-bond interaction network between $R^{3.50a}$ and $D/E^{6.30a}$, aligned with variable residues at positions 3.54b and 6.35b that do not form interactions in GCGR and CRFR1 crystal structures. $Y^{3.55b}$ in TM3, a part of a conserved $E^{3.50b}XXXXY^{3.55b}$ motif in aGPCRs, which is present in 21 out of 33 human aGPCRs [2], is however aligned with $Y^{3.51a}$ which is part of a conserved $D/ER^{3.50a}Y^{3.51a}$ motif in rhodopsin family GPCRs [60] (Fig. 3). Glutamate-like GPCRs contain a conserved charged lysine residue ($K^{3.50c}$) in TM3 that is structurally aligned with the charged $E^{3.50b}$ residue in secretin family GPCRs and most aGPCRs and forms an ionic/H-bond interaction network with a conserved glutamate residue in TM6 ($E^{6.35c}$) in mGluR1 [43] and mGluR5 crystal structures [61], which resembles the intracellular ionic locks observed in rhodopsin ($R^{3.50a}$ and $D/E^{6.30a}$) and secretin ($E^{3.50b}$ and $H^{2.50b}$, present in most aGPCRs) family crystal structures. In the secretin family GCGR crystal structure, $Y400^{7.57b}$ forms an H-bond with $T351^{6.42b}$ and $E245^{3.50b}$ (Fig. 4c) in a conformation that rhodopsin family GPCRs is linked to activation and interaction with the G protein via the structurally aligned $Y^{7.53a}$ residue at the intracellular end of TM7 [62–64]. Seven aGPCRs contain the same Tyr residue at position 7.57b, while 16 other aGPCRs contain a homologous Phe (2) or His (14) residue at this position (Fig. 3). For rhodopsin family GPCRs, the role of $Y^{7.53a}$ became evident from mutagenesis studies [65, 66] and the crystal structure of the active state rhodopsin in complex with a peptide derived from the C-terminus of the Galpha subunit [64]. In the GCGR crystal structure [11] $E406^{7.63b}$ located at the start of helix 8 forms an H-bond network with $R173^{2.46b}$ (Fig. 4c), a residue that is conserved in secretin family GPCRs and present in about half of the aGPCRs. $R173^{2.46b}A$ is one of the eleven thermostabilizing mutations in another GCGR crystal structure [40]. Seven human aGPCRs contain a positively charged residue at position 2.46b and a negatively charged residue at the position aligned with $E406^{7.63b}$ in GCGR (i.e. five positions from the conserved $C^{7.58b}$ residue), namely, ADGRB1–3 (BAI1–3), ADGRD1–2 (GPR133, GPR144) and ADGRA1–2 (GPR123, GPR124), suggesting that a similar H-bond network may be present in these aGPCRs.

2.4 TM4 Bulge: TM2, TM3 and TM4

TM4 of aGPCRs and secretin family GPCRs contains a conserved $GW/Y^{4.50b}GxP$ motif, at the same position as the highly conserved $W^{4.50a}$ in rhodopsin family GPCRs (Fig. 3) that is proposed to form a cholesterol binding site [67]. Despite structural differences in the $GW^{4.50b}GxP$ region in the GCGR and CRFR1 crystal structures, all structures show that $W^{4.50b}$ stabilizes the TM4 bulge by interacting with $Y^{3.38b}$, $W^{3.46b}$ and $N^{2.52b}$, and identical/homologous residues are conserved in most aGPCRs (e.g. $F484^{3.38b}$, $W492^{3.46b}$, $N446^{2.52b}$ in GPR56, Table 2, Fig. 4d). The interaction sites of $W^{4.50b}$ with TM2 and TM3 are located close to residues that are part of the transmission switch ($M/L^{3.47b}$, Fig. 4b) and ionic lock ($H^{2.50b}$, Fig. 4c) in secretin and adhesion family GPCRs.

2.5 Extracellular Loops

The extracellular loops 1 (ECL1) and 3 (ECL3) of aGPCRs are of similar short length as the ECL1 and ECL3 of glutamate family GPCRs [43], but significantly shorter than the ECL1 of rhodopsin family GPCRs [58], secretin-like receptors (including CRFR1 [41] and GCGR [11]) and frizzled [44] family GPCRs (Fig. 3). All GPCR families contain a disulphide bond between a cysteine residue in extracellular loop 2 ($C^{45.50}$) and a cysteine residue in TM3 ($C^{3.29b}$ in aGPCRs and secretin family GPCRs (Table 2), $C^{3.25a}$ in rhodopsin, $C^{3.29c}$ in glutamate and $C^{3.25f}$ in frizzled family GPCRs) [53]. It should be noted however that several GPCRs do not contain such disulphide bond [53], including the crystallized S1P1 and LPA1 receptors which form an internal disulphide bridge in ECL2 [68, 69]. The length of the ECL2 region upstream from $C^{45.50}$ (i.e. from TM4 to the disulphide bridge) is at least two residues longer in aGPCRs, and also the downstream ECL2 length (i.e. from the disulphide bridge to TM5) is variable between secretin family and adhesion family members. Especially ADRGR1–7 has significantly longer (upstream in most cases, also downstream) ECL2 length than the GCGR and CRFR1 crystal structure templates (Fig. 4). Nevertheless $W^{45.51}$, located one position downstream from $C^{45.50}$, is conserved between secretin family and adhesion family GPCRs, and this residue plays a role in peptide ligand binding (and/or structural integrity of ECL2) in several secretin family GPCRs [11, 45]. As Fig. 3 shows, we can indeed observe that the ECL2 sequence length of aGPCRs is quite comparable to the one of secretin receptors and contains a cysteine residue at a position comparable to CRFR1 and GCGR. As this cysteine is forming a disulphide bond to $C^{3.50a}$ and $C^{3.50b}$, it introduces an important structural constraint for the folding of ECL2. However, several members of the family exhibit extended lengths of ECL2, while still containing only a single cysteine.

3 Druggability of aGPCRs

Rational design of aGPCR ligands that interact with a 7TM binding cavity relies on good receptor models. As proven in the last decades, rhodopsin family members have been successfully targeted by a variety of molecules binding to either orthosteric or allosteric binding sites in the 7TM bundle. Likewise, glutamate GPCRs that possess a large extracellular Venus flytrap domain can be allosterically targeted in their 7TM regions. In previous years, aGPCRs have been successfully targeted (at their extracellular domains) with aGPCR-specific antibodies. Interestingly, some of these antibodies modulate aGPCR functioning, resulting in therapeutic relevant outcomes: monoclonal antibodies against the NTF of ADGRE1 (EMR1) [70] and ADGRE5 (CD97) [71] are affective in eosinophilic disorders and inflammatory disease models, respectively. Thus far, only ADGRG3 (GPR97) was shown to be activated by a drug molecule (i.e. the corticosteroid beclomethasone dipropionate) [23]. Improved insights in aGPCR 7TM organization, structure and potential ligand interaction sites will form the basis to target aGPCRs 7TM regions

in a pharmacological relevant manner. As presented in Sects. 1 and 2, secretin family GPCRs represent a template for the structural fold of aGPCRs and hence are considered as a useful structural template to characterize the 7TM binding pocket of aGPCRs as described below.

3.1 Comparison of 7TM Pocket of Secretin Family GPCRs and aGPCRs

Mutations of residues at positions $Y^{1.43b}$, $Y/H^{1.47b}$, $R/K^{2.60b}$, $V^{2.46b}$, $I/K^{2.67b}$, $D^{2.68b}$, $L/V^{2.71b}$, $Q^{3.37b}$, $W^{45.51}$, $F/Y/I/T^{5.35b}$, $W^{5.36b}$, $I^{5.38b}$, $R^{5.40b}$, $E^{6.53b}$, $F^{6.56b}$, $R^{7.35b}$, $L^{7.39b}$, $D^{7.42b}$ and $L^{7.43b}$ have been shown to affect peptide ligand binding and/or potency in multiple secretin family GPCRs, including GCGR [13, 72–74], CRFR1 [41, 75] GLP-1 [13, 76–80], GIP [81, 82], secretin [83, 84], $VPAC_1$ [85, 86] and PTH1 [87, 88] receptors (Fig. 3). These residues line the proposed peptide ligand binding site in the 7TM of GCGR and CRFR1 crystal structures [11, 41] and are expected to line the 7TM binding site of aGPCRs as well based on conservation of residues that determine the TM fold (e.g. $G^{1.46b}$, $G^{7.49b}XGXP$, $P^{5.46b}$, $G^{6.50b}$, $G^{7.50b}$). In most aGPCRs, large-/medium-sized residues at position 1.44b (Y/W/H/N/Q) may also be accessible from this binding site, as this residue is located next to a small residue (T/S) at position 1.43b. In the same way $E/N/D/Q^{2.61b}$ may be accessible from the 7TM pocket of aGPCRs as this residue is located next to the relatively small $A/S^{2.62b}$, while $L/V/I^{6.57b}$ located next to $G/A^{6.56b}$ may be accessible from the aGPCR 7TM pocket as well. Several of the polar residues that line the 7TM binding site of secretin family GPCRs are hydrophobic residues in most aGPCRs ($A/L^{2.60b}$, $I^{2.68b}$, $I/V/L/A^{5.40b}$, $W^{6.53b}$, $F^{7.42b}$), while there are no conserved hydrophobic binding pocket residues in secretin family GPCRs that are polar residues in most aGPCRs (Fig. 3). Assuming that aGPCRs and secretin-like receptors share a similar large and open 7TM binding site (Figs. 1–4), the putative 7TM pockets of aGPCRs contain less buried polar groups than in related secretin-like receptors. The implications of this with respect to druggability of the 7TM pocket of aGPCRs are discussed in Sect. 3.2.

3.2 Druggability 7TM Pocket of aGPCRs

Despite the lack of sequence conservation, comparison of secretin family members CRFR1 and GCGR structures with those of rhodopsin, glutamate and frizzled family GPCR shows that the orientations and positions of TM helices are conserved [11, 40, 41]. This common fold is stabilized by similar contacts between TM helices in both families, but involves distinct patterns of conserved residues in rhodopsin family [42] and secretin family [11] GPCRs. The distances between the extracellular ends of TM2 and TM7 and TM3 and TM7 of CRFR1 (PDB, 4K5Y) and GCGR (PDB, 4L6R, 5EE7) are however (among) the largest observed in GPCR structures. Consequently, the orthosteric 7TM pockets of these two receptors are wider and deeper than those of any rhodopsin family member [11]. In addition, several

residues located deep in the orthosteric pocket between TM1 (1.47b) and TM2 (2.60b) play an important role in peptide ligand binding in secretin family GPCRs, while the corresponding structurally aligned residues (positions 1.43 and 2.53) are *not* involved in ligand binding in rhodopsin family receptors. Figure 5 shows a comparison of the 7TM pocket of the available secretin family GPCR crystal structures and two representative rhodopsin family GPCRs, CXCR4 and H_1R. The CXCR4 structure is the only GPCR crystal structure in which a (non-peptide) ligand (IT1t) binds only in the subpocket between TM1–3 and TM7 (PDB, 3ODU) [48] that is also primarily targeted by the chemokine ligand vMIP-II (PDB, 4RWS) [89]. The peptide ligand CVX15 targets an overlapping but distinct binding site region in CXCR4 located between TM3 and TM7 (PDB, 3OE0) [48], which is occupied by non-peptide ligands in all other rhodopsin family GPCR crystal structures [42, 90] and is also targeted in the ligand-bound 7TM crystal structures of glutamate family GPCRs (mGluR1, mGLuR5) [43, 61, 91] and frizzled family GPCRs (SMO) (Fig. 3) [44, 92]. The H_1R ligand doxepin binds in a pocket that is located closest to the cytoplasm of all the ligand binding sites observed in rhodopsin family GPCR crystal structures (PDB, 3RZE) [47]. The crystal structure of CRFR1 revealed an unexpected small-molecule binding pocket located in the cytoplasmic half of the receptor, more than 7 Å further down than the doxepin binding site in the rhodopsin family GPCR H_1R (Fig. 5). CP-376395 binds in this druggable site defined by residues of TM3, TM5 and TM6 showing a combination of hydrophobic and hydrophilic features compatible with drug-like small organic molecules. In this region, the sequence identity in secretin family GPCRs is remarkably high (Fig. 3). Of the 14 residues directly interacting with CP-376395, 7 are identical in CRFR1 and GCGR, and 8 are conserved/similar among aGPCRs. Among them is $N^{5.50b}$, which forms an essential hydrogen bond with the ligand, while CRFR1 and most aGPCRs share homologous residues at positions $Y/F^{3.44b}$, $M^{3.47b}$, $V/I^{5.46b}$, $L/V/I^{5.47b}$, $L^{6.45b}$, $L^{6.46b}$, $L^{6.49b}$ and $G^{6.50b}$ which provide hydrophobic interactions with the antagonist. A second recently solved crystal structure of GCGR revealed another unexpected allosteric small-molecule binding pocket in the secretin family of GPCRs at an interface between the cytoplasmic end of TM5, TM6, TM7 and the membrane bilayer (Figs. 4d and 5) [40]. In this GCGR crystal structure (PDB, 5EE7), the small-molecule allosteric antagonist Mk-0893 forms H-bonds with the side chains of residues $R346^{6.37b}$, $K349^{6.40b}$, $S350^{6.41b}$ and $N404^{7.61b}$ and makes apolar interactions with $L329^{5.61b}$, $F345^{6.36b}$, $A348^{6.39b}$, $K349^{6.40b}$, $L352^{6.43b}$, $T353^{6.44b}$, $L399^{7.56b}$, $L403^{7.60b}$ and four co-crystallized oleic acid molecules. Mk-0893 and chemically similar GCGR antagonists (large, hydrophobic, negatively charged) are proposed to adopt similar binding modes and prevent glucagon-induced activation of GCGR by restriction of the outward helical movement of TM6 required for G-protein coupling [40]. Most of the residues comprising this shallow pocket at the membrane interface at the outside of GCGR are not conserved among aGPCRs (Fig. 3). Several aGPCRs share $N^{7.61b}$ that forms an H-bond with the carboxylic acid moiety of Mk-0893 in GCGR, and few aGPCRs contain $K^{6.40b}$ that forms an H-bond with the amide carbonyl and

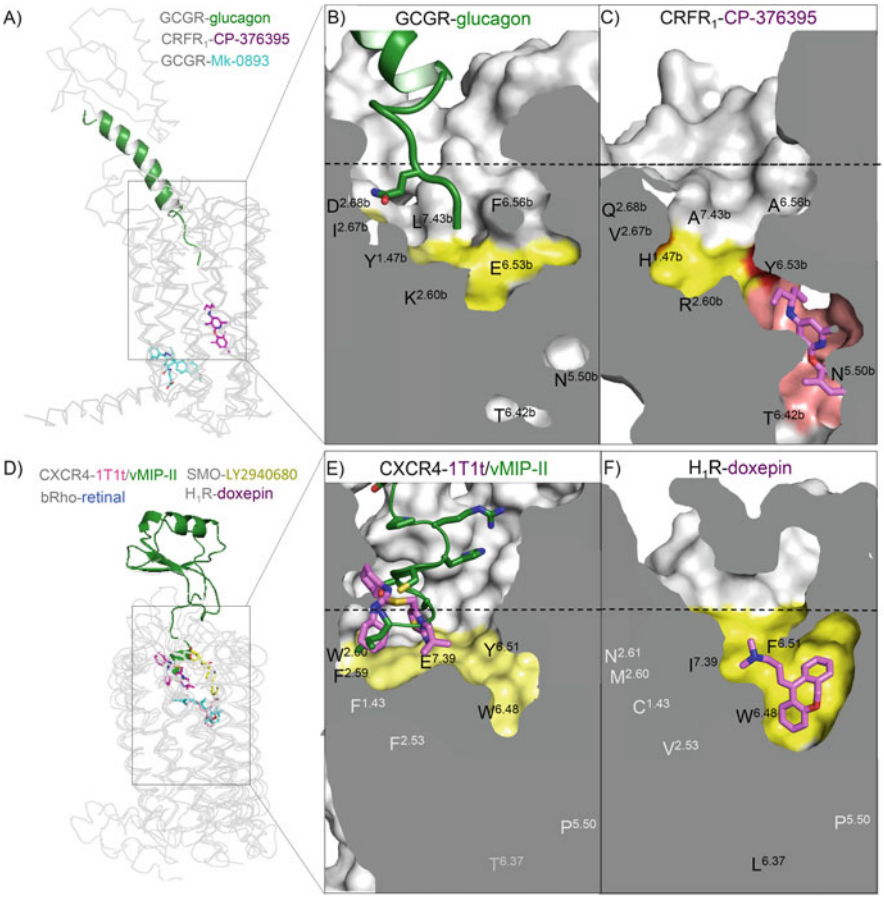

Fig. 5 Structural alignments of (**a**) secretin family (GCGR [11, 40] and CRFR1 [41]) and (**d**) rhodopsin (bRho [46], H_1R [47], CXCR4 [48, 88]), frizzled (SMO [44]) and glutamate family (mGluR1 [43]) crystal structures, and comparison of druggable binding sites of (**a**) glucagon receptor (GCGR; PDB, 4L6R), (**b**) corticotropin-releasing factor receptor 1 (CRFR1; PDB, 4K5Y), (**e**) CXC-chemokine receptor 4 (CXCR4; PDB, 3ODU, 4RWS) and (**f**) histamine H_1 receptor (H_1R; PDB, 3RZE). GCGR and CRFR1 are secretin family GPCRs that share sequence similarity and structural fold with aGPCRs (see Fig. 3 for corresponding sequence alignment). The surfaces of binding sites that are considered druggable are coloured *yellow* (orthosteric pocket) and salmon (CP-376395 binding pocket). Of nine structurally aligned residues, the residues that line the binding pocket shown in the figures are labelled *black*; the residues that are not part of the depicted pockets are labelled *light grey*. The approximate position of the extracellular membrane boundary is shown as a *dashed black line*. The binding mode of glucagon is derived from an experimentally validated full-length secretin family GCGR structure combining structural and experimental information from the GCGR 7TMD crystal structure (PDB, 4L6R), the GCGR ECD structure (PDB, 4ERS) and the ECD structure of GLP-1-bound GLP-1R (PDB, 3IOL), complemented by site-directed mutagenesis, electron microscopy, HDX and cross-linking studies [11–13]. Carbon atoms of peptide ligands (glucagon in the full-length GCGR model and vMIP-II in the CXCR4 crystal structure) and non-peptide ligands (IT1t in the CXCR4 crystal structure and doxepin in the H_1R crystal structure) are coloured *green* and *magenta*, respectively

makes apolar contacts with the naphthalene and phenylacetamide moieties of Mk-0893. None of the aGPCRs however combines these residues with a positively charged (R/K) and a small polar residue (S/T/N) at positions 6.37b and 6.41b ($R346^{6.37b}$ and $S350^{6.41b}$ in GCGR) that is required to allow the negatively charged beta-alanine group of Mk-0893 to target the intracellular pocket of GCGR. GPR110, GPR111 and GPR115, for example, share $R^{6.37b}$ and $K^{6.40b}$ in combination with $S/N^{6.41b}$ but contain a negatively charged D/E residue aligned with $N404^{7.61b}$ of GCGR. Although it is unlikely that aGPCRs can be targeted via the same ligand binding mode observed in the Mk-0893-bound GCGR crystal structure, similar hydrophobic, charged allosteric binding sites at extrahelical interface with the membrane may be accessible in aGPCRs as well. The buried orthosteric site of H_1R represents an excellent example of a druggable pocket, whereas the open binding region of CXCR4 is more challenging from a drug design perspective (Fig. 5) [93]. The orthosteric sites of both GCGR and CRFR1 are similar to CXCR4 and open and mostly occupied by bulk-like solvent, with only a single druggable site at the bottom of the pocket. This hotspot in the 7TM pocket of secretin family GPCRs is lined with residues that play an important role in peptide ligand binding, representing a mix of polar (e.g. $Y^{1.47b}$, $R/K^{2.60b}$, $E/Y^{6.53b}$, $D/N^{7.42b}$) and apolar ($I/V^{2.67b}$, $F^{6.56b}$, $L^{7.43b}$) residues (Fig. 4). In contrast, aGPCRs contain only hydrophobic ($I/L/V^{1.47b}$, $I/L^{2.60b}$, $W^{6.53b}$, $F^{7.42b}$) or smaller ($G/A^{2.68b}$, $T/A^{6.56b}$, $T/A^{7.43b}$) residues at the positions surrounding these hotspots and lack other conserved polar interaction sites in the 7TM pocket, suggesting that this binding pocket is even less druggable than in secretin family GPCRs.

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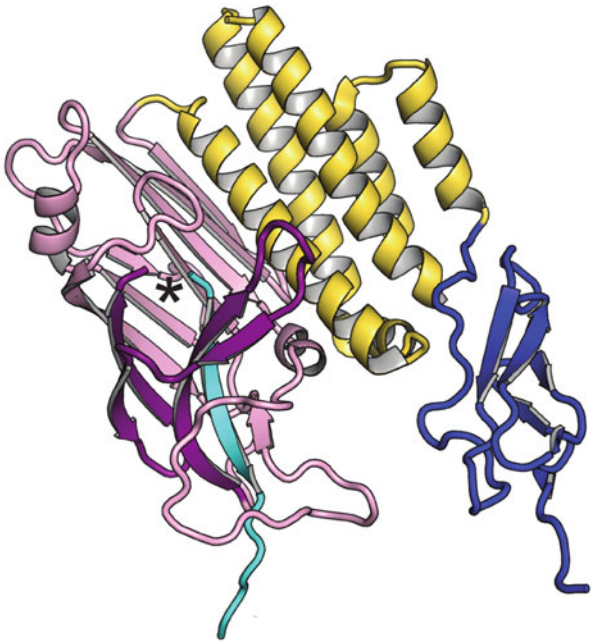
Understanding the Structural Basis of Adhesion GPCR Functions

Demet Araç, Norbert Sträter, and Elena Seiradake

Graphical Abstract



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Contents

Abstract

Unlike conventional G-protein-coupled receptors (GPCRs), adhesion GPCRs (aGPCRs) have large extracellular regions that are autoproteolytically cleaved from their membrane-embedded seven-pass transmembrane helices. Autoproteolysis occurs within the conserved GPCR-Autoproteolysis INducing (GAIN) domain that is juxtaposed to the transmembrane domain and cleaves the last beta strand of the GAIN domain. The other domains of the extracellular region are variable and specific to each aGPCR and are likely involved in adhering to various ligands. Emerging evidence suggest that extracellular regions may modulate receptor function and that ligand binding to the extracellular regions may induce receptor activation via multiple mechanisms. Here, we summarize current knowledge about the structural understanding for the extracellular regions of aGPCRs and discuss their possible functional roles that emerge from the available structural information.

Keywords

GAIN • Horm • Olfactomedin • Lectin • Stachel • Tethered agonist • Unc5 • FLRT • Super-complex • Latrophilin

1 Introduction

The aGPCR ectodomain consists of an N-terminal region with a family-specific set of domains, which probably mediate interactions with binding partners, followed by a conserved region that in almost all aGPCRs contains a GPCR-Autoproteolysis INducing (GAIN) domain just upstream of the seven-transmembrane helix (7TM) domain (Fig. 1) [1]. Direct experimental structural information on the architecture and mode of action of aGPCR ectodomain is still scarce. However, crystal structures are available for the hormone receptor (HormR) and GAIN domains of B3 and L1 [1]. Conformational changes induced by ligand binding must be transmitted to the 7TM domain. This is most likely the crucial function of the GAIN

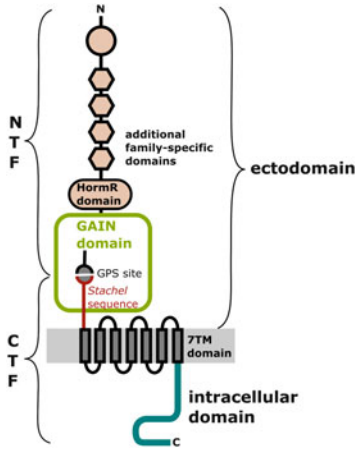

Fig. 1 Scheme of the architecture of a prototypical aGPCR. The GAIN domain contains an autoproteolytic cleavage site (GPS site), resulting in an N-terminal fragment (NTF) and a C-terminal fragment (CTF). As the N-terminal part of the *Stachel* sequence (sequence between the GPS site and the start of the 7TM domain) is tightly bound to the GAIN domain, NTF and CTF do not dissociate. The HormR domain is present in many, but not all, aGPCRs

domain. Liebscher et al. [2] demonstrated that the sequence stretch after the GPS autoproteolytic cleavage site of the GAIN domain activates the GPR126 (ADGRG6) and GPR133 (ADGRD1) receptors in a sequence-specific manner if the rest of the GAIN domain is missing. This sequence stretch has been designated as the *Stachel* sequence. In addition, peptides corresponding to the *Stachel* sequence could also activate variants of these receptors lacking the GAIN domain and *Stachel* sequence with sequence specificity but relatively low affinity. A similar mechanism of activation by the region following the GPS cleavage site was also demonstrated for ADGRG1 (GPR56), ADGRF1 (GPR110), ADGRG5 (GPR114), and ADGRG2 (GPR64) [3–5]. These studies demonstrated that aGPCRs are activated by a tethered agonist which resides in the GAIN domain.

Latrophilin is the only aGPCR whose N-terminal ligand-binding domains have been determined structurally. Nuclear magnetic resonance (NMR) models of the N-terminal lectin domain [6], a recent crystal structure of lectin and olfactomedin domains [7] and first ligand-complex structures [8–10], have provided exciting first insights into the structural repertoire of the N-terminal aGPCR domains. These suggest that the N-terminal domains of latrophilin have at least two distinct functions: They allow tethering of the ectodomain to specific extracellular ligands through high-affinity protein-protein interaction. In addition, they encode a structural mechanism for ligand-induced receptor clustering, thereby controlling the multimerization state of the aGPCR latrophilin. In the following, we summarize the key features of the available crystal structures of aGPCR ectodomains as well as some insights obtained from sequence comparisons to related structurally known domain folds.

2 The GAIN Domain

2.1 A Conserved Domain in All aGPCR Extracellular Regions

Unlike other GPCRs, aGPCRs have large extracellular regions that are autoproteolytically cleaved from their seven-pass transmembrane regions at a conserved GPCR proteolysis site (GPS) [11, 12] (see also [13]). The GPS is well described and recognized as a vital unit for receptor function [14–16]. However, a recent study showed that—as opposed to the previous belief—the GPS does not constitute a functional folded domain by itself, but rather is an integral part of a much larger novel domain that is termed the GPCR-Autoproteolysis INducing (GAIN) domain [1]. Of great importance, the tethered agonist *Stachel* peptide resides within the GAIN domain (see also [17, 18]).

Strikingly, the GAIN domain is shared by all human aGPCRs (except GPR123/ADGRA1 which has only a short sequence of ~20 residues before the first transmembrane helix). GAIN domain is unique in that it is the only domain that exists in all members of the aGPCRs in humans indicating an essential role in aGPCR function. Moreover, database searches revealed that all five members of another human protein family, the polycystic kidney disease proteins, an unrelated family of membrane proteins that are also autoproteolyzed, contain GAIN domains. In addition, primitive organisms, such as *Dictyostelium discoideum* that arose early in evolution before animals emerged, encode GAIN domains although they lack most other autoproteolytic domains, important adhesion and signaling domains, and critical signaling pathways. Among all domains that are found in aGPCRs, LRR (leucine-rich repeats), EGF (epidermal growth factor), and TSP (thrombospondin) are the only other domains that exist in such primitive organisms. These results showed that the GAIN domain is widespread and conserved in higher eukaryotes as well as in ancient organisms. Intriguingly, analysis of numerous sequences also revealed that the GAIN domain always immediately precedes the first transmembrane helix by a short linker of about seven residues raising the question whether the GAIN domain regulates receptor signaling via intramolecular interactions with the nearby transmembrane helices.

2.2 Structure of the GAIN Domain

The crystal structures of GAIN domains from two distantly related aGPCRs, latrophilin 1 (LPHN1/ADGRL1) and brain angiogenesis inhibitor 3 (BAI3/ADGRB3), revealed a novel fold that was previously unidentified. The GAIN domain (of LPHN1) contains an N-terminal subdomain A that is composed of six alpha helices and a C-terminal subdomain B that is composed of a twisted beta sandwich including 13 beta strands and two small helices (Fig. 2). The last five beta strands of subdomain B constitute the GPS motif. The GPS motif is the most conserved region of the GAIN domain (and thus it was chronologically noticed first and was mistakenly called a domain). The conservation of primary sequence of the

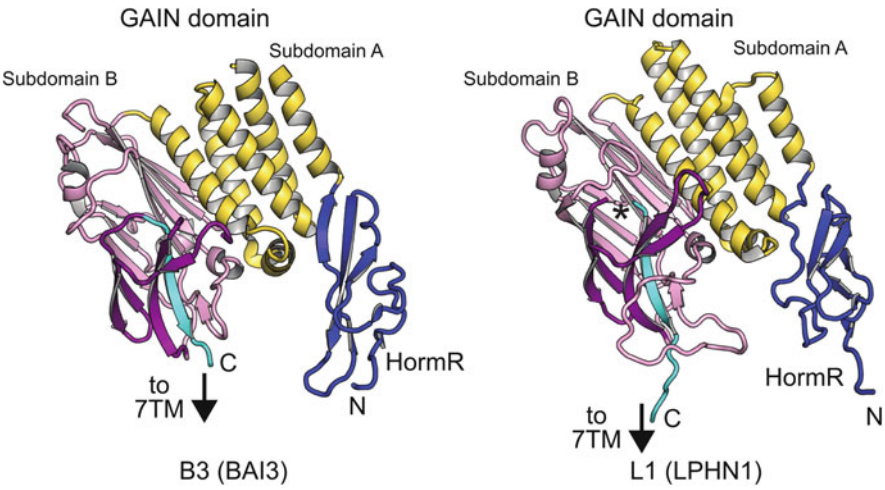

Fig. 2 Atomic structures of the GAIN and HormR domains. Cartoon representations of the uncleaved ADGRB3 GAIN and HormR domains (*left*, PDB ID 4DLO) and the cleaved ADGRL1 GAIN and HormR domains (*right*, PDB ID 4DLQ) revealed the novel GAIN domain fold. GAIN subdomain A and subdomain B are colored *yellow* and *light-pink*, respectively. The GPS motif (*magenta*) is an integral part of subdomain B. *Stachel* peptide (*cyan*) corresponds to the last beta strand of the GAIN domain and is deeply buried within the GAIN domain. Autoproteolysis (indicated by *asterisk*) occurs between the last two beta strands of the GAIN domain cleaving, but not releasing the *Stachel* peptide. The HormR domain (*blue*) has a rigid orientation relative to the GAIN domain in both ADGRL1 and ADGRB3

GAIN domains increases from the N-terminus to the C-terminus. In spite of the low sequence identity between LPHN1 and BAI3 GAIN domains (24 %), the very high similarity of the GAIN domain structures indicate that the three-dimensional structure is conserved more strictly than primary sequence throughout evolution.

2.3 Autoproteolysis Cleaves Off the *Stachel* Peptide But Does Not Release It from the GAIN Domain

GAIN domain is an autoproteolytic fold that is both required and sufficient for autoproteolysis, whereas the GPS motif without the rest of the GAIN domain is not functional. Autoproteolysis occurs in the short loop between the last two beta strands of the GAIN domain and cleaves the C-terminal beta strand from the rest of the domain. Indeed, the C-terminal beta strand of the GAIN domain corresponds to the *Stachel* peptide and acts as the tethered agonist that activates the aGPCR (Fig. 2). However, autoproteolysis does not cause the dissociation of the *Stachel* peptide from the rest of the GAIN domain, and it is unclear how it can be exposed to the transmembrane region to activate the receptor. The *Stachel* peptide is highly conserved and has a hydrophobic nature (TNFAVLM in LPHN1) and is involved in

approximately 15 strong hydrogen bonds and numerous hydrophobic interactions that tightly keep it in the GAIN domain. It is suggested that mechanical force applied on the extracellular domains may lead to shedding of the extracellular regions and release of the *Stachel* peptide from the GAIN domain enabling the *Stachel* peptide to activate the receptor (see also [17–20]).

2.4 Possible Roles of the GAIN Domain in aGPCR Activation

It is possible that, in addition to modulating *Stachel* peptide exposure to the transmembrane region, the GAIN domain is involved in more complicated mechanisms for regulating aGPCR function. Analysis of disease-causing mutations in different GAIN domain-containing proteins revealed that GAIN domains are hot spots for disease mutations. The GAIN domains of CL/BAI, polycystic kidney disease, and GPR56 and CL3 proteins are mutated in cancer [21], polycystic kidney disease, bilateral frontoparietal polymicrogyria, and attention deficit hyperactivity disorder [22], respectively. Monitoring the effect of polycystic kidney disease and cancer mutations on autoproteolysis and plasma membrane localization (thus presumably proper protein folding) of the receptors suggested that the mechanisms by which GAIN domain mutations lead to different diseases might be various. Thus, the GAIN domain might be involved in many functions in addition to autoproteolysis such as binding to other proteins, regulating receptor signaling via intramolecular interactions with the transmembrane helices, etc. (see also [19]).

The extracellular regions of several aGPCRs were suggested to regulate aGPCR activation. For example, the deletion of the N-terminal fragment (NTF) of GPR56 exhibits enhanced signaling ability when compared to the wild-type GPR56, and thus, the extracellular region is proposed to have an inhibitory role on receptor activation [23, 24]. The observation that the GPR126 GAIN domain binds to laminin suggests that GAIN domain functions as a protein-binding domain in addition to an autoproteolytic domain pointing to a multipurpose role in aGPCR function. However, other mechanisms by which aGPCRs are regulated and which domains of their very large extracellular regions are involved in their downstream signaling are unclear.

3 The HormR Domain

The hormone receptor (HormR) domain is the second most frequently observed domain in aGPCRs (found in 12 out of 33 human aGPCRs, specifically in LPHN1–3, GPR124/ADGRA2, GPR125/ADGRA3, CELSR1–3/ADGRC1–3, GPR113/ADGRF3, BAI1–3/ADGRB1–3). HormR domain-containing aGPCRs are homologous to family B GPCRs that always include an HormR domain. The crystal structures show that the HormR domains of LPHN1 and BAI3 are ~70-residue domains formed by two antiparallel β-sheets with conserved disulfide

bonds and tryptophan residues (Fig. 2) and yielded a low RMSD (1.1 Å) in spite of the low sequence identity (24 %). Since no hormone ligand has yet been found for aGPCRs, it has been believed that the HormR domain in aGPCRs may not be a true hormone-binding region. Remarkably, a DALI search revealed that N-terminal hormone-binding extracellular domain of the corticotrophin-releasing factor receptor (CRFR) (PDB ID: 3EHU) is strikingly similar to the HormR domains of LPHN1 and BAI3 yielding RMSDs of 0.7 Å and 1.1 Å, respectively, in spite of the low sequence identity (29 %) [25]. The unusually high structural similarity of the HormR domains of LPHN1 and BAI3 to that of the CRF receptor raised the possibility that aGPCRs can be real hormone receptors.

Superposition of the LPHN1 structure with the structure of the CRFR HormR domain bound to corticotrophin-releasing factor (CRF), a 41-amino acid peptide hormone, showed that a similar hormone could not bind to LPHN1 HormR because the GAIN domain is blocking the homologous hormone-binding site on the HormR domain of LPHN1 [1]. Clearly, hormone binding would require a conformational change in LPHN1 to expose the putative hormone-binding site (such as the reduction of one of the conserved disulfide bonds to switch to an open conformation). In family B GPCRs, the HormR domain precedes the transmembrane helices and is juxtaposed to the membrane. According to the two-domain model of family B GPCR activation, the interaction of the extracellular hormone-binding domain with the hormone promotes the interaction of the hormone with the transmembrane helices, leading to the activation of the receptor [26]. However, the mechanism of aGPCR activation upon hormone binding may be different, because the GAIN domain lies between the HormR domain and the transmembrane helices.

4 Olfactomedin and Lectin Domains of Latrophilins

Based on phylogenetic information of the 7TM parts, aGPCRs have been categorized into nine subfamilies [27] (see also [13]). First structural information on the N-terminal ligand-binding domains has become available for the subfamily I aGPCR member latrophilin. The three members of the latrophilin (LPHN, ADGRL) aGPCR subfamily have conserved domain architecture including N-terminal lectin domain, olfactomedin domain, serine/threonine-rich region, HormR domain, juxtamembrane GAIN domain, 7TM, and intracellular domains (Fig. 3a). The LPHN ectodomains have been reported to interact with various ligands including alpha-latrotoxin [11, 28], a potent toxin produced by the black widow spider, teneurin/ODZ family proteins [29], neurexins [30], and the single-pass transmembrane fibronectin-leucine-rich repeat protein FLRT3 [31]. Importantly, these interactions are mediated chiefly via the N-terminal domains of the latrophilin ectodomain. The first extracellular domain of an aGPCR to be determined at high resolution was the murine LPHN1 lectin domain: Nuclear magnetic resonance (NMR) revealed a compact structure, decorated by largely ordered loops, which harbor a weak glycan-binding site of unknown biological significance

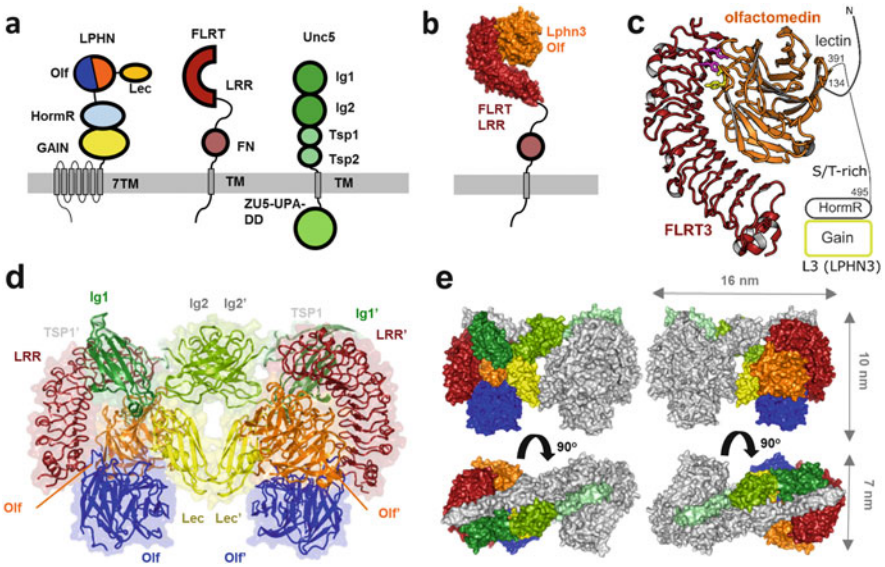

Fig. 3 Structures of latrophilin ectodomain interactions. (**a**) Domain overview of LPHN, FLRT, and Unc5. *Lec* lectin domain, *Olf* olfactomedin domain, *Horm* hormone domain, *GAIN* GPCR-Autoproteolysis INducing domain, *7TM* GPCR domain, *LRR* leucine-rich repeat domain, *FN* fibronectin domain, *Ig1* immunoglobulin domain 1, *Ig2* immunoglobulin domain 2, *TSP1* thrombospondin domain 1, *TSP2* thrombospondin domain 2, *DD* death domain. (**b**) FLRT3 LRR: LPHN3 Olf complex [9]. (**c**) Zoomed view of the FLRT3 LRR: LPHN3 Olf complex [9]. (**d**) The super-complex formed by extracellular domains of FLRT2 (LRR), Unc5D (Ig1, Ig2, TSP1), and LPHN3 (Lec, Olf) reveals a large assembly with an unusual stoichiometry (2:2:4) [10]. (**e**) The super-complex shown in panel (**d**) consists of two pseudosymmetric halves, joined together by the antiparallel arrangement of Unc5D

[6]. More recently, X-ray crystallography revealed the structure of LPHN3/ADGRL3 lectin plus olfactomedin domains. The olfactomedin domain has a five-bladed beta-propeller structure with tightly bound, presumably structural metal ions at its center [7–9]. The olfactomedin domain of LPHN3 has been shown to promote glutamatergic synapse formation by interacting with FLRT3 [31]. Surface mutagenesis and X-ray crystallography approaches demonstrated an extended binding surface across the "top" of the domain, which interacts with the concave surface of the horseshoe-shaped human FLRT2/3 leucine-rich repeat domains [7–9] (Fig. 3b, c). The crystal structures of FLRT3 leucine-rich repeat domain in complex with isolated LPHN3 olfactomedin domain revealed a simple 1:1 mode of interaction [8, 9]. The formation of a ternary complex between LPHN3, FLRT3, and another receptor involved in axon guidance, Uncoordinated-5B/D (Unc5B/D), which does not interact with LPHN3 in the absence of FLRT, was demonstrated suggesting LPHN3 may function via interacting with multiple ligands simultaneously [9, 10]. Surprisingly, crystal structures showed that the addition of Unc5D to the FLRT2 leucine-rich repeat domain and LPHN3 lectin-olfactomedin

domains resulted in an unexpected large assembly with a 2:2:4 stoichiometry [10] (Fig. 3d). A defining characteristic of the "super-complex" is that it demonstrates a molecular mechanism for high-affinity receptor oligomerization via the extracellular regions of the three proteins: FLRT2 acts as an initial scaffold by providing the previously described distinct high-affinity binding sites for LPHN3 and Unc5D [8, 9, 32]. This minimal ternary complex triggers the docking of a second copy of LPHN3 onto a newly formed binding site provided by FLRT2 and the first copy of LPHN3 (olfactomedin and lectin domains). The resulting tetrameric arrangement further dimerizes via the Unc5D Ig2 and TSP1 domains, thus forming an octameric super-complex. Unc5D adopts an antiparallel arrangement that holds together the sandwich formed by FLRT2 and four copies of LPHN3 (Fig. 3e). Sequence analysis revealed that other homologues of the Unc5 and FLRT family (Unc5A,B,C; FLRT1,3) are likely to produce smaller ternary complexes, including only one copy of each FLRT and Unc5 and two copies of LPHN [10].

What may the functions of different LPHN-FLRT and LPHN-FLRT-Unc5 assemblies be in the neural system, where these receptors are expressed in overlapping regions? Cell aggregation experiments have suggested that the interaction between full-length LPHN3 and FLRT3 occurs in trans [9], which is in agreement with previous cell stripe assay data [7] and the suggested adhesive role at synapses [31]. Given the recent speculation that mechanical force on the ectodomain may play a key role in activation of aGPCRs, it is interesting to consider that the FLRT3-expressing cell may exert force on the LPHN3-expressing cell and thereby activate LPHN3 in glutamatergic synapse formation. In contrast to the adhesive LPHN:FLRT interaction, the 1:1 Unc5:FLRT interaction triggers strong repulsion of radially migrating cells in the developing cortex and also induces neurite repulsion/collapse in vitro [32, 33]. These apparently opposing functions (FLRT:LPHN-adhesion, FLRT:Unc5-repulsion) make the ternary super-complex functionally intriguing. Pull-down experiments using murine cortical lysates are in agreement with the ternary complex forming in vivo, and recent stripe assay data suggested that the co-expression of Unc5D in FLRT2-expressing cells reduces their adhesive response to externally presented LPHN3 protein [10]. Thus, the ternary complex is likely to play distinct roles in vivo, possibly involving functions that require higher-order receptor multimerization.

5 Ectodomain Structures of Subfamily II–IX aGPCR

In contrast to subfamily I aGPCR, no crystal structures are available for the ectodomains except for the HormR and GAIN domains, as discussed above. In this section, we will focus on the domains which are located at the N-terminal side of the HormR and GAIN domains.

Subfamily II (E) aGPCRs contain one EGF-like domain at the N-terminus followed by one, four, or five calcium-binding EGF-like domains (Fig. 4a). EGF-like domains are found in many extracellular domains of membrane-bound proteins, and they often form repeats that fold to a linear solenoid architecture. Each

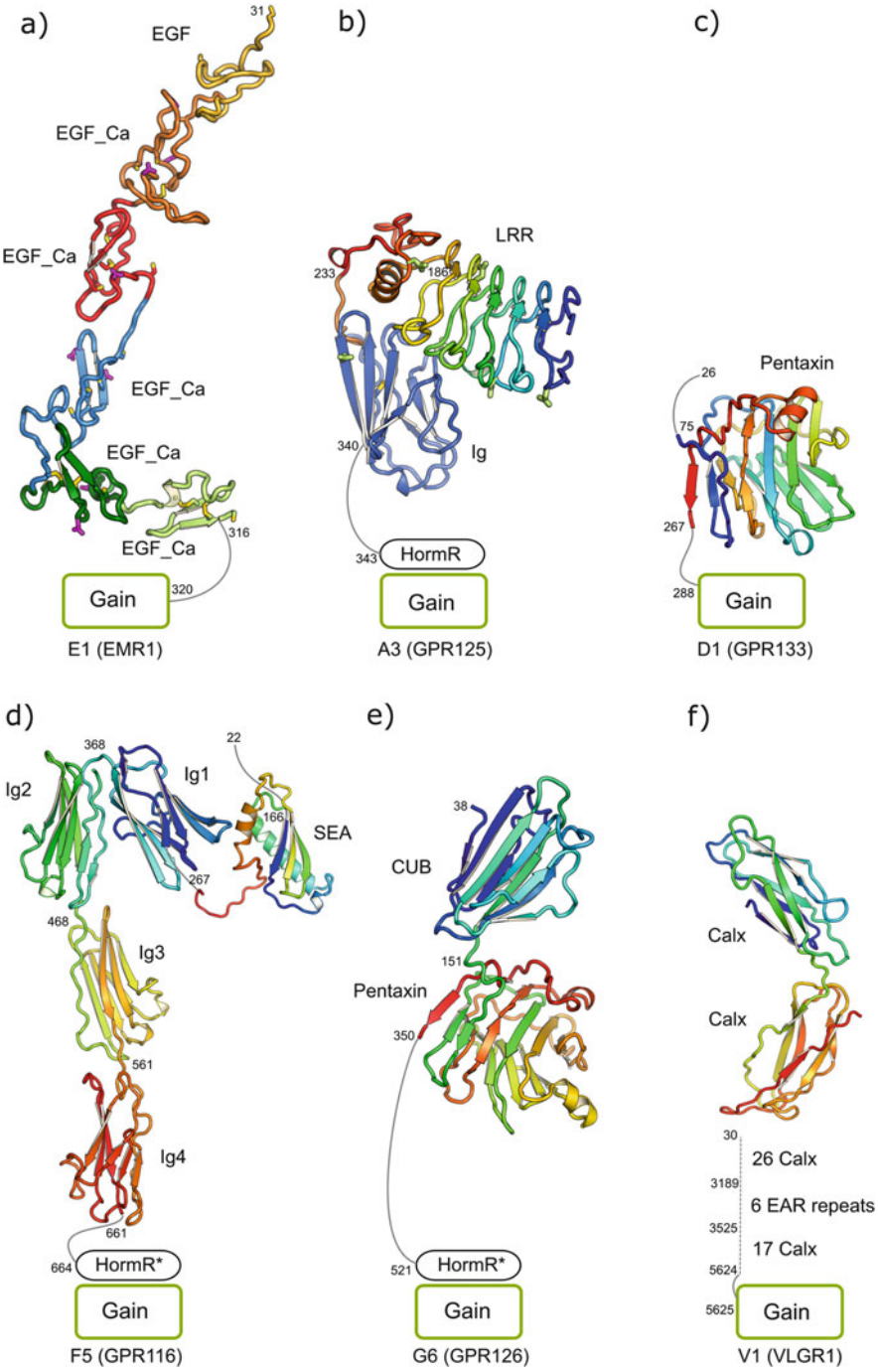

Fig. 4 Models of selected aGPCR ectodomains. (**a**) ADGRE1 (EMR1) of subfamily II aGPCR, (**b**) ADGRA3 of subfamily III aGPCR, (**c**) ADGRD1 of subfamily V aGPCR, (**d**) ADGRF5 (GPR116) of subfamily VI, (**e**) ADGRG6 (GPR126) of subfamily VIII, aGPCR, and (**f**)

EGF domain is typically cross-linked by three intradomain cystine bridges [35]. The calcium-binding EGF-like domains have a size of ~45 residues and typically contain a single calcium-binding site of the consensus sequence D-X-D-Q/E-C [36]. The ectodomain of L4 (ELTD1/ADGRL4) of receptor subfamily I also contains an EGF and an EGF_Ca domain, and it thus resembles more of a subfamily II aGPCR concerning the ectodomain structure.

Subfamily III consists of three receptors, of which A1 (GPR123) is unique in that it does not contain a GAIN domain but only a ~20-residue-long peptide sequence before the start of the first transmembrane helix. Interestingly, this length corresponds to the typical length of the *Stachel* sequence. The other two receptors of subfamily III, A2 (GPR124) and A3 (GPR125), share 42 % sequence identity displaying homology over the whole sequence range. They consist of a leucine-rich repeat (LRR) domain at the N-terminus, followed by an immunoglobulin (Ig) domain and HormR domain before the GAIN domain (Fig. 4b).

The three subfamily IV receptors share about 35 % sequence identity and have a similar domain structure. At the N-terminus, they contain a region of 237 (C1), 150 (C2), and 292 (C3) residues with low sequence similarity to each other and no predictable domain structures. This region is followed by nine cadherin domains and in total eight calcium-binding EGF domains, two laminin G domains and one laminin EGF domain (Fig. 5a). Each of the domains usually has a compact structure, and the termini are positioned at opposite ends of the domain (except for the laminin G domains) to form an extended chain of domains. This chain may be bent, but it is usually not folded itself into a compact ectodomain structure. For the C1–C3 ectodomains, an extended conformation of the domains would have a length of around 70 nm (in addition to the ~240 residues at the N-terminus and the GAIN/HormR domains). However, the arrangement of the individual domains cannot be predicted with confidence, and the structure in Fig. 5a is meant to illustrate the approximate architecture of the domains forming these large ectodomains.

The two receptors D1 (GPR133) and D2 (GPR144/ADGRD2) of subfamily V aGPCR share only 20 % sequence identity for the complete ectodomain, and both contain a pentaxin domain (Fig. 4c). A HormR domain appears not to be present in the subfamily V receptors.

Subfamily VI comprises five receptors with quite diverse ectodomain structures compared to the subfamilies already described. F3 is the only receptor in this

Fig. 4 (continued) ADGRV1 (VLGR1) of subfamily IX. Domain structures have been modeled with phyre2 [34], and groups of domains have either been arranged in an arbitrary orientation to form an extended chain or are based on a multidomain template structure, if available. The overall sequence identity for many of these models to the template is less than 20 %. Therefore, this figure illustrates the predicted domain architecture of the aGPCR ectodomains shown here, but accurate models for most of the ectodomains cannot be obtained based on current structural information. HormR* indicates that, while the domain is HormR-like in sequence, it is only distantly related to the structurally characterized HormR domains and probably contains only one of the two conserved disulfide bridges

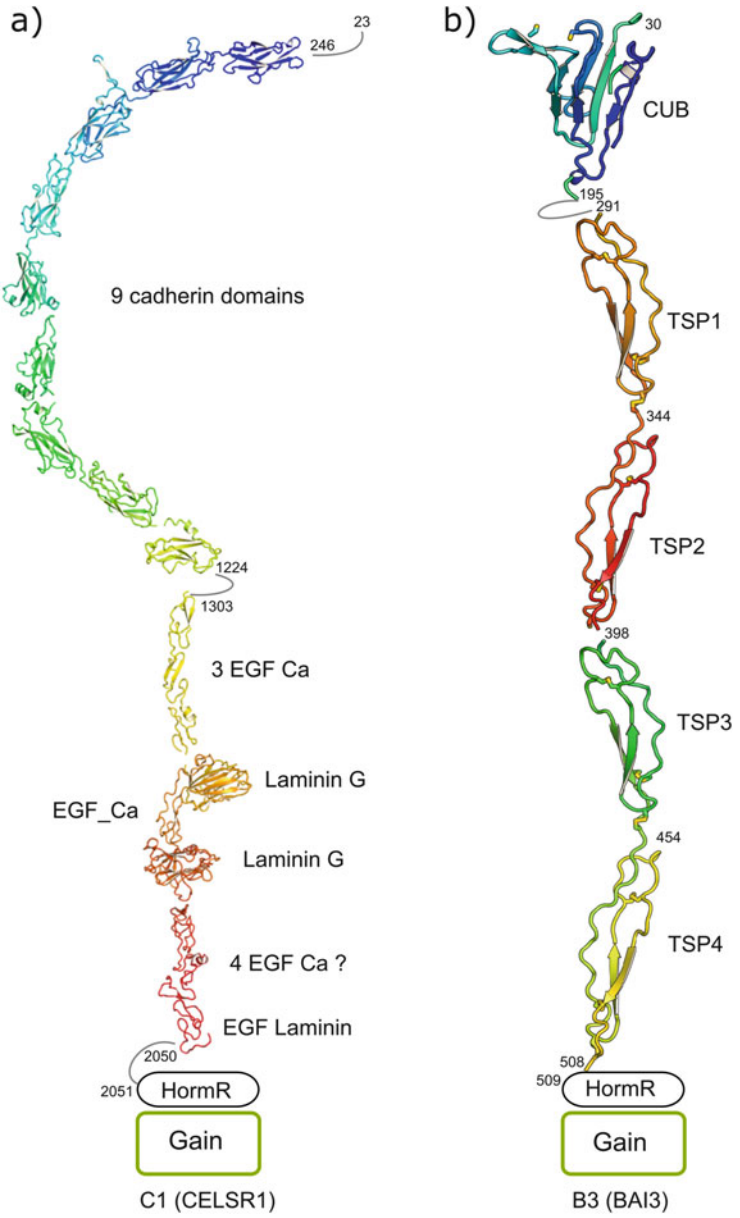

Fig. 5 Models of the ectodomain architecture of C1 and B3. (**a**) ADGRC1 of subfamily IV aGPCR and (**b**) ADGRB3 of subfamily 7. See Fig. 4 legend for further information

subfamily which contains a clearly identifiable HormR domain containing both conserved disulfide bridges. However, also for the other four receptors, a HormR-like fold is probably present, with at least one disulfide bridge conserved. F2 and F4

contain rather short ectodomains comprising only the HormR/GAIN domains. In addition, F2 contains an N-terminal region of less than 100 residues with no predicted homologous domain structures. F5 contains the largest ectodomain in this receptor subfamily (Fig. 4d). It comprises ~140 residues at the N-terminus, with no identifiable homologous folds, followed by a SEA domain (named after its occurrence in the three proteins: sperm protein, enterokinase, and agrin), four Ig-like domains, and the HormR/GAIN domains. F1 also contains an N-terminal fold which is related to that of F5, followed by a SEA domain and the HormR/GAIN domains. It thus lacks the Ig domains. F3 contains the N-terminal fold followed by two Ig domains and the HormR/GAIN domains.

In subfamily VII, the three receptors ADGRB1–3 all contain a HormR domain and upstream four or five thrombospondin (TSP) domains (Fig. 5b). The N-terminal regions of ~250–270 probably contain a CUB domain (for complement C1r/C1s, Uegf, Bmp1) in B1 and B3. As detailed above, the structure of the HormR/GAIN domain of B3 has been elucidated (Fig. 2a). The very N-terminal sequence RCSEQRCP of the crystallized construct probably belongs to the preceding TSP domain, as indicated by sequence comparisons.

Subfamily VIII comprises seven receptors. Some of these do not contain complete GAIN domains. This is most obvious for G5 (GPR114) and G3 (GPR97), which have ectodomains of only 260 and 280 residues, respectively, and a sequence comparison indicates that the first three helices of the helical GAIN subdomain A are missing. Likewise, for G1 (GPR56) and G2 (GPR64), only the C-terminal part of subdomain A appears to be conserved. These receptors contain longer N-terminal regions, but these probably do not correspond to the first three helices of GAIN subdomain A. Domain folds cannot be clearly identified for these sequence regions. In contrast, G4 (GPR112), G6 (GPR126), and G7 (GPR128) contain complete GAIN domains and probably also HormR-like domains with two (G6 and G7) or one (G4) disulfide bridge conserved. G6 contains a CUB and a pentaxin domain at the N-terminus (Fig. 4e). G4 also contains a pentaxin domain at the N-terminus, followed by a long stretch of more than 2000 residues with no significant homology to known domain folds.

Subfamily IX comprises only one receptor, but it has the largest ectodomain with 5878 residues. In addition to the GAIN domain, it probably contains in total 43 Calx-beta motifs, which have been characterized as a tandem repeat in the cytoplasmic domains of Calx sodium-calcium exchangers (Fig. 4f). After the 16th Calx domain, six EARs (epilepsy-associated repeats) are inserted. Each motif contains four β-strands, and it has been speculated that these form a seven-bladed β-propeller, as other proteins usually contain seven EARs.

6 Concluding Remarks

A flurry of recent structural data has recently been published on the conserved C-terminal regions of aGPCRs and the family-specific N-terminal domains of LPHN3. The conserved GAIN domain, harboring the autoproteolytic GPS motif,

is of key interest with regard to the recently established activation mechanism of aGPCRs via mechanical force [2, 3, 37, 38]. The ligand-binding domains are thought to act as anchors, enabling mechanical tethering to other cell surface receptors/ligands or matrix proteins through high-affinity interaction. Many aGPCRs are orphan receptors still, but insights into the structures of LPHN3 N-terminal domains have revealed first examples for such a tethering mechanism, using the binding partner FLRT. High-affinity interaction with FLRT is ensured through an extended and highly specific binding interface. Recent structural information [10] also revealed a surprising novel mechanism for higher-order LPHN multimerization via the N-terminal domains, when in complex with two receptors, FLRT and Unc5. This exciting finding suggests that the N-terminal ligand-binding domains have functions beyond mediating simple adhesive interactions and that, at least in the case of LPHNs, they likely determine the receptor's multimerization state. GPCR multimerization is an emerging theme in the field and has so far been suggested for a series of non-aGPCRs [39]. The functional consequences of aGPCR multimerization and how this compares mechanistically with the multimerization seen for other GPCRs via their transmembrane domains remain to be explored.

Taken together, this chapter has aimed to summarize the significant recent progress made with understanding the structural/functional properties of aGPCR extracellular domains, a rapidly progressing field that is receiving increasing attention.

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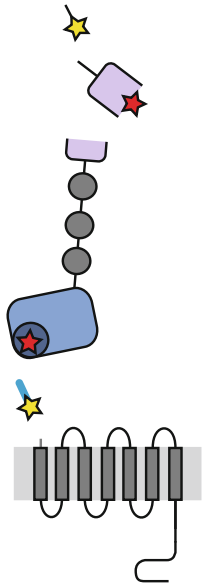
Control of Adhesion GPCR Function Through Proteolytic Processing

Matthias Nieberler, Robert J. Kittel, Alexander G. Petrenko, Hsi-Hsien Lin, and Tobias Langenhan

Graphical Abstract



Proteolytic processing events in adhesion GPCRs. aGPCRs can undergo multiple autoproteolytic (*red asterisks*) and proteolytic processing events by exogenous proteases (*yellow asterisks*) that may be involved in signaling events of the receptors.

Contents

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Abstract

Proteolytic processing is an unusual property of adhesion family G protein-coupled receptors (aGPCRs) that was observed upon their cloning and biochemical characterization. Ever since, much effort has been dedicated to delineate the mechanisms and requirements for cleavage events in the control of aGPCR function. Most notably, all aGPCRs possess a juxtamembrane protein fold, the GPCR autoproteolysis-inducing (GAIN) domain, which operates as an autoprotease for many aGPCR homologs investigated thus far. Analysis of its autoproteolytic reaction, the consequences for receptor fate and function, and the allocation of physiological effects to this peculiar feature of aGPCRs has occupied the experimental agenda of the aGPCR field and shaped our current understanding of the signaling properties and cell biological effects of aGPCRs. Interestingly, individual aGPCRs may undergo additional proteolytic steps, one of them resulting in shedding of the entire ectodomain that is secreted and can function independently. Here, we summarize the current state of knowledge on GAIN domain-mediated and GAIN domain-independent aGPCR cleavage events and their significance for the pharmacological and cellular actions of aGPCRs. Further, we compare and contrast the proteolytic profile of aGPCRs with known signaling routes that are governed through proteolysis of surface molecules such as the Notch and ephrin pathways.

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Keywords
Adhesion GPCR • GAIN domain • GPS • Proteolysis • Autoproteolysis

1 Forms of Proteolytic Processing Events in aGPCRs

1.1 GAIN-Mediated GPS Cleavage

One of the structural and functional hallmarks of aGPCRs is the juxtamembrane localization of a highly conserved GPCR proteolysis site (GPS) motif (Fig. 1) [1–3]. All aGPCRs, except GPR123/ADGRA1, contain the GPS motif [1]. Proteolytic modification of aGPCRs was first reported for CD97/ADGRE5 in 1996 by Kelly and colleagues [4]. They revealed a novel two-subunit structure of CD97, consisting of an extracellular fragment and a seven-transmembrane (7TM) fragment derived from a proprotein precursor. Petrenko et al. later identified the cleavage site and coined the term GPS to describe the proteolytic processing of CIRL/latrophilin/ADGRL1 [5, 6].

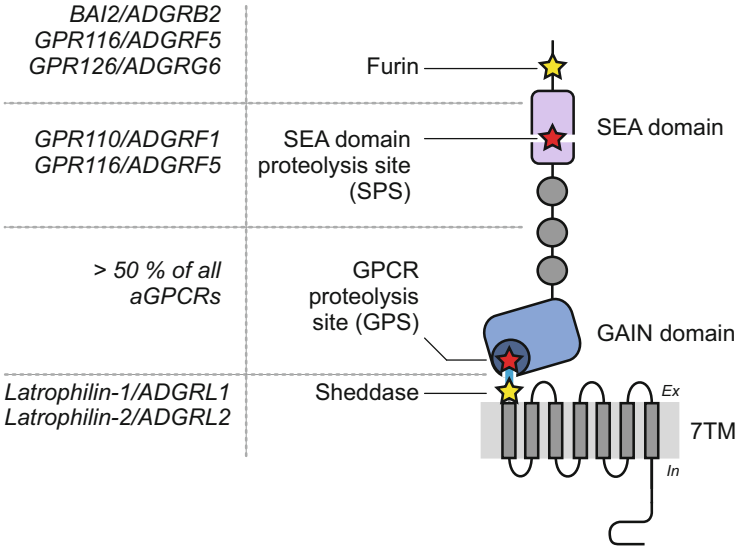

Fig. 1 Proteolytic processing events of adhesion GPCRs. aGPCRs can undergo multiple autoproteolytic (*red asterisks*) and proteolytic processing events by exogenous proteases (*yellow asterisks*) that may be involved in signaling events of the receptors. The most prevalent cleavage of aGPCR family occurs at the GPCR proteolytic site (GPS; *dark blue circle*) and is catalyzed by the GPCR autoproteolysis-inducing (GAIN) domain (*light blue*). Another type of autoproteolysis is governed by the SEA domain (*pink box*) and shares similarities with GAIN domain cleavages. aGPCRs can also be substrates for proteases and release parts of the ECD. Exemplary aGPCR homologs and associated proteolytic processing events are indicated on the *left*

The GPS motif of ~50 amino acids contains a highly conserved tripeptide cleavage sequence and several canonical cysteine and tryptophan residues [2]. Moreover, the 6–8 residues C-terminal to the cleavage site are usually small and hydrophobic [7]. The cleavage tripeptide almost always starts with His, followed by Leu/Ile and Ser/Thr, with proteolysis occurring between Leu/Ile and Ser/Thr (HL/I↓S/T) [2, 3]. Most interestingly, GPS proteolysis is not executed by any proteinases, but is brought about by an autocatalytic mechanism analogous to that of *hedgehog* morphogens [8, 9] and Ntn-hydrolases [10–14]. It is concluded that the GPS proteolytic reaction is most likely initiated by the deprotonation of the hydroxyl group of the P^{+1} residue (Ser/Thr) by the P^{-2} His residue. This is followed by a *cis*-nucleophilic attack on the α-carbonyl carbon of the P^{-1} Leu/Ile residue, producing a tetrahedral intermediate. An ester intermediate is subsequently generated via an N→O acyl shift. Finally, the attack by H_2O cleaves the ester bond splitting the receptor into two protein fragments (Fig. 2) [11]. The two fragments usually do not separate after proteolysis, but instead associate non-covalently to form a mature heterodimeric receptor complex on the cell surface [6, 15]. Interestingly, the GPS motif is absolutely necessary for proteolysis, but is insufficient to mediate the autoproteolytic reaction on its own [7].

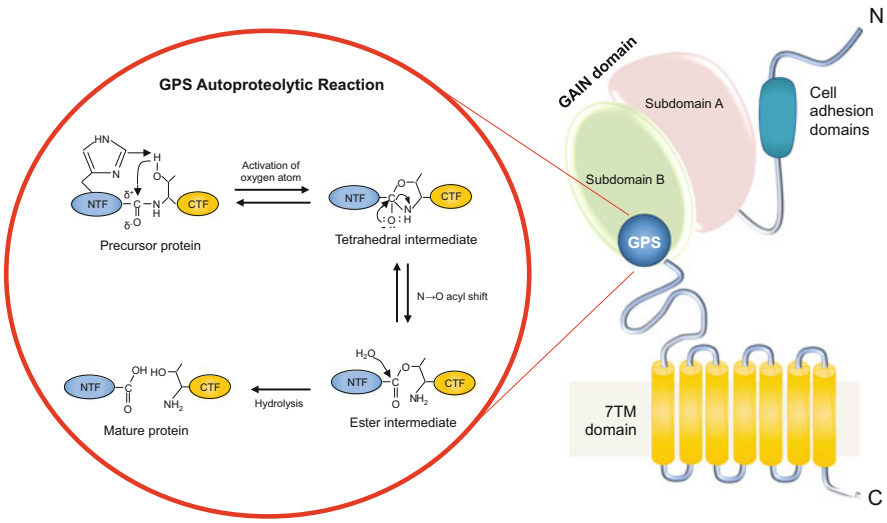

Fig. 2 The GAIN domain and GPS autoproteolysis of adhesion GPCRs. A schematic diagram of an aGPCR is shown. Located at the C-terminal half of the NTF, the GAIN domain is divided into the subdomain A (*pink*) and subdomain B (*light green*). The GPS motif (*blue*) is a part of subdomain B. The proposed mechanism of the GPS autoproteolytic reaction is shown inside the *red circle*. A His or another general base withdraws a proton from the hydroxyl group of a Ser or Thr at position +1. The resulting negatively charged oxygen makes a nucleophilic attack on the carbonyl group of the residue at position −1 (e.g., a Leu), yielding a tetrahedral intermediate and subsequently an ester intermediate. The resulting ester is then hydrolyzed to produce the NTF and CTF that form the mature protein

Interestingly, while it is well accepted now that the GPS proteolysis is a self-catalytic intramolecular reaction, the proteolytic efficiency is not always complete. Indeed, both processed and unprocessed GPR56/ADGRG1 and polycystin-1 receptors have been detected in vivo [16–18]. Moreover, crystals of two structurally similar GPS-containing fragments of CIRL/latrophilin and BAI3/ADGRB3 were described, the former in a completely cleaved and the latter in a non-cleaved conformation [19]. Hence, it is suggested that the GPS domain-containing receptors might adopt receptor folding conditions that either promote or demote GPS cleavage depending on cell types and cellular environments [20, 21].

One critical factor regulating the GPS proteolysis is found to be the first step of N-glycosylation during receptor biosynthesis in the ER [20, 21]. Other potential factors include the specific conditions of aGPCR expression, including the cell type and expression levels. While in the brain tissue only the cleaved form of CIRL/latrophilin is detected, its heterologous expression in transfected cells yields only a minor portion of the processed form that depends on the cell line used [5, 15]. Interestingly, the receptor cleavage yield in transfected cells can be regulated by pharmacological agents such as PMA and ionomycin, regulators of the protein kinase pathway, suggesting the existence of intracellular signaling mechanisms to fine-tune the autoproteolysis [22]. As with any chemical reaction, one can also anticipate a contribution of local pH and ionic changes, as well as a direct involvement of available nucleophilic molecules that serve as cofactors.

Pulse-chase experiments and use of various recombinant receptors and protein trafficking inhibitors have identified the ER lumen as the major subcellular localization of GPS proteolysis [2, 4, 11, 15]. However, due to the highly regulated nature of the GPS proteolytic reaction mentioned above, it is likely that GPS proteolysis could also occur at a later time point during protein maturation or perhaps even at the cell surface. Indeed, cell type-specific location of the GPS cleavage was reported for polycystin-1, whose proteolysis may occur within the ER or post-ER [21].

Recent structural analyses finally delineate the peculiar requirement and characteristics of GPS autoproteolysis. Crystallization of two aGPCRs, Latrophilin-1/ADGRL1 and BAI3, identifies a much larger extracellular GPCR autoproteolysis-inducing (GAIN) domain (~320 residues) that is sufficient and minimally required for GPS autoproteolytic reaction [19] (see also [23]). In fact, the GPS motif is an integral part of the GAIN domain. The crystal structure of the GAIN domain shows a subdomain A of 6 α-helices and a subdomain B consisting of a twisted β-sandwich of 13 β-strands and 2 small α-helices [19, 24]. The GPS motif is enclosed in the last five β-strands of subdomain B, and the cleavage takes place in a short kinked loop between the last two β-strands (Fig. 2) [19, 24]. The proper folding of the GAIN domain, hence the arrangement of the scissile bond in a unique configuration, provides an essential environment for the GPS autoproteolytic reaction. Due to the lack of the conformational constraint and chemical environment required for the proteolytic reaction, the GPS motif alone cannot mediate autoproteolysis. In addition, the cleaved last β-strand is tightly embedded within the rest of the GAIN domain, hence it is energetically unfavorable for the two fragments to dissociate [19, 24].

Interestingly, phylogenetic analysis shows that the GAIN domain is evolutionarily conserved from tetrahymena to mammals. In fact, it is believed that the GAIN domain is one of the most evolutionarily ancient and functional autoproteolytic protein folds identified to date [19, 24]. The close proximity to the TM region and the unique structural requirement for the GPS autoproteolytic reaction all suggest an important role for the GAIN domain in aGPCR function [25]. Furthermore, the GAIN domain is also present in all members of human polycystic kidney disease 1 (PKD1) protein family, suggesting a much wider usage of this novel domain in receptor biology [19, 24]. Indeed, sequence mutations in the GAIN domain have been linked to various human diseases, a clear indication of its role in regulating receptor activities [17, 24, 26, 27]. How the GAIN domain-mediated autoproteolysis may regulate receptor signaling and function will be discussed in the later sections.

1.2 Other Autoproteolytic Cleavages of aGPCRs

Apart from the GPS autoproteolysis, additional autoproteolytic reactions were noted for certain aGPCRs. Abe et al. showed that Ig-Hepta (GPR116/ADGRF5) undergoes two specific proteolytic events in the extracellular region [28]. One of the proteolytic sites identified is at the GPS motif, while the other is at the SEA module located at the N-terminus of the receptor (Fig. 1). Identified first in three different proteins (sea urchin sperm protein, enterokinase, and agrin) [29], the SEA module is a conserved extracellular protein motif of ~80–110 residues usually found in O-glycosylated mucin-like membrane proteins such as MUC1, MUC3, MUC12, MUC13, and MUC17 [30–32]. A highly conserved G↓S[V/I]VV sequence is identified as the SEA domain proteolysis site (SPS) [32].

Interestingly, the SEA module-mediated proteolysis shares many similar characteristics with GPS autoproteolysis. First, although some SEA module-containing molecules are soluble proteins, the SEA module is mostly found in cell-surface proteins and is located at the extracellular region of the molecule, near or close to the TM region [32]. Second, the proteolytic modification takes place within the ER during early protein biosynthesis. Third, proteolysis only proceeds when the P^{+1} cleavage site is a residue containing a terminal hydroxyl group (Ser and Thr) or thiol group (Cys) [33]. Fourth, proteolysis at the SEA domain is an autocatalytic intramolecular reaction likely mediated by a series of nucleophilic attacks and the formation and hydrolysis of an ester intermediate via an N→O acyl shift and H_2O, respectively [33–35]. Fifth, the autoproteolytic reaction is achieved by conformational strain and requires strict and proper protein folding [34, 36, 37]. Finally, the resulting cleaved fragments remain associated non-covalently following proteolysis [33, 35].

Two aGPCRs, GPR110/ADGRF1 and GPR116, are known to contain both the SEA module and the GAIN domain [1]. Indeed, multiple proteolytic modifications of GPR116 have been identified, leading to the formation of a mature receptor with many non-covalently associated fragments [28, 38]. GPR116 has been linked to a

number of physiological and pathological processes such as pulmonary surfactant homeostasis, insulin insensitivity, and breast cancer metastasis [39–44]. However, the role of autoproteolysis in the SEA module and GAIN domain in GPR116 function has yet to be investigated. Little is known regarding the proteolytic modification of GPR110.

1.3 Cleavage of aGPCRs by Other Proteases

With aGPCR research on the rise, more and more homologs are identified as targets of classical proteases such as furin or matrix metalloproteinase (MMP; Fig. 1). These include BAI1/ADGRB1, BAI2/ADGRB2, GPR116, GPR126/ADGRG6, and Latrophilin-1/ADGRL1 [38, 45–48]. Furin, a subtilisin-like proprotein convertase, is a calcium-dependent serine endoprotease enriched in Golgi and is involved predominantly in intracellular protein processing within the secretory pathway [49]. Consistent with previous reports, the proteolytic site of BAI2, GPR116, and GPR126 by furin was identified right after an Arg residue of a consensus furin-cleavage sequence [38, 47, 48]. Interestingly, these furin-cleavage sites are all located at the extracellular region N-terminal to the GPS and SEA domain. One exception is the furin processing of Latrophilin-1, which occurs before an Arg residue located C-terminal of the GPS motif within the CTF [46]. The furin-cleaved aGPCR fragment was shown to either remain associated with the rest of the molecule (GPR116) or released to the extracellular milieu (BAI2, GPR126, Latrophilin-1). The functional significance of the furin-mediated proteolysis of aGPCRs is currently unknown, but additional functions exerted by the shed receptor ectodomain remain a possibility. Modulation of aGPCR activity by furin-mediated shedding is also an alternative.

BAI1, initially identified as a brain-specific p53-regulated gene, is highly expressed in normal but not tumor brain cells [50, 51]. GPS proteolysis of BAI1 released a 120 kDa thrombospondin type-1 repeat (TSR)-containing "vasculostatin" fragment with anti-angiogenic and anti-tumorigenic function [50]. Later studies revealed another extracellular cleavage mediated by MMP-14 at a more N-terminal region, producing a 40 kDa (vasculostatin-40) fragment also with very potent anti-angiogenic activity [45]. In fact, the second cleavage of BAI1 is processed by a two-step protease activation cascade in which the latent MMP-14 is activated by furin [45]. Interestingly, the generation of vasculostatin-120 by GPS autoproteolysis is not a prerequisite for vasculostatin-40 production by MMP-14. Hence, intra- and extracellular proteolytic processing of BAI1 to distinct ectodomain fragments by GPS autoproteolysis and MMP-14, respectively, represents important activation and regulatory mechanisms for the BAI1 receptor function [45].

Another interesting example of aGPCR cleavage involving a sheddase is the dissociation of a CIRL/Latrophilin-1 two-subunit complex at the cell surface that results in the secretion of its ectodomain that contains the intact GAIN domain (Fig. 1). About 5 % of the endogenous brain-expressed CIRL/latrophilin undergoes

this processing. The soluble receptor form is comprised of NTF linked to a small peptide fragment of CTF. This peptide was identified by mass spectrometry indicating the location of the second cleavage site at the border between the GAIN domain and the 7TM core. Similar processing was also shown for CIRL-2/ADGRL2 [46].

2 Biological Effects Controlled Through aGPCR Proteolysis

The consequences and roles of the autoproteolytic processing of aGPCRs have been under intense scrutiny since its discovery. Multiple experimental approaches have been implemented to grasp this biochemical peculiarity of aGPCRs, and several conclusions have been drawn from the results. We will discuss the most popular ones below.

2.1 Trafficking

Several cell physiological consequences have been ascribed to the autoproteolytic processing of aGPCRs at the GPS. Insights into these features derived from studies of aGPCR and polycystin-1 homologs, in which the consensus site was mutated at different positions in order to disable the autocatalytic reaction. The Latrophilin-1 homolog with a GPS disrupting mutation was the first receptor that was scrutinized this way. It was noted that the GPS-deficient Latrophilin-1 variant did not traffic to the cell surface lending support to a model, in which the posttranslational cleavage event may function as a maturation signal during the biosynthesis of the receptor molecule in the ER [15]. Later on, this hypothesis was further explored in several other aGPCRs and polycystins returning mixed results: while impeded surface expression was found for proteolysis-deficient versions of Latrophilin-1 [15] and GPR126 [52], no such effect was noted for polycystin-1 [17], GPR133/ADGRG1 [53], and the nematode latrophilin homolog LAT-1 [54]. Also Latrophilin-1 was reprobed and several GPS cleavage mutations did not affect cell-surface transport of the receptor [19].

Also the GAIN-mediated cleavage of polycystin-1 has drawn interest to its physiological requirement, and its investigation contributed insights into the role of the proteolysis event. An allele of *PKD1*, which encodes for a cleavage-deficient polycystin-1 product, leads to strong hypomorphic phenotypes that manifested through defects in the development of kidney tubules [18] (see below). Follow-up work on this effect suggests that the CTF of polycystin-1 may act as a cofactor that is required for membrane trafficking of the NTF. The NTF subsequently detaches from the CTF, but remains associated to the membrane, probably through other surface receptors [55]. Similar findings were obtained for the NTF of the aGPCR Latrophilin-1, whose CTF may also exist as a separate protomer at the cell surface [56].

Hence, it remains controversial whether GPS autoproteolysis is functioning as a gatekeeping step in the biosynthesis and maturation of aGPCRs. One solution to this puzzle may be offered by the observation that several potentially GPS-disabling mutations rather lead to reduced stability and unfolding of the GAIN domain and consequently do not traffic properly to the cell membrane. Further, GPS cleavage appears to be dependent on cell context and other posttranslational modifiers such as glycosylation [17, 20]. Therefore, recombinant expression of aGPCRs in heterologous cell lines—the classical test system utilized for cleavage assays—may not provide the necessary cofactors or conditions that are required for efficient GAIN proteolysis.

2.2 Terminating Adhesion

An obvious role for the autoproteolytic cleavage of aGPCRs is one that has remained unexplored thus far. Movements during proliferation, migration, polarity establishment, but also postmitotic motion of cells or their context impose considerable forces on cells, which are counteracted by adhesion molecules such as cadherins, laminins, or integrins [57].

In this vein, aGPCRs possess an extensive repertoire of adhesion domains that are located in the ectodomain of most of the receptor homologs (see also [23]). aGPCRs are exposed to and likely engage in binding events with adhesive partner molecules that are affixed either within the extracellular matrix lattice or anchored on opposite cell surfaces [25]. Thus, autoproteolytic cleavage of aGPCRs may determine a threshold for forces transmitted onto the receptor expressing cells, above which the NTF and CTF are separated and relieved of their adhesive interaction. Such a role was suggested for other surface-mounted molecules such as mucins (see above), which line the surface of mucous epithelia. By means of an autocatalytically active SEA domain, potentially damaging shear forces that endanger the epithelial barrier are limited to the energy that is necessary to split the two non-covalently bound cleavage fragments of mucins [35].

2.3 Triggering Metabotropic Signaling

With the advent of molecular models on the activation mechanism of aGPCRs, and their suspected role as mechanoreceptors, receptor autoproteolysis receives increasing attention as a potentially crucial component in these processes.

As discussed in detail in [58, 59], several aGPCRs possess a tethered agonist that is an integral part of the receptor molecule. Structure-function studies of GPR56 implied that the NTF of an aGPCR exerts an inhibitory role on the metabotropic and biological activity of its CTF. This conclusion was based on receptor variants that either contained a shortened or no NTF at all, which displayed increased activation of cellular behaviors [44] and downstream effectors [60], respectively. These observations were explained by two models: either the NTF directly suppresses

metabotropic activity of the CTF consistent with the function of a tethered inverse agonist, or alternatively, the NTF counteracts the activity of a tethered agonist of the CTF [61]. Both models account for the disinhibiting effects of NTF removal. Studies on LAT-1 (see also [62]) provided evidence for the latter model. A panel of LAT-1 receptor variants was scored for their capacity to rescue the penetrant developmental lethality caused through removal of the *lat-1* gene in *C. elegans*. In the course of this study, it was noted that neither a receptor that lacks the 7TM domain nor a full-length chimeric version containing a foreign GPS motif of the GAIN domain was able to remedy the lethal effects of *lat-1* deletion. However, when both receptor variants were co-expressed, they complemented each other intermolecularly to reestablish the full biological functionality of the wild-type receptor. The conclusion drawn from this set of experiments suggested that the GPS motif interacts with the 7TM domain in an agonistic fashion [54].

Further investigations unveiled the molecular underpinnings of this effect and supplied further evidence for the model that aGPCR signaling can be activated through a tethered agonist. The stalk region that links the GPS with the first TM helix, a peptide of approximately 15–25 amino acids in length depending on individual receptor homologs, comprises an agonistic activity that stimulates metabotropic signaling of aGPCRs. When truncated receptor versions that lack the NTF are expressed, the agonist (termed *Stachel*; German: sting, or alternatively *stalk*) is exposed and conceivably interacts with the 7TM continuously leading to high signaling activity as observed before. Receptor layouts that lack the entire ECD (i.e., including the *Stachel*/*stalk*), however, are muted, but can be reactivated by high amounts of soluble *Stachel*/*stalk* peptide indicating that the tethered agonist is necessary and sufficient for receptor activation. This was shown first for GPR126 and GPR133 [52] and subsequently for additional receptors including GPR56 [63], GPR64/ADGRG2 [64], GPR114/ADGRG5 [65], Latrophilin-1, and LAT-1 [66].

Interestingly, the agonistic property of the peptide appears to reside in its N-terminal half [65], which also represents the last beta-sheet of the GAIN domain that is severed through the autocatalytic event from the much larger rest of the fold. In cleavage-competent receptor homologs, the *Stachel*/*stalk* therefore constitutes the very beginning of the CTF, which also mediates the non-covalent lock between NTF and CTF that results in the heterodimeric configuration in which aGPCRs are found on the cell membrane [19].

How is exposure of the *Stachel*/*stalk* enacted under physiological conditions? As the agonist is buried inside the GAIN domain, the simplest mode would see the NTF removed through a combination of firm ligand engagement with the extracellular adhesion domains through which mechanical force is transmitted onto the NTF that pulls it off the CTF. This way, the *Stachel*/*stalk* sequence would become instantly exposed. Corroboration of the interplay between mechanical challenge and transmembrane signal transduction has recently been found in EMR2/ADGRE2: Boyden et al. identified two kindreds that displayed symptoms of severe vibratory urticaria, a condition associated with degranulation of mast cells upon dermal challenge with physical force. In this study, an autosomal-dominant missense mutation in EMR2/ADGRE2 was shown to underlie these effects. In vitro

experiments with mast cells transfected with the mutated receptor variant indicated that removal of the NTF through vibratory shear stress was increased [67]. This is consistent with model in which elevated exposure of the tethered agonist (*Stachel*/stalk) triggers subsequent downstream signaling events and is further discussed in [68]. Also for other protease-activated membrane receptor systems, e.g., the Notch-DSL pathway (see below), similar mechanisms, executed through proteolysis by an exogenous protease, were proposed [69].

In this context, GAIN autoproteolysis would be an essential precondition for the liberation of the tethered agonist upon mechanical stimulus encounter and a satisfactory explanation for its evolutionary conservation. However, also non-cleavable aGPCRs appear to possess agonistic activity in the *Stachel*/*stalk* peptide and are sensitive to mechanical stimulation, at least in vitro, as recently shown for GPR114 [65]. To complicate matters, recent studies indicate that aGPCR engage in *Stachel*-independent metabotropic (CTF-dependent) signaling [70], which is discussed in detail in [59].

Currently, there is no obvious explanation for how the encounter between the agonist and the 7TM may be facilitated assuming that the available GAIN domain structures are representing the physiological conformation of the fold (see also [58, 71]). Alternatively, there exist steric layouts of the GAIN domain that allow access of the *Stachel*/*stalk* to its cognate 7TM interface even if the agonist is an integral part of a contiguous polypeptide chain rather than released through the autoproteolytic cleavage. Such conformations are subject to future investigations and will help answering the question for the role of aGPCR autoproteolysis.

2.4 Liberation of NTF for Cell-Non-autonomous Effects

An interesting addition to the cell-autonomous information fed into the Notch-expressing cell, the Notch-DSL interactions also appear to drive cell-non-autonomous events in the ligand-expressing cells. Also this feature of the Notch pathway may compare to properties of several aGPCR homologs and their capacity to not only act as signal sensors but also senders of information. A well-studied example is the effect of the N-terminal fragment (NTF) of Gpr126/ADGRG6 during mouse and zebra fish heart development [72, 73]. Gpr126 is expressed by endocardial cells but not cardiomyocytes and is essential for cardiac mitochondrial function and trabeculation of the heart. Genetic structure-function studies have indicated that the C-terminal fragment of Gpr126, which contains the metabotropic signaling unit of the aGPCR [52], is dispensable for these effects while they critically depend on the NTF of Gpr126. Interestingly, this requirement is shared by endocardial cells and cardiomyocytes, of which the latter do not express the receptor molecule. Immunolocalization studies further detailed that Gpr126 may work in a paracrine mode to exert its function on cardiomyocytes, possibly by shedding its NTF and thereby governing the development of these cells in a cell-non-autonomous fashion [72].

2.4.1 Split Personality Hypothesis

While the NTF and CTF of most aGPCRs are associated non-covalently, it was found that the two fragments could also be expressed separately on the cell surface as independent entities in some aGPCRs such as Latrophilin-1 and EMR2/ADGRE2 [56, 74, 75]. The so-called split personality hypothesis was coined to reflect the fact that the NTF remains free even though the CTF is pulled down exhaustively by immunoprecipitation [56, 76]. Furthermore, expression of the CTF-truncated recombinant Latrophilin-1 was found to remain tethered on the cell membrane. Most interestingly, the NTF could be efficiently removed from the membrane without solubilizing any CTF when cells were treated with perfluorooctanoic acid, a weak detergent that does not disrupt the lipid bilayer. These results strongly suggest that some NTF is self-anchored on the membrane independently of the CTF.

Indeed, subsequent studies showed that membrane localization of the two fragments does not completely overlap and the fragments could even be internalized independently [56]. Further, it is possible to detect ligand-induced interaction of individual NTF and CTF from the same receptor molecule fused with different tags (so-called homogeneric heterodimers), or even from two distinct aGPCRs (heterogeneric heterodimers; e.g., $NTF^{Latrophilin\text{-}1}$::$CTF^{GPR56}$ or NTF^{EMR4}::CTF^{EMR2}) [56, 74, 75]. For EMR2, it was shown that the NTF and CTF were differentially distributed in lipid raft microdomains and ligation of the NTF by EMR2-specific monoclonal antibodies induced the translocation and interaction of NTF with CTF to the lipid rafts for receptor activation and signaling [74]. Consistent with these findings, GPS proteolysis of aGPCRs could possibly create diverse functional receptor complexes by cross association of independent NTFs and CTFs of different aGPCRs.

Several possibilities exist as to how such molecular cross-chimerization may come about. Receptor fragments may either recombine after GAIN cleavage at the GPS. For this scenario GPS cleavage is absolutely necessary. Alternatively, aGPCRs may form heterodimers at the level of the 7TM domain, ECD, or ICD that may lead to crosswise pulldown results interpreted as heterogeneric heterodimer formation. Only in one study thus far, these possibilities were tested by the use of GPS cleavage-incompetent receptor forms, which still showed co-immunoprecipitation [77]. The authors thus concluded that homo- and heterogeneric cross talk of aGPCRs is likely the result of receptor oligomerization that does not involve NTF-CTF re-pairing at the GPS, but rather the lateral interaction of several aGPCR molecules.

Future investigations will need to further define the properties of GPS proteolysis for separate fates and biological activities of aGPCR fragments.

3 Similarities and Differences to Other Proteolysis-Dependent Signaling Pathways

aGPCRs are by far not the only group of biomolecules whose actions are controlled through proteolytic cleavage (Fig. 3, Table 1). Here, we only concentrate on those that are governed by the proteolytic processing of surface receptors. However, we note that also a wealth of other biological signals depend on the proteolytic activation of precursor states of intracellular or secreted substrates. This includes the shedding of N-terminal signal peptides through signal peptide peptidases or the functionalization of prohormones and proenzymes into active molecule species, such as proinsulin in pancreatic β cells or serine proteases in the gastrointestinal system, respectively.

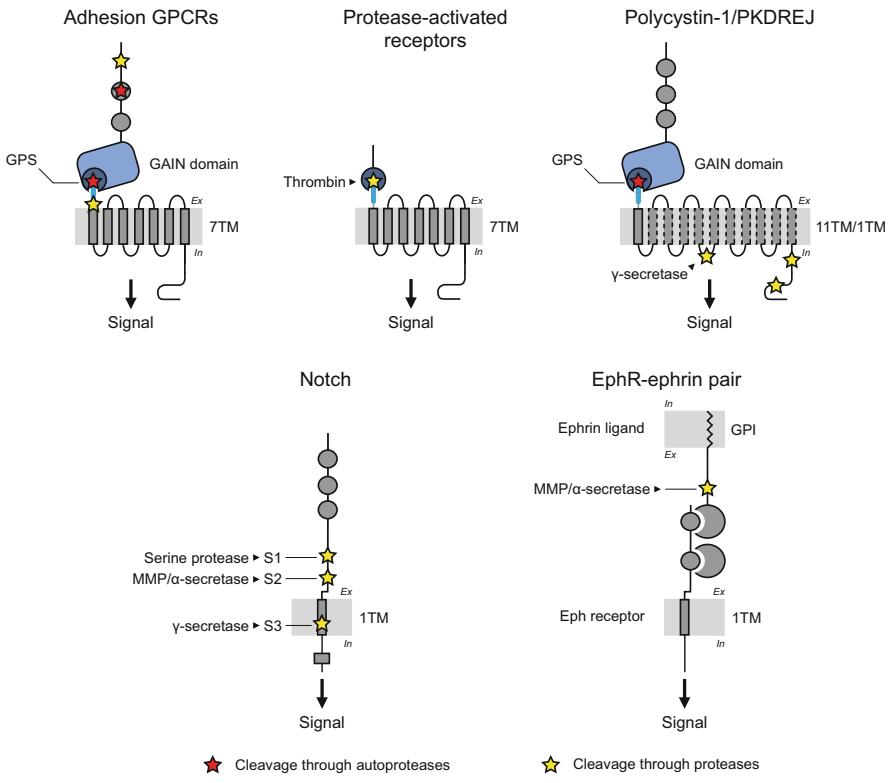

Fig. 3 Molecular pathways controlled though proteolytic processing. aGPCR processing through self-cleavage and cleavage by proteases is implicated in several biological properties of these receptors including critical steps in their signaling cascade. In this respect, aGPCRs may share signaling principles with other receptor systems that rely on proteolysis to trigger and/or transduce extracellular events into intracellular information. These encompass, among others, the protease-activated receptor group of GPCRs, polycystin-1/PKDREJ, Notch, and ephrin receptor families of cell-surface receptors

Table 1 Signaling pathways executed and/or modulated through non-GAIN-domain-mediated proteolysis

Substrate	Serine protease	MMP/ α-secretase	β-secretase	γ-secretase	Selected physiological effects of proteolysis
EGF, TGF-α, neuregulins, etc.		●			Shedding of substrate for EGFR: cell growth, migration, proliferation, differentiation [92, 93]
TNF-α		●			Shedding of substrate for cytokine receptor: inflammation, apoptosis, necrosis [94, 95]
APP		●		●	Physiological tasks: learning, memory, synaptic plasticity (LTP) [96–98]
			●	●	Pathological effects: accumulation of Amyloid-β protein in Alzheimer's Disease [98, 99]
Notch	●	●		●	Binary developmental commands involved in cell fate decisions; NCID: transcriptional regulation [100] of inner ear development [101], pancreatic β cell production [102], specification of goblet cells in the intestine [103], various functions in B and T cell development [104, 105], etc.
N-cadherin		●		●	Cell-cell adhesion [106]; β-catenin transcriptional control: neuronal differentiation, synaptogenesis, axonal remodeling (synaptic plasticity) [107]; cell proliferation and cell survival (cancer) [106]
Ephrin-A2		●			Contact-mediated axon repulsion [108, 109]
Neuroligin-1		●		●	Negative regulation of spine remodeling at excitatory synapses [110]
Neurexin-3β		●		●	Glutamatergic synapse assembly and possibly also function [111, 112]
PAR1	●				Fibrin conversion (platelet aggregation), endothelial cell activation, vasoconstriction/vasodilation, immune regulation [81, 82, 113]
BAI1/ADGRB1		●			Release of vasculostatin-40: inhibition of angiogenesis [45]
BAI2/ADGRB2	●				Unknown [48]
GPR116/ADGRF5	●				α-Fragment: unknown role in cellular signaling [38]
LPHN1/ADGRL1	●				Regulation of surface expression, generation of soluble receptor forms [46, 77]
GPR126/ADGRG6	●				Release of secreted ligand which regulates myocardial energy metabolism, contractility, and trabeculation [47, 72]

3.1 Protease-Activated Receptors (PARs)

Apart from aGPCRs, there are also other members of the GPCR superfamily that require cleavage for their biological activity, e.g., for the initiation of their signaling cascade. Thus far, four protease-activated receptors have been identified, PAR1–4 [78], each following a canonical activation principle. PAR1 is considered the prototype receptor of the family. It is activated by the serine protease thrombin, a key regulator of platelet aggregation, endothelial cell activation, and further vascular effects [79–81].

For activation of human PAR1, thrombin cleaves the ectodomain of the receptor at a specific recognition site (LDPR↓S) that is located at position 41 of the receptor molecule [82]. The resulting new N-terminus contains a tethered agonist that becomes unmasked upon the proteolytic event (Fig. 3, Table 1). The agonist physically interacts with the 7TM domain of PAR1 and activates its signaling cascade. Furthermore, synthetic peptides comprising the first six residues of the unmasked tethered agonist are capable of activating PAR1, even without prior receptor cleavage [82]. Additionally, genetic exchange of the cleavage site, e.g., to a trypsin cleavage site, resulted in receptor activation through trypsin under heterologous expression conditions [83, 84], confirming the hypothesis that the role of thrombin comprises the exposure of the receptor's tethered agonist [85, 86].

An activation mechanism which shares similarities with the proteolytic activation of PAR1 has recently been unraveled for GPR126, GPR133 [52], and further aGPCRs [63–65] (see above). However, several differences to PAR activation have to be considered: while PAR1 processing through the exogenous thrombin protease directly leads to the exposure of its tethered ligand, GAIN domain-mediated GPS cleavage of aGPCRs alone may not be sufficient to unmask the *Stachel*/*stalk* agonist of selected aGPCRs as the cleavage fragments remain attached to each other. Further structural changes in their extracellular domain, e.g., through ligand binding to the adhesion domains within the receptor ectodomain similar to the situation of the Notch receptor, and/or mechanical removal of the NTF may be required for *Stachel*/*stalk* exposure and aGPCR activation [87–89].

However, studying properties of PAR receptors may reveal additional parallels to the signaling paradigm(s) utilized by aGPCRs. For example, rapid phosphorylation-dependent internalization of activated PAR molecules and subsequent lysosomal degradation terminate PAR1 signaling [90, 91]. At least one aGPCR study suggests that ligand contact and mechanical challenge of CD97 trigger removal and degradation of the receptor's CTF, thereby providing means to quench signaling through aGPCRs [87].

3.2 Notch

Developmental signals governed through the activation of the Notch receptor are arguably the best researched and understood functions that result from proteolytic processing of a receptor molecule. The Notch receptor consists of the single-pass

transmembrane protein and contains a species-specific array of up to 36 epidermal growth factor-like (EGF) repeats strung along the length of its extensive ectodomain. Through the ectodomain, the receptor interacts with DSL ligands (Delta, Serrate/Jagged, LAG-2), which themselves are large type I transmembrane molecules mounted on neighboring cell surfaces to the Notch-bearing cell [114, 115], thereby resembling the interaction scenario of several aGPCRs and their cellular ligands, e.g., CD97 with CD55 [116] or Latrophilins with FLRTs, teneurins, and neurexins [117–119].

An important consequence imposed by the positional Notch receptor-ligand configuration is the restriction of signaling events to cellular neighbors [120], which may also figure in the physiological roles of aGPCRs. This restriction is critical for the developmental switches governed by the Notch pathway, as it regulates binary cell fate decisions during embryogenesis, organogenesis, and cell differentiation, and many examples across the tree of metazoan life bear witness to the generality of this concept [104, 121]. In the classical paradigm of the Notch receptor-DSL ligand interplay, two daughter cells deriving from a precursor blastomere inherit equal amounts of both Notch and DSL. Engagement of Notch and ligand at the cell contact faces initiates an iteratively looping feedback cycle, which culminates in downregulation of the receptor in only one of the two cells. In the 'winner cell', the intracellular actions of Notch repress a proneural gene battery and drive it into the epidermal cell lineage. Conversely, the cell that has lost Notch on its surface becomes a neuronal precursor cell [122].

Intriguingly, the activation of the Notch receptor molecule is the consequence of a cascade of at least four cleavage events that sequentially process the receptor molecule along its N→C axis (Fig. 3, Table 1). First, after biosynthesis and en route to the cell surface, the receptor is cleaved by a furin-like convertase at the S1 cleavage site severing most of the receptor's ectodomain including the ligand-binding EGF repeats from a fragment holding the juxtamembrane, transmembrane, and intracellular receptor portion. S1 cleavage, however, does not result in physical separation of the cleavage fragments as they form a heterodimer held together through non-covalent interactions [123, 124], resembling the situation of aGPCRs that have undergone GAIN autoproteolysis but appear as heterodimers at the plasma membrane [4, 125].

The second proteolytic step occurs at the S2 site, which is positioned C-terminal to the S1 site just above the transmembrane helix. Before activation, the S2 site is protected by an arrangement of three LIN-12/Notch (LNR) repeats that are grouped around the cleavage site blocking access for the cognate S2 metalloproteases Kuzbanian and TACE/ADAM10 [126, 127]. When Notch engages with its DSL ligand, DSL endocytosis is thought to generate mechanical forces pulling at the receptor molecule, which eventually leads to conformational unwinding of the LNR repeats and exposure of the S2 site and its cleavage [128, 129]. While S1 cleavage is dispensable for Notch function [130], S2 cleavage appears as the gatekeeping step in Notch activation rendering the pathway a developmental command control system that may be triggered by mechanical input and may thus share similarities with the role of aGPCRs in development.

After S2 proteolysis, the remaining transmembrane-intracellular fragment of the Notch receptor [Notch extracellular truncated (NEXT)] undergoes further regulated intramembrane cleavage (RIP) catalyzed by the γ-secretase complex. This large multi-protein enzyme cleaves the NEXT intermediate at two further sites inside the membrane (S3 and S4 sites) [126, 127, 131, 132]. Ultimately, S3/S4 proteolysis results in the release of the Notch intracellular domain (NICD), which heteromerizes with DNA-binding and transcriptional activation partners and travels into the nucleus, where the complex controls the expression of target genes [133–136].

3.3 Ephrins

Apart from Notch, proteolysis through ADAM10 assumes a central position in the processing of a number of other neuronal proteins like APP, N-cadherin, neuroligins, or ephrins [96, 106, 108, 110]. Among those, the proteolytic activation and physiological relevance of the signaling mode of ephrin-A2 are exemplary (Fig. 3, Table 1).

Ephrin-A2 is a GPI-anchored molecule that is cleaved by ADAM10 upon binding its endogenous receptor EphA3. The binding and cleavage event consequently disrupts the cell-cell contact mediated through the Eph/ephrin interaction [108]. Upon the formation of the ligand-receptor complex, the molecular recognition motif in ephrin-A2 is rendered accessible for ADAM10, which then associates with this complex and cleaves ephrin-A2 in a *trans* mode, as protease and substrate are expressed in different cells [137, 138]. It appears that this mechanism ensures the exclusive cleavage of receptor-bound ligands [137]. Following the proteolytic rupture of the intercellular connection, the Eph/ephrin complex is rapidly internalized into the receptor expressing cell [138], which has been shown for ephrin-A5, a related member of the ephrin family [139]. Blocking the Eph/ephrin complex binding site of ADAM10 using specific monoclonal antibodies resulted in impaired internalization and EphA3-mediated cell function, suggesting a physiological role for the cleavage [140].

Ephrins and their Eph receptors are generally involved in the guidance of cell migration and neural development, tissue separation, and synaptic plasticity [139, 141], but also in extraneuronal processes including vascular development, epithelial cell response, and inflammation [142–144]. One particular physiological function of ephrin-A2 is the control of axon guidance [108] and involves proteolysis through ADAM10. Migrating EphA3-presenting axons come in contact with cells expressing ephrin-A2. Upon this encounter, the EphA3-positive neurites are actively repelled by the proteolytic disruption of the Eph/ephrin connection and thereby lead to axon withdrawal and precise spatio-mechanical control of neurogenesis [108, 145]. Inside the cell, regulation of this signal is mainly communicated through the intrinsic tyrosine kinase activity of ephrin receptors. Phosphorylation-dependent activation of EphA3 triggers a conformational change shifting the kinase domain away from the plasma membrane into its active form

[146, 147], where it no longer obstructs the alignment with ADAM10 and therefore allows ephrin shedding [146, 148]. Accordingly, EphA3 mutants carrying a constitutively released kinase domain showed increased ephrin cleavage by ADAM10, even when kinase function was disabled [146]. Thus, tyrosine kinase activity of ephrin receptors is an intracellularly regulated means to switch between cell-cell repulsion (high activity) and cell-cell adhesion (low activity) [146, 148, 149].

This binary signaling of Eph receptors may bear functional and cell biological similarities to aGPCRs. Their variety of extracellular adhesion motifs are predestined for intercellular cell-cell interactions like those observed for Eph/ephrin, although the majority of aGPCRs are still orphaned without known ligands or intracellular interactors [1]. aGPCR-ligand complexes could conceivably be shed involving an exogenous protease (see discussion about furin-mediated cleavages of individual aGPCR homologs above). Equally possible, mechanical force may solely govern receptor fragment (NTF-CTF) separation at the breakpoint originating from receptor autoproteolysis at the GPS. This way, aGPCR-expressing cells may be able to switch from an adhesion to signaling state.

3.4 Polycystins

PKD1 and *PKD2* are genes encoding polycystins, which are multitransmembrane proteins with a large amino-terminal extracellular domain [150]. *PKD* mutations have been demonstrated to cause one of the most common genetic diseases worldwide, the autosomal-dominant polycystic kidney disease (ADPKD) [151], which is characterized by the formation of multiple fluid-filled cysts that lead to renal failure in patients [152]. Loss-of-function mutations in polycystin-1 (*PKD1*) are responsible for a vast majority of ADPKD cases with physiological functions of *PKD1* found in cell adhesion and cell junction formation [153, 154]. Consistent with these findings, polycystin-1 appears involved in mechanical coupling between cells and in the regulation of tubular lumen diameter along the nephron [155].

Interestingly, polycystin-1 shares several structural features with aGPCRs (Fig. 3). They possess a multi-pass transmembrane domain (with 11 instead of 7 helices), an extended ectodomain with arrays of PKD motifs, and most notably a GAIN domain [19]. Similar to aGPCRs, polycystin-1 undergoes autoproteolysis resulting in the generation of an NTF and CTF [17], which remain non-covalently attached after cleavage. The physiological role of the polycystin-1 GPS cleavage is unknown. However, cleavage-deficient mutants exhibit impaired function in vitro [17] and in vivo [18]. A *PKD1* GPS proteolysis-deficient mouse mutant shows abnormal renal development after the first days of postnatal life apparent in reduced size and weight, as well as grossly enlarged cystic kidneys. Furthermore, the mutation is lethal within 6 weeks after birth presumably due to renal insufficiency [18]. This is only partly compatible with defects displayed by $PKD1^{-/-}$ mice, which show severe embryonic phenotypes and die already a few hours after birth [156]. Therefore, it was concluded that GPS cleavage of polycystin-1 is required for postnatal renal maturation, while it is not essential for

embryonic nephrogenesis [18]. This is supported by the fact that cleaved and uncleaved polycystin-1 can coexist under physiological conditions [155]. Interestingly, polycystin-1 is also substrate to proteolytic events in addition to GAIN domain autoproteolysis, as it, too, is cleaved by the γ-secretase complex, resembling S3 and S4 proteolyses of the Notch receptor [157, 158].

4 Conclusions

aGPCRs are by far not the only group of biomolecules whose actions are controlled through proteolytic cleavage. Here, we only concentrated on those that are governed by the classical proteases and autoproteolytic events. As shown here, autoproteolysis and proteolytic cleavage seem neither mutually exclusive nor are their functional implications in aGPCRs sufficiently understood. Considering the tremendous number of surface receptors controlled through proteolysis, and the requirements for their function, there is no doubt that elucidation of the physiology of aGPCRs requires further investigation of their proteolytic properties. This should include a better understanding how GAIN domain-mediated cleavage is involved in receptor signaling and resolve the question if and how it may be modulated, e.g., through allosteric mechanisms. Further interest should be directed toward the study of other aGPCR domains that entertain non-GAIN domain autoproteolytic steps and the role of other proteases in the processing of the receptor molecule and pin down their physiological roles in receptor trafficking, cell adhesion, metabotropic, and non-cell-autonomous signaling. The structural and physiological properties of other surface molecule systems including the PAR, Notch, ephrin, or polycystin pathways should be considered as examples of how proteolytic processing can shape the function of receptor modules. The extensive body of work accumulated on their biological significance can instruct new experimental avenues and working models that should be explored in the quest to elucidate the interplay between the proteolytic processing of aGPCRs and their diverse signaling profiles.

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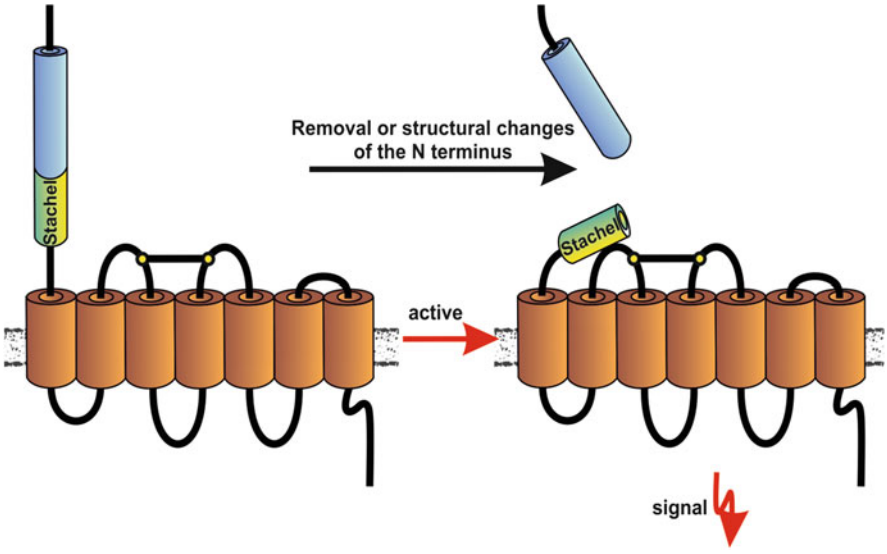
Tethered Agonism: A Common Activation Mechanism of Adhesion GPCRs

Ines Liebscher and Torsten Schöneberg

Graphical Abstract



Adhesion GPCRs harbor a tethered agonist sequence (reproduced from [24])

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Contents

Abstract

As the past years have seen a magnificent increase in knowledge on adhesion GPCR (aGPCR) signal transduction, the time had come to fill the gap on how these receptors can be activated. Based on experimental observations that deletion of the ectodomain can induce signaling, the idea arose that aGPCRs, just like other atypical GPCRs, may harbor a tethered agonist sequence. In this chapter, we describe the recent findings and characteristics of this agonist, called the *Stachel* sequence, and discuss potential mechanisms that cause liberation of this encrypted sequence. Further, we provide perspectives for application of *Stachel*-derived synthetic peptides in future studies of aGPCR function.

Keywords

Adhesion GPCR • Signal transduction • Tethered agonism • Activation mechanism • Peptide agonist

1 Adhesion GPCRs Harbor an Encrypted Agonistic Sequence, the *Stachel* Sequence

In classic pharmacology, an agonist (activating ligand) is a compound that specifically binds to its receptor, induces conformational changes (activation), and produces a biological response. This liganded and unliganded conformational stage of the receptor is formalized as:

$L + R \Leftrightarrow LR$, where the quotient of k_{on} and k_{off} is the equilibrium constant K_a. The reciprocal of K_a, the dissociation constant K_d, is a direct measure of the receptor-agonist affinity. The kinetics of most agonist-receptor pairs follows this equilibrium where the agonist reaches its receptor by diffusion and attaches to the binding pocket. However, a few exceptions exist from this classical model. Rhodopsin, a light-sensitive G-protein-coupled receptor (GPCR), is the most prominent example. Rhodopsin and other opsins contain a chromophore as a prosthetic group and are specialized for the detection of quanta of light. Here, the ligand is covalently bound to the receptor, and a single photon isomerizes the 11-*cis*- to *trans*-

retinal triggering receptor conformation changes and G-protein activation [1]. At least during rhodopsin activation, agonist diffusion and concentration are not part of the process allowing extreme fast off/on kinetics.

The concept of covalently carrying an agonist that isomerizes or is exposed by an external signal is brought to perfection in protease-activated receptors (PAR). PAR1 is a GPCR that is classically activated through cleavage of the N-terminal ectodomain by the serine protease thrombin exposing an agonistic amino acid sequence (SFLLRN) within the N terminus [2]. The agonist is covalently bound during activation, but it is not a prosthetic group as in the case of rhodopsin. Here, cleavage seems to isomerize, relax, or expose the peptidic agonist into the activating conformation. Not only specific cleavage by thrombin but also by other proteases such as trypsin and plasmin activates PARs [3]. Interestingly, matrix metalloproteases (MMP-1 and MMP-13) cleave the N-terminal ectodomain of PAR1 at noncanonical sites, which results in distinct tethered ligands that activate G-protein signaling pathways [4]. An integration of diverse signals into a defined biological response, e.g., during platelet activation and aggregation, is most probably the biological meaning behind this stimulus diversity.

All adhesion GPCRs (aGPCRs) have a large ectodomain providing manifold interaction possibilities with other molecules (see also [5]). One can speculate that the ectodomain may serve as an integrator for diverse signals. This concept, however, would require a tethered agonist as in the case of rhodopsin and PARs. This chapter features the discovery of such internal agonistic sequences—termed *Stachel* sequence—in aGPCRs and discusses the mechanisms of exposing, isomerizing, or relaxing this tethered agonist.

1.1 Identification of the *Stachel* Sequence

Taken the enormous pharmacological potential of aGPCRs, the identification of the endogenous agonists or molecules that modulate their activation levels was a major task in this field. However, the screen for ligands was limited by the unknown signal transduction of aGPCRs. This knowledge is a prerequisite to establish reliable readout systems of receptor activation.

Its structural relation of the 7TM to other GPCRs suggested G-protein coupling and a number of studies tested for the capability of aGPCRs to activate these classical signaling cascades. Overexpression of receptor variants has been used to identify the signaling pathways of several GPCRs because of increased basal activity in heterologous expression cell system [6–8]. The same approach was applied to identify the G-protein-coupling abilities of several aGPCRs: GPR56/ADGRG1 [9, 10], CD97/ADGRE5 [11], GPR133/ADGRD1 [12], GPR114/ADGRG2, GPR97/ADGRG3, GPR110/ADGRF1, GPR115/ADGRF4 [13], BAI1/ADGRB1 [14], GPR126/ADGRG6 [15], VLGR1/ADGRV1 [16], GPR64/ADGRG2 [17, 18], and LAT-1/ADGRL1 [19]. Interestingly, even with these readout systems at hand, the search for endogenous ligands did not yield any activating compounds for a long time.

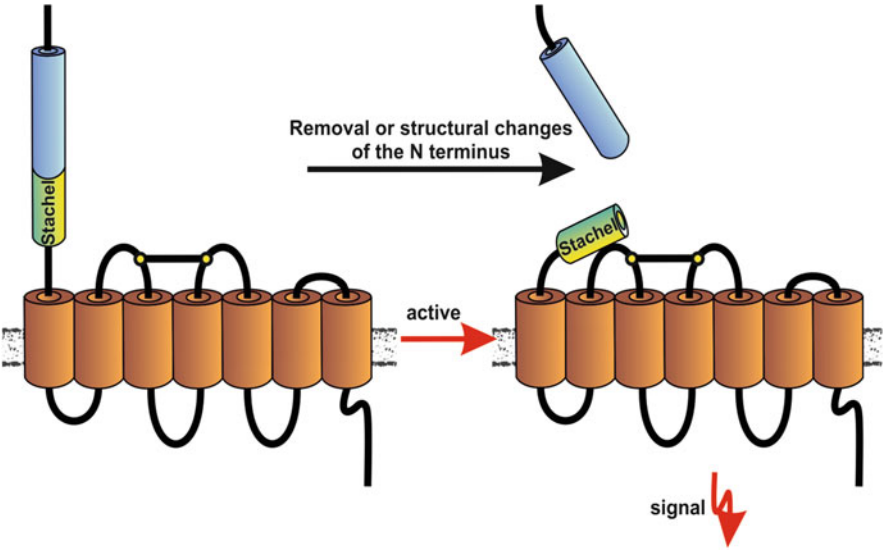

Fig. 1 Adhesion GPCRs harbor a tethered agonist sequence (reproduced from [24])

An important contribution to the identification of the aGPCR activation mechanism was the observation that receptor activation was seen upon deletion of the N-terminal fragment (NTF) of BAI2/ADGRB2 [20], GPR56 [21, 22], and CD97 [11]. Two possible scenarios were suggested to explain this deletion-induced activation [23]: (1) the N terminus acts as an inhibitor of the constitutively active C-terminal fragment (CTF) or (2) the residual part of the N terminus contains an agonistic sequence. To shed light on the underlying mechanism, the NTFs of two aGPCRs, GPR126 and GPR133, which had been shown to couple to the G_s protein/adenylyl cyclase pathway were deleted, and, as expected, subsequent increases in their signaling activity were seen [24]. However, upon deletion of the amino acids N-terminally of the transmembrane helix 1 (TM1) that remained in the CTF, receptor activity was abolished, favoring a tethered agonist model over an inhibitory NTF. These data suggested the presence of a tethered agonist between the natural cleavage site and the N terminus of TM1. Indeed, synthetic peptides derived from this sequence were capable of activating the inactive receptor mutants as well as the full-length GPR126 and GPR133 (Fig. 1). Based on the exposed position of this region after removal of the NTF and its expected piercing interaction with the potential binding pocket within the 7TM region, the tethered agonist sequence was named the "*Stachel*" sequence, which is the German word for "stinger." Mutational analysis of CTF mutants and derived peptides revealed that an exchange of the first amino acid of the tethered agonist to alanine is tolerated for sustained receptor function, while positions 2–7 are essential for CTF mutant and peptide activity. In line with these results, a mutant GPR126 zebrafish was designed, which lacked the amino acids 4 and 5 after the cleavage site of the GPS within the full-length receptor

[24]. This mutant zebrafish exhibited the same phenotype as the previously published complete GPR126 gene knockout zebrafish strain [25] presenting with a pronounced myelination defect and so-called puffy ears. All these in vitro and in vivo data support the hypothesis that the residual part of the N terminus in the CTF contains an agonistic sequence.

Later a tethered agonist sequence was identified for several other aGPCRs, namely GPR110 and GPR56 [26], GPR64 [17], GPR114 [27], and LAT-1 [19]. However, a recent study indicated that CTF mutants of GPR56 and BAI1 do not exclusively elicit constitutive activity [28] (see also [29]). While GPR56 CTF showed increased activity in a serum response factor reporter assay, all other CTF constructs examined did not show elevated activity in other activity measures. Whether these findings hold true upon stimulation with agonistic peptides needs to be determined since the repertoire of the receptors' signal transduction may significantly differ between agonist and mutationally induced activity as shown for the aGPCR GPR64 [17].

1.2 Structure and Genomic Organization of the *Stachel* Sequence

At the current state, there is no X-ray crystal structure of a full-length aGPCR available. However, partial structures of aGPCR ectodomains are solved [30, 31] (see also [5]). There are two crystal structure models available for LAT-1 and BAI3 that reach from the functional "sticky domains" to TM1, thereby including the mucin-like stalk region, the highly conserved GPCR proteolytic site (GPS), and the *Stachel* sequence [30]. Since it was shown that this crystallized domain is required and sufficient for cleavage within the GPS, it was called the GPCR autoproteolysis-inducing (GAIN) domain. Projecting our functional data onto these crystal structures, it is conceivable that the *Stachel* sequence matches the most C-terminal β-strand of the GAIN domain. Interestingly, this β-strand is encapsulated in a β-sandwich of 12 additional β-strands [30]. The crystal structures suggest that the *Stachel* sequence is buried between those β-strands prohibiting the exposure of the agonistic sequence within the full-length receptor. As the *Stachel* sequence presents with an assembly of hydrophobic amino acids (Fig. 2), exposition of this region to a hydrophilic environment is unfavorable and most probably supports interaction with its hypothetical binding pocket, whose exact position needs yet to be determined.

One important factor for *Stachel*-mediated receptor activation is the length of the β-strand. Screening of peptide libraries varying in length revealed active peptides of the *Stachel* region between 7 and 18 amino acids (see below). This is in accordance with our mutagenesis-based findings that amino acids 2–7 are essential for activity in GPR126 (see above). We therefore defined the first 8 amino acids of the *Stachel* sequence as the core region. Interestingly, the core regions of the *Stachel* sequences are always encoded by a single exon in a module-like fashion (Fig. 2). There are natural splice variants differing in the length of the *Stachel* sequence. For example, there are two isoforms of GPR114, which diverge only in the existence of a single

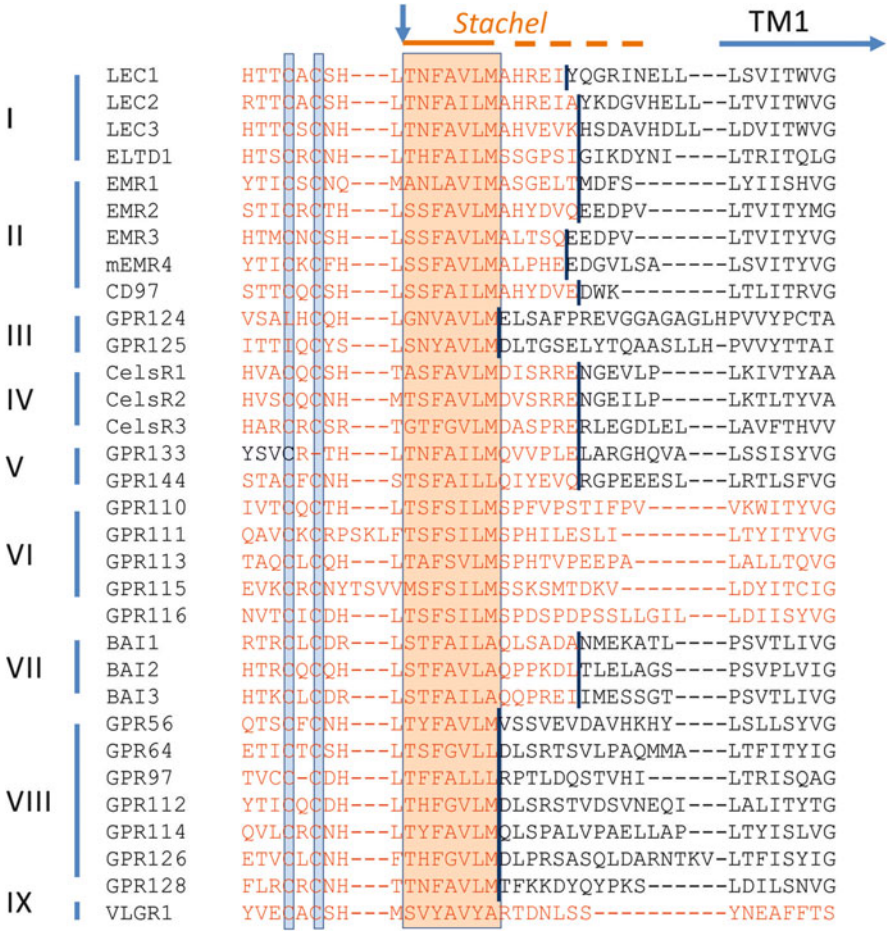

Fig. 2 *Stachel* sequence of adhesion receptors. An alignment of the amino acid sequences of all human adhesion GPCRs in close proximity to the transmembrane helix 1 (TM1) is shown (most of the N terminus is not included in the alignment). Two highly conserved cysteine residues N-terminal of the potential autocleavage site (*arrow*) are boxed in *blue*. The core sequence of the *Stachel* sequence is boxed in *orange*. Many active peptides derived from this region are longer than the core *Stachel* sequence and are marked by a *broken orange line*. Interestingly, in several families, the C terminus of the *Stachel* core sequence is defined by an exon/intron boundary (*black line*; see text). The *Stachel* core sequences are encoded by a single exon (*red characters*). Note: GPR123 was not included in the alignment since this sequence does not show any relevant sequence similarities in this region

glutamine within the *Stachel* sequence, yet display vast differences in basal full-length and mutant activity levels [27]. The isoform with the glutamine elicits very high basal activity, which can be increased through deletion of the NTF, while the isoform without the glutamine has low basal activity levels which cannot be elevated

further. Deletion of the glutamine within the synthetic peptide does not affect its activation potential. As this amino acid is only important within the receptor structure, it can be assumed that it plays a role in positioning the *Stachel* sequence relative to the 7TM (see also [32]). The shortened agonistic sequence upon amino acid deletion is not able to reach its binding pocket, thereby failing to activate the receptor. This hypothesis is supported by the fact that the deletion of the adjacent leucine showed the same lack of activation, while both mutants are expressed in the cell membrane and can be activated through external peptide application [27].

Taking a closer look at the genomic structure of aGPCRs, it became evident that the GPR114 isoforms are in fact two splice variants as the glutamine is positioned at the beginning of exon 8 [27]. Here, alternate splicing can be seen as a regulatory event in order to control GPR114 function. It is therefore not surprising that the distribution of the variants shows tissue specificity. Analysis of the whole aGPCR family revealed that both families III (except GPR123, which has no GPS) and VIII have an intron/exon transition at the same location within the highly conserved *Stachel* sequence (Fig. 2), supporting the idea of an evolutionary conserved way of regulating receptor activity.

1.3 Proposed Mechanisms of *Stachel* Release

While the existence of activating peptides derived from the *Stachel* sequence is accepted, the concept of how the liberation of this sequence should happen in vivo is still a matter of debate. Current models favor an activation scenario in which the ligand binding to the NTF together with the exertion of mechanical stress will lead to its removal and subsequent exposition of the tethered agonist [33, 34], and the presumptive function of aGPCRs as mechanosensors is extensively discussed in [35]. This hypothesis is supported by the fact that GPR126 and GPR114 can be activated in vitro through mechanical forces like shaking and vibration [27, 33]. - Shaking-induced activation depends on a functional *Stachel* sequence because no mechanical activation is seen when inactivating mutations are introduced into the core *Stachel* sequence. The in vivo correlate to mechanical activation of GPR126 is the polymerization of its ligand laminin 211, which, when impaired, mimics the phenotype of the receptor knockout in zebrafish [33]. Further hints for the above suggested activation scenario come from a study that observed an enrichment of the NTF of GPR56 in supernatant after receptor stimulation with its ligand collagen III [36]. There is further in vivo evidence that aGPCRs are involved in mechanosensing. Thus, signaling of GPR56 regulates muscle hypertrophy associated with mechanical overload in mice, [37] and latrophilin/CIRL mediates perception of tactile, proprioceptive, and auditory stimuli in *Drosophila* [38].

Although binding of extracellular proteins to the NTF and the removal of the autocleaved NTF by mechanical forces are an intriguing activation model, it does not explain how non-cleavable receptors get activated through their tethered agonist or how shaking of the receptor without the addition of the ligand can already lead to an increase in signaling. It further fails to explain how it is possible that the same

aGPCR that is thought to be activated through NTF removal after ligand binding can be directly activated through a different ligand. For example, the other known ligand of GPR126, collagen IV, has been shown to induce cAMP production on GPR126-expressing cells without exposure to mechanical forces [39]. Also, GPR56 has been shown to be internalized with its ligand transglutaminase 2 (TG2) meaning that there are also no cleavage or shedding processes involved since TG2 binds within the NTF of GPR56 [40].

The existence of an autoproteolytic event and its biological function has probably been one of the most controversially discussed facts in the aGPCR field in the past years and is discussed in detail in [41]. With the initial discovery of this event for latrophilin 1 (CIRL) [42] and further detailed description of the mechanism underlying this process [43], it had been assumed that the majority of aGPCRs should undergo this process and that it is a specific requirement for proper receptor function [44]. However, several studies have questioned this assumption as a general feature of aGPCRs. As for the cleavage event, there are aGPCRs that have a disrupted motif (GPR111 and GPR115; see Fig. 2) and that are therefore accepted to be non-cleavable [45]. BAI1 has a classic cleavage site, but it was shown that cleavage of this receptor does not occur in HEK293 cells but in malignant melanoma cells (discussed in [46]). Of note, BAI1 is properly expressed in the cell membrane even without cleavage. The question how an autoproteolytic event can be cell-type specific has not been answered, yet. For many other aGPCRs, it has never been tested whether cleavage actually occurs. Several studies show that cleavage is dispensable for proper receptor function [12, 47]. Interestingly, GPR114 displays high constitutive activity [13] but is not cleaved [13, 27]. Yet, GPR114 contains a tethered agonist sequence and can be activated through *Stachel* sequence-derived peptides. These findings are not compatible with a scenario in which the tethered agonist is liberated through the removal of the NTF. The high constitutive activity of GPR114, which mutually depends on the intactness of the *Stachel* sequence [27], is also not compatible with the current crystal structure of the GAIN domain [30]. Since the *Stachel* sequence is shielded by β-sheets in this structural model, it would be blocked from interacting with the 7TM to mediate the high level of basal activity. Current functional data rather favors a scenario where the *Stachel* sequence is prebound in the binding pocket of the 7TM. Upon binding of an extracellular ligand and/or exposure to mechanical stress, the inactive conformation of the *Stachel* sequence then changes into an active conformation. In the constitutively active isoform of GPR114, the *Stachel* sequence seems to be already in its active conformation. Such isomerization-induced activation of a GPCR is found in rhodopsin but needs to be proven for aGPCRs, e.g., by crystallizing full-length receptors with or without activating ligands bound to the ectodomain.

1.4 Tethered Agonism: A Common Activation Mechanism in GPCR?

As introduced above the existence of an agonistic sequence that is bound to its receptor is rare but has been shown for rhodopsin-like GPCRs. The prototypic and most intensively studied GPCR, rhodopsin, is just one example. Here, the chromophore 11-*cis*-retinal, a prosthetic group, is covalently linked to the apoprotein opsin. When exposed to light, 11-*cis*-retinal isomerizes to an all-*trans* state. The resulting metarhodopsin II confers then the signal via transducin into the cell [48]. In the case of the PARs, the receptors harbor a tethered agonist within their N termini, which is released through enzymatic cleavage of the sequence N-terminal. As in aGPCRs, this agonist sequence can be mimicked using synthetic peptides [49]. For PARs it is not clear whether the tethered ligand is prebound and only isomerizes. Very recently we have shown that glycoprotein hormone receptors (GPHRs), also members of the rhodopsin-like GPCR family, are activated by an internal agonist upon glycoprotein hormone binding [50]. Similar to findings on aGPCRs, studies have shown that removal of the large ectodomain can lead to GPHR activation. For example, proteolytic cleavage of the GPHR ectodomain by trypsin or artificially generated ectodomain deletions and truncations increased TSH receptor activity [51–53]. We have shown that deletion of the entire ectodomain did not activate the LH/hCG receptor which supported an alternative hypothesis of an "intramolecular agonistic unit" where an internal agonist within the ectodomain is exposed upon ligand binding [54, 55]. This "intramolecular agonistic unit" was later identified as p10 region, integrating the activating actions of glycoprotein hormones, mutations, and autoantibodies to trigger GPHR signal transduction [50].

Reflecting the findings with aGPCRs and other GPCRs which contain internal agonists, one can speculate that attachment of large ectodomains to the conserved 7TM structure of GPCRs has the general functions:

1. To allow for G-protein signaling for very large proteins (e.g., matrix or cell-bound proteins) which do not fit into the classical 7TM binding pocket
2. To integrate diverse extracellular signals (different proteins, ions, mechanical forces) into the same G-protein signaling pathway of a given cell/tissue

Latter point is further diversified by the fact that alternative splicing allows for modularly defining the signals that may interact with the ectodomain and, as shown for GPR114, fine-tunes the basal activity of a given receptor. It is therefore conceivable that aGPCRs might present a collection of all mechanisms known to activate the tethered agonist sequence. Current knowledge allows for the conclusion that the majority, if not all aGPCRs, carries a tethered agonist sequence, but future studies will have to focus on unraveling the molecular mechanisms exposing the tethered agonist.

2 Synthetic Peptides Derived from the *Stachel* Sequence as Tools to Modulate aGPCR Function

2.1 Pharmacologic Properties of the Peptide Agonists

Short peptide sequences mimicking the *Stachel* sequence have been shown to activate aGPCRs in vitro. The length of the most efficient peptides varies between 7 and 18 amino acids (pGPR126, 16 AA [24]; pGPR133, 13 AA [24]; pGPR110, 12 AA [26]; pGPR56, 7 AA [26]; pGPR64, 15 AA [17]; pGPR114, 18 AA [27]; pLPHN-1, 12 AA [19]). It is noteworthy that the activating peptide of GPR56 differs from all other peptide agonists in a way that it is by far the shortest peptide identified and that it was the only peptide out of the whole library tested on GPR56 that elicited some activation [26]. All other libraries featured at least one more peptide with similar activity levels. The *Stachel* sequences are highly conserved regions of aGPCRs which explain that peptides can activate more than one species. Thus, synthetic peptides have been shown to activate different receptor orthologs like human, mouse, and zebrafish. For example, a peptide derived from the human GPR126 *Stachel* sequences is able to activate also the zebrafish ortholog, and a peptide derived from the human GPR133 *Stachel* sequences can also activate the mouse GPR133 [24]. Yet, no cross-species activity was found for peptides derived from LPHN-1 of rat and *C. elegans* [19].

As the *Stachel* sequence is highly hydrophobic, the resulting peptides are similarly difficult to dilute in assay reagents making it sometimes hard to work with them. However, within the peptide library derived from the same receptor, there has been no correlation between the activity induced by and the solubility of the peptide. Another obstacle in working with these peptides is that they require large amounts in order to elicit significant activation levels in vitro with EC_{50} values between ~80 and 400 μM. The reason for this low affinity interaction can be explained by the physiologic 1:1 stoichiometry of the tethered agonist and the 7TM interface which makes high affinity dispensable. Similarly, an effective concentration of 0.4 mM of the tethered agonistic peptide was determined for PAR1 [56]. Even as such high concentrations are needed, the interaction of each peptide with its derived receptor seems to be highly specific for a given aGPCR [17, 24]. However, we cannot exclude that peptides derived from *Stachel* sequences of closely related aGPCRs can cross-activate. The *Stachel* sequences within but also between aGPCR groups are very similar (see Fig. 2). Future analyses will study possible cross activations in more detail.

2.2 Synthetic Peptides as Tools for Ex Vivo and In Vivo aGPCR Studies

Despite the difficulties that arise from the properties of the synthetic peptides, they are resourceful tools to study aGPCR function ex and in vivo. They have been shown to rescue pathological phenotypes in receptor-deficient animal models.

Thus, a GPR126 hypomorph zebrafish showed significant improvement of the myelination deficit upon treatment with 100 μM of peptide solution [24]. Further, the lethality phenotype of LAT-1 mutant nematodes was rescued with the same peptide concentration [19].

The low solubility and affinity of most *Stachel* sequence-derived peptides limit in vivo studies. Chemical modification of the peptides may help to improve their solubility. Investigations are launched which systematically test modifications of amino acids in order to increase peptide affinity to the 7TM. This may also help to define the 7TM binding pocket.

Due to the peptide nature and the high concentration needed for stimulation, in vivo experiments essentially require the development of more potent and specific small molecule ligands. As in the case of the GPHRs [57–59], such small molecule compounds may bind in the 7TM at ortho- but also allosteric positions and modulate 7TM activity. Beclomethasone which apparently activates GPR97 [13] is a promising example for such small molecule screening strategies.

3 Summary and Future Perspectives

Based on the currently available information, activation of several aGPCRs is conducted via a tethered agonist sequence, which resides within the very N-terminal part of the CTF. It is conceivable that this could be applicable for all members of the aGPCR class, yet it still needs to be proven for the majority of aGPCRs. While synthetic peptides that are mimicking the agonistic sequence can readily activate the receptor they are derived from, the question remains how this would be exercised under physiological conditions. According to the available crystal structure, the *Stachel* sequence, being equivalent to the last β-strand of the GAIN domain, is embedded in other β-strands shielding it from exposure to the 7TM interface. It is estimated that only structure-disruptive forces could liberate this sequence. This notion is supported by the fact that the combination of mechanical force and ligand addition lead to receptor activation which was not elicited under ligand addition itself. This model, however, requires a cleavable receptor. Taken that there are several non-cleavable receptors out of which at least one (GPR114) is highly constitutively active; it is eminent that there have to be other activation scenarios that need to be considered. The isomerization of a prebound agonist is one possible scenario. However, future studies are needed to answer this question. Especially the establishment of crystal structures that encompass the full-length aGPCR in its active and inactive state should provide clarity.

Even though the molecular characterization of aGPCR function has leaped forward tremendously in the past years, there are still several gaps to close in our understanding. First of all, the intracellular pathways following receptor activation need to be unraveled for the remaining orphan aGPCRs. Further, after looking at activation mechanisms, the processes involved in ending the signaling cascades need to be examined more closely. Interaction with β-arrestin and ubiquitination has only been shown for BAI1 [14], and studies dealing with internalization of

aGPCRs are effectively missing. Knowing that there is most likely an interaction of aGPCRs with β-arrestin, the question arises if these receptors just like rhodopsin-like GPCRs have biased ligands. Is it possible that endogenous ligands under different mechanical conditions could activate distinct signaling cascades? In order to answer that question, it is mandatory to determine and define the specific kinds of mechanical stress that are needed to elicit receptor activation.

As peptides are not ideal activators for in vivo studies for several reasons (low affinity, solubility, and degradation issues), the development of small molecule agonists, antagonists, and inverse agonists is decisive. Another aspect for future experiments is to identify the potential of *trans* signaling for pharmacological studies. In vivo models are already distinguishing between the consequences of a signal that is mediated by the CTF within the cell (*cis* signaling) and the effects transduced by the NTF (*trans* signaling) [33, 47, 60]. In vitro studies could test for auto- or cross activation of purified functional domains of the NTF of aGPCRs. Such an approach was already undertaken for processed parts of the NTF of GPR116 [61]. Here, a purified part of the NTF, called the alpha fragment, was used to show influence on the expression levels of intracellular signaling molecules. As most aGPCRs display highly modular ectodomains, it is conceivable that similar observations can be seen in the future.

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Versatile Signaling Activity of Adhesion GPCRs

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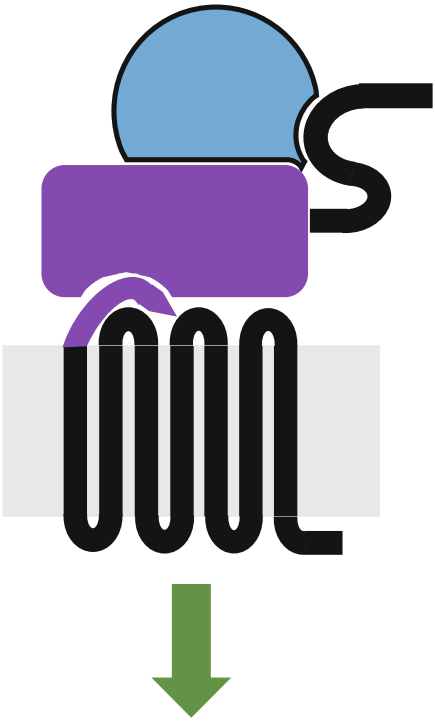
Graphical Abstract



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Contents

Abstract

The adhesion G protein-coupled receptors (aGPCRs) are a family of 33 receptors in humans that are widely expressed in various tissues and involved in many diverse biological processes. These receptors possess extremely large N-termini (NT) containing a variety of adhesion domains. A distinguishing feature of these receptors is the presence within the NT of a highly conserved *G*PCR *a*utoproteolysis-*in*ducing (GAIN) domain, which mediates autoproteolysis of the receptors into N-terminal and C-terminal fragments that stay non-covalently associated. The downstream signaling pathways and G protein-coupling preferences of many aGPCRs have recently been elucidated, and putative endogenous ligands for some aGPCRs have also been discovered and characterized in recent years. A pivotal observation for aGPCRs has been that deletion or removal of the NT up the point of GAIN cleavage results in constitutive receptor activation. For at least some aGPCRs, this activation is dependent on the unmasking of specific agonistic peptide sequences within the N-terminal stalk region (i.e., the region between the site of GAIN domain cleavage and the first transmembrane domain). However, the specific peptide sequences involved and the overall importance of the stalk region for activation can vary greatly from receptor to receptor. An emerging theme of work in this area is that aGPCRs are capable of versatile signaling activity that may be fine-tuned to suit the specific physiological roles played by the various members of this family.

Keywords

Adhesion • Receptor • GPCR • G protein • Signaling • Activity • Agonist • Ligand • Arrestin • Pathway

1 Adhesion G Protein-Coupled Receptors Are a Diverse Group of Self-Cleaving Cell Surface Receptors

G protein-coupled receptors (GPCRs) form the largest superfamily of cell surface signaling proteins in vertebrates [1]. Within this superfamily, the adhesion GPCRs (aGPCRs) represent the second largest family, encompassing 33 receptors in humans. These receptors are broadly expressed in different tissues and involved

in many diverse processes including neural development, immunity, myelination, and angiogenesis [2]. Via their large extracellular N-termini (NT), which range from 200 to 5600 amino acids in length and harbor a variety of adhesion domains, aGPCRs are thought to survey the surrounding cellular environment and transduce signals from the extracellular milieu into intracellular signaling [3]. The tremendous diversity in the NT regions of aGPCRs has led to the further categorization of the 33 aGPCRs into 9 distinct subfamilies [4] (see [5]). A new nomenclature for aGPCRs based on these subfamilies was recently approved by IUPHAR [4], and both the new names (all starting with "ADGR") and the traditional names for each receptor will be used in this review. The most commonly shared protein-protein interaction domains found in the N-termini of each of the nine aGPCR families are olfactomedin (OLF) and rhamnose-binding lectin-like (RBL) domains (subfamily I); epidermal growth factor (EGF)-like repeats (subfamily II); leucine-rich repeats (LRRs; subfamily III); cadherin repeats (subfamily IV); pentraxin domains (PTX; subfamily V); sea urchin sperm protein, enterokinase, and agrin (SEA) domains (subfamily VI); thrombospondin type 1 repeats (TSRs; subfamily VII); pentraxin domains (subfamily VIII); and calx-β repeats (subfamily IX) [4] (see [6]).

A unique feature of the aGPCRs is their autoproteolytic activity at a membrane-proximal motif of the NT called the GPS or *G*PCR *p*roteolysis *s*ite motif [7, 8] (see also [6, 9]). This ~50-amino acid, cysteine- and tryptophan-rich motif is located within a much larger functional domain that is both necessary and sufficient for aGPCR self-cleavage called the *G*PCR *a*utoproteolysis-*in*ducing (GAIN) domain [10]. The GAIN domain is the only commonly shared domain in the NT of aGPCRs (with the exception of ADGRA1/GPR123) [11]. Moreover, the GAIN domain is also one of the most ancient domains found in aGPCRs, existing in the genomes of more primitive organisms such as *Dictyostelium discoideum* and *Tetrahymena thermophila* [10, 12]. Structural studies by Arac and colleagues showed that the GAIN domain stays intact following cleavage through an extensive network of hydrogen bonding and hydrophobic side-chain interactions [10]. These insights confirmed prior biochemical observations that autoproteolysis does not necessarily result in the dissociation of the N-terminal fragment (NTF) and C-terminal fragment (CTF) that result from GAIN domain cleavage of a given aGPCR.

2 Evidence for G Protein-Mediated Signaling by Adhesion GPCRs

Notwithstanding their N-terminal diversity, all members of the aGPCR family share a similar seven-transmembrane (7TM) domain architecture, which is the molecular signature of GPCRs. However, in the early years of aGPCR research, it was not known whether these proteins were bona fide GPCRs. In studies that were facilitated by the serendipitous discovery of a potent and high-affinity agonist, ADGRL1 (latrophilin-1) was one of the first aGPCRs characterized in terms of its signaling activity [13]. It was found that α-latrotoxin (α-LTX), a component of black widow spider venom, stimulated increases in intracellular cAMP and IP3 levels in ADGRL1-transfected COS7 cells in a receptor-dependent manner

[14]. However, in addition to binding to ADGRL1, α-LTX can also form calcium-permeable pores in the plasma membrane and trigger exocytosis [15]. Therefore, a mutant version of the toxin was generated, α-LTX^{N4C}, which does not cause exocytosis but still binds to and activates ADGRL1 [15]. Further studies showed that ADGRL1 could activate phospholipase C (PLC) and increase intracellular Ca^{2+} within minutes of α-LTX^{N4C} treatment, suggesting coupling of the receptor to $G\alpha_q$ [16]. Moreover, ADGRL1 could be co-purified with $G\alpha_o$ [14, 17] and $G\alpha_{q/11}$ [17] using α-LTX affinity chromatography.

Unlike ADGRL1, the majority of aGPCRs do not have known ligands. Thus, a common method of discerning the signaling pathways downstream of aGPCRs has been to overexpress the receptors in heterologous systems and measure their constitutive activities in assays of specific G protein signaling. For example, overexpression of ADGRG1 (GPR56), a receptor that is critically involved in the development of the cerebral cortex [18, 19], was shown to robustly stimulate the activation of RhoA via coupling to the $G\alpha_{12/13}$ signaling pathway [20, 21]. Subsequent studies have demonstrated that ADGRG1 expression can upregulate the activity of a variety of downstream transcription factors, including NFκB [22], PAI-1 [22], TCF [22], SRE [20, 23–25], SRF [26], and NFAT [23, 26]. Other outputs influenced by ADGRG1 include PKCα [27], VEGF [25], and TGFα shedding [26]. In addition to these results, other lines of evidence supporting receptor G protein coupling have been provided by several groups. For example, it was demonstrated that $G\alpha_{q/11}$ could be co-immunoprecipitated with ADGRG1 in heterologous cells [28]. This interaction, however, depended on the presence of the tetraspanin CD81, which may act as a scaffold for the ADGRG1/$G\alpha_{q/11}$ signaling complex. In agreement with these data, stimulation of ADGRG1 in U87-MG cells was found to raise intracellular Ca^{2+} levels in a manner that was blocked by YM-245890, an inhibitor of $G\alpha_{q/11}$-mediated signaling [29]. Additionally, ADGRG1 has been shown to activate $G\alpha_{13}$ in a reconstituted GTPγS-binding assay [24], and an association between ADGRG1 and $G\alpha_{13}$ has also been shown via a co-immunoprecipitation approach [26].

In addition to ADGRG1, evidence for G protein coupling has also been provided for several other members of aGPCR subfamily VIII. For example, ADGRG2 (GPR64) expression in transfected cells has been demonstrated to stimulate the SRE and NFκB pathways [30], raise intracellular cAMP, and elevate IP3 levels in the presence of the chimeric G protein $G\alpha_{qi4}$, suggesting promiscuous coupling to both $G_{\alpha s}$ and $G_{\alpha i}$ [31]. Similarly, it was shown that overexpression of ADGRG3 (GPR97) in HEK293 cells stimulated IP3 accumulation only in the presence of chimeric G protein $G\alpha_{qo3}$, which converts $G\alpha_o$ signaling into $G\alpha_q$ activity, suggesting natural coupling of the receptor to $G\alpha_o$ [32]. ADGRG5 (GPR114) overexpression was shown to potentiate cAMP levels, an effect that could be blocked via knockdown of endogenous $G\alpha_s$ or overexpression of the chimeric G protein $G\alpha_{qs4}$, which converts $G\alpha_s$ signaling into $G\alpha_q$-mediated activity [33]. Another member of the subfamily, ADGRG6 (GPR126), which plays an important role in regulating peripheral nerve myelination [34], was also found to raise intracellular cAMP [35–37] as well as stimulate IP3 accumulation in the

presence of chimeric G proteins to redirect either $G_{\alpha s}$ or $G_{\alpha i}$ activity toward $G_{\alpha q}$ pathways [36]. Thus, both ADGRG2 and ADGRG6 may couple to $G\alpha_s$ to raise cAMP levels while also exhibiting coupling to other G proteins to mediate pleiotropic effects on cellular physiology.

ADGRB1 (BAI1), a receptor that regulates phagocytosis [38–41], myogenesis [42], and synaptic plasticity [43, 44], has been shown to constitutively activate RhoA [45], Rac1 [41], ERK [45], SRF [26], NFAT [26], and TGFα shedding [26] when overexpressed in heterologous cells. ADGRB1 signaling to most of these downstream readouts can be greatly attenuated by co-expression of the RGS domain of p115-RhoGEF, suggesting a predominant coupling of the receptor to $G\alpha_{12/13}$. These functional data are consistent with co-immunoprecipitation data revealing the existence of cellular complexes between ADGRB1 and $G\alpha_{12/13}$ [26]. Expression of ADGRB2 (BAI2), a close relative of ADGRB1, was found to also stimulate the NFAT pathway and additionally induce IP3 accumulation in HEK293 cells, indicating a likely coupling to $G\alpha_{q/11}$ [46].

ADGRE2 (EMR2), a receptor highly enriched in immune cells, was demonstrated to stimulate IP3 accumulation in transiently transfected HEK293 cells, indicative of $G_{\alpha q}$ coupling [32]. Expression of another receptor from the same subfamily, ADGRE5 (CD97), was found to activate the SRE pathway in transfected COS7 cells in a manner that was sensitive to the presence of RGS-p115-RhoGEF, suggesting receptor coupling to $G\alpha_{12/13}$ [47]. Receptors ADGRF1 (GPR110) and ADGRF4 (GPR115) were both shown to stimulate IP3 accumulation in transiently transfected HEK293 cells [32]. In separate studies that confirmed some of these findings, ADGRF1 was shown to activate $G\alpha_q$ in a GTPγS assay [24].

ADGRV1 (VLGR1), a receptor that has a crucial role in hearing and vision and whose dysfunction is associated with the human disease known as Usher syndrome, was shown to inhibit isoproterenol-induced cAMP levels in HEK293 cells, indicative of $G\alpha_i$ coupling [48]. Moreover, co-expression of the chimeric G protein $G\alpha_{qi5}$ was able to reroute receptor activity toward a $G\alpha_{q/11}$ readout (NFAT activation), thereby providing further evidence for $G\alpha_i$ coupling. In contrast, expression of ADGRD1 (GPR133) has been shown to raise cAMP levels in multiple studies [32, 37, 49]. Moreover, it was demonstrated that ADGRD1-mediated cAMP elevation could be blocked by knocking down $G\alpha_s$ [32].

3 Ligands for Adhesion GPCRs

Potential ligands have been identified for a number of members of the aGPCR family (Table 1). As mentioned previously, α-LTX is a high-affinity agonist of ADGRL1 that has been shown to stimulate several readouts of receptor activity. Another reported ligand for ADGRL1 is teneurin-2, a large (~2800 residue) glycoprotein with a single transmembrane region that is found predominantly in the brain [50]. Teneurin-2 was first identified as a binding partner of ADGRL1 through pull-down studies in which rat brain lysates were subjected to α-LTX affinity chromatography [50]. Treatment of cultured neurons expressing ADGRL1 with a soluble,

Table 1 Adhesion GPCR ligands and/or agonists

Receptor	Ligand	Binding region	Downstream activity
Family I			
ADGRL1	α-Latrotoxin	NT (GAIN domain)	Increased cAMP [14], IP3 [14], Ca^{2+} [16], and PLC activation [16]
ADGRL1	Teneurin-2	NT	Increased Ca^{2+} in cultured hippocampal neurons [50]
ADGRL1	Neurexin1α	NT	Regulation of α-latrotoxin-mediated glutamate release [51]
ADGRL3	FLRT3	NT	Regulation of synaptic density [52]
ADGRL3	FLRT2	NT (OLF domain)	Regulation of cell adhesion/repulsion [53]
Family II			
ADGRE2	NT antibody (2A1)	NT	Increased production of inflammatory cytokines [54]
ADGRE2/ ADGRE5	Chondroitin sulfate	? (likely NT region)	Mediates cell adhesion [55]
ADGRE5	CD55	NT (EGF domains)	Alteration in ADGRE5 NT-CTF interaction [56]
ADGRE5	α5β1/αvβ3	NT	Mediates endothelial cell migration [57]
ADGRE5	CD90	NT	Mediates cell adhesion [58]
Family V			
ADGRD1	Stalk peptide(s)	? (likely 7TM region)	Increased cAMP levels [37]
Family VI			
ADGRF1	Stalk peptide(s)	? (likely 7TM region)	Increased GTPγS binding [24]
Family VII			
ADGRB1	Phosphatidylserine	NT (TSR domains)	Enhanced Rac1-dependent uptake of apoptotic cells [39]
ADGRB3	C1ql1	NT (CUB domain)	Regulation of dendritic spine density [59]
ADGRB3	C1ql3	NT (TSR domains)	Regulation of synaptic density [60]
Family VIII			
ADGRG1	Tissue transglutaminase 2	NT (STP region)	Regulation of VEGF secretion [27]
ADGRG1	Collagen III	NT (aa 27–160)	Stimulation of RhoA activation [61]
ADGRG1	NT antibody	NT	Stimulation of SRE and RhoA activity [20]
ADGRG1	Stalk peptide(s)	? (likely 7TM region)	Stimulation of SRE luciferase [24]
ADGRG2	Stalk peptide(s)	? (likely 7TM region)	Increased cAMP and IP3 accumulation [31]
ADGRG3	Beclomethasone dipropionate	?	Increased GTPγS binding [32]

(continued)

Table 1 (continued)

Receptor	Ligand	Binding region	Downstream activity
ADGRG5	Stalk peptide(s)	? (likely 7TM region)	Increased cAMP levels [33]
ADGRG6	Collagen IV	NT (CUB and PTX domains)	Increased cAMP levels [35]
ADGRG6	Laminin-211	NT (aa 446–807)	Increased cAMP levels upon mechanical shaking [62]
ADGRG6	Stalk peptide(s)	? (likely 7TM region)	Increased cAMP levels [37] and IP3 accumulation when co-expressed with chimeric Gqi [36]

? unavailable

C-terminal fragment of teneurin-2 was found to trigger the release of intracellular Ca^{2+}, possibly through a G protein-dependent mechanism [50]. In another study, coculturing cells expressing either ADGRL1 or teneurin-2 resulted in the formation of large cell aggregates, indicating that the specific interaction between the two proteins may mediate cell adhesion [64]. In the brain, ADGRL1 and teneurin-2 are enriched in the presynaptic and postsynaptic membranes, respectively. The extracellular NT of ADGRL1, however, may be large enough to span the synaptic cleft to mediate interneuronal contact through its high-affinity interaction with teneurin-2.

ADGRL1 has also been shown to interact with neurexin, a presynaptic protein implicated in synaptogenesis and function [65]. Neurexin is a binding partner of α-LTX, as is ADGRL1 [66]. A particular neurexin isoform (1α) binds α-LTX in a Ca^{2+}-dependent fashion, while the α-LTX-ADGRL1 interaction is Ca^{2+} independent [66]. Interestingly, in the absence of Ca^{2+}, knockdown of neurexin in cultured hippocampal neurons significantly diminished the α-LTX response compared to wild-type neurons, suggesting that while ADGRL1 and neurexin can independently associate with α-LTX, their interaction may synergistically enhance α-LTX-induced signaling by ADGRL1 [51]. Moreover, coculture of cells expressing either ADGRL1 or neurexin resulted in numerous cell aggregates, providing evidence that the interaction promotes adhesion complexes [67]. More work must be done, however, to demonstrate whether neurexins directly stimulate receptor signaling activity.

The fibronectin leucine-rich repeat transmembrane (FLRT) proteins are an additional class of ligands for ADGRL1 and the related receptor ADGRL3 (latrophilin-3) [52]. Direct interactions between the NT of ADGRL3 and FLRT3 were demonstrated in a non-cell-based assay [52]. In vivo, both proteins are enriched in cell-to-cell junctions and regulate synaptic density [52]. In another study, a high-affinity interaction was demonstrated for ADGRL3 and FLRT2 [53]. This interaction was found to be mediated by the OLF domain on the ADGRL3 NT and, intriguingly, promoted either adhesion of FLRT2-expressing HeLa cells or repulsion of FLRT2-expressing cultured cortical neurons. These

results potentially highlight the influence that cellular environment may have on the relationship between receptor and ligand. At present, however, there is no evidence that FLRT proteins can directly instigate signaling by the latrophilin receptors.

The association between ADGRE5 and CD55 was one of the first confirmed protein-protein interactions involving an aGPCR [68]. This interaction was found to be mediated by the EGF domains on the receptor's NT [69]. Recently, it was shown that CD55 does not modulate ADGRE5-mediated signaling to ERK or Akt [56]. It remains to be determined whether CD55 can modulate other receptor-controlled pathways, such as perhaps the RhoA signaling pathway. ADGRE2 is a close relative of ADGRE5 with highly homologous EGF domains, but nonetheless ADGRE2 has been found to have a much lower binding affinity for CD55 than ADGRE5 [70]. Both ADGRE5 and ADGRE2 have also been shown to bind to extracellular matrix (ECM) components known as chondroitin sulfates [55]. These interactions are generally low affinity and Ca^{2+} dependent and have not yet been demonstrated to instigate G protein-mediated signaling for either receptor.

A number of ligands have been identified for subfamily VII aGPCRs. ADGRB1 was found to bind externalized phosphatidylserine on apoptotic cells through the thrombospondin type 1 repeat domains on its NT [38]. This interaction promoted the engulfment of the apoptotic cells in a mechanism reliant on the adaptor protein ELMO1 and signaling by the small GTPase Rac1 [38]. Another receptor from this subfamily, ADGRB3 (BAI3), was shown to bind to C1q-like (C1ql) proteins [60, 71]. Similar to the interaction of ADGRB1 and phosphatidylserine, the interaction between ADGRB3 and C1ql3 was found to be mediated by thrombospondin repeats on the receptor's NT [60]. In cultured neurons, submicromolar C1ql3 treatment significantly reduced synaptic density, an effect readily blocked by exogenous addition of purified ADGRB3 NT [60]. In a similar study, it was shown that ADGRB3 binds C1ql1 via its N-terminal CUB domain and that both proteins were necessary for normal spine density of cerebellar neurons [59]. Furthermore, the interaction between C1ql1 and ADGRB3 was demonstrated to regulate pruning in mouse cerebellum, with knockout of either protein resulting in severe motor learning deficits [72]. Future studies in this area will likely examine whether C1ql proteins have similar binding affinities for other members of subfamily VII and whether those interactions can stimulate receptor-mediated activity.

Several ligands have been identified for ADGRG1, including tissue transglutaminase 2 (TG2), a major cross-linking enzyme of the extracellular matrix implicated in cancer progression [63, 73]. TG2 binds a ~70-residue region on the NT of ADGRG1; deletion of this TG2-binding region was found to enhance receptor-mediated VEGF production in vitro and significantly increase tumor growth and angiogenesis in vivo, whereas expression of the wild-type receptor reduced both measures [27]. In a more recent study, it was demonstrated that the antagonistic relationship between ADGRG1 and TG2 may be attributed to internalization and lysosomal degradation of extracellular TG2 in a receptor-dependent mechanism [74]. It is unclear at present whether interaction with TG2 stimulates G protein-mediated signaling by ADGRG1.

Collagen III is another ligand for ADGRG1 [61]. ADGRG1 loss-of-function mutations cause the human disease bilateral frontoparietal polymicrogyria (BFPP). Patients with BFPP have a cortical malformation due to aberrant neural stem cell migration [75]. Remarkably, knockout of collagen III in mice results in a cortical phenotype similar to that observed in mice lacking ADGRG1 as well as human BFPP patients [75]. Collagen III binds a ~130-residue region in the distal half of the receptor's NT [76]. Moreover, nanomolar concentrations of collagen III have been shown to significantly reduce migration of mouse neurospheres (masses of cells containing neural stem cells) in a receptor-dependent fashion [61]. Biochemical studies revealed that collagen III could stimulate RhoA signaling in a mechanism dependent on receptor expression and likely mediated by $G\alpha_{12/13}$ [61].

Another subfamily VIII receptor, ADGRG6, has also been shown to be stimulated by collagen interactions, albeit with a distinct type of collagen. The association between ADGRG6 and collagen IV was found to be mediated by a region of the ADGRG6 NT containing the CUB and PTX domains [35]. Furthermore, the association was shown to be specific, as other types of collagen, including collagen III, did not bind the receptor. In heterologous cells, collagen IV stimulated receptor-dependent cAMP elevation. The half-maximal effective concentration for this response was 0.7 nM, indicating that collagen IV is a potent agonist for ADGRG6.

An additional ligand for ADGRG6 is laminin-211, an extracellular matrix protein that is involved in Schwann cell development and peripheral nervous system myelination [62]. Interestingly, laminin-211 was found to antagonize receptor-mediated cAMP elevation in a dose-dependent fashion in heterologous cells. Furthermore, cAMP inhibition was due to antagonism of receptor-mediated $G\alpha_s$ activity rather than through differential activation of $G\alpha_i$. Remarkably, laminin-211 treatment under the condition of mechanical shaking had the opposite effect of boosting receptor-mediated cAMP levels. Thus, laminin-211 may serve as a unique ligand that can differentially modulate receptor activity depending upon other physical cues and mechanical forces in the extracellular environment.

Most of the putative aGPCR endogenous ligands described thus far are large, ECM-derived molecules. Nonetheless, it has been shown that small molecules can be developed as aGPCR ligands. For example, screening studies revealed beclomethasone dipropionate as a ligand for ADGRG3 [32]. Beclomethasone dipropionate is a glucocorticoid steroid that can stimulate ADGRG3 with nanomolar potency. The region of the receptor that interacts with beclomethasone is unknown, but considering the molecule's hydrophobicity, it would not be surprising if it were found in future studies to directly interact with the receptor's 7TM region to modulate receptor activity.

An intriguing observation made for several aGPCRs has been that these receptors may be activated by antibodies directed against their NT regions. Antibodies may be able to mimic the binding of endogenous ligands to aGPCRs and thus may represent powerful research tools for studying aGPCR signaling, especially for those receptors with no identified ligands. An N-terminal activating antibody of ADGRG1 was first described in 2008 by Itoh and colleagues. Studies in

heterologous cells revealed that antibody treatment could dose-dependently stimulate receptor signaling in the SRE luciferase assay (a commonly used assay for $G\alpha_{12/13}$ activity) [20]. Moreover, stimulation was readily blocked by exogenous addition of the receptor's NT, which presumably competed for antibody binding. Moreover, in a later study it was shown that other newly generated N-terminal antibodies for ADGRG1 could inhibit cell migration in a manner that was sensitive to inhibition of either $G\alpha_q$ or $G\alpha_{12/13}$ signaling [29]. In another example, an antibody directed against the N-terminal region of ADGRE2 was shown to dose-dependently increase inflammatory cytokine production in receptor-mediated neutrophil activation [54].

Given the importance of aGPCR N-termini in mediating binding to extracellular ligands, it is perhaps not surprising that mutations to the aGPCR N-termini can oftentimes lead to loss of receptor function and human disease. For example, there are several reported N-terminal disease-causing mutations to ADGRG1 that result in reduced plasma membrane expression of the receptor [77, 78] and/or disruption of the receptor's ability to bind collagen III [76]. Another prominent example is of ADGRV1, where several NT mutations cause cochlear and retinal defects in humans [79]. Moreover, missense NT mutations to ADGRC1 (CELSR1) impair surface trafficking of the protein and are implicated in a severe neural tube defect in humans known as craniorachischisis [80].

4 Adhesion GPCR Models of Activation

With the idea that aGPCR ligands mainly bind to the large extracellular NT regions and that the NT regions are cleaved in the GAIN domain and may be removed at some point following ligand binding, a number of groups have generated truncated versions of aGPCRs lacking most of their NT regions up to the sites of predicted GAIN cleavage. The first studies of this type were performed independently for a trio of receptors—ADGRB2 [46], ADGRG1 [21], and ADGRE5 [47]—and in each case the truncation was found to result in a substantial increase in the receptors' constitutive signaling activity. Subsequently, this phenomenon has been reported for a number of other aGPCRs, including ADGRB1 [45], ADGRG6 [35], ADGRG2 [30, 31], ADGRD1 [37], ADGRF1 [24], and ADGRV1 [48]. In light of these findings, a general model of aGPCR activation was proposed wherein the tethered NTF behaves as an antagonist of CTF-mediated signaling, with N-terminal deletion mimicking ligand-mediated removal of the NTF to result in receptor activation [81]. This model of activation, termed the *disinhibition model*, was a general model that left open the mechanistic question of precisely how removal of aGPCR NT regions might activate receptor signaling.

Subsequently, a more mechanistically specific model of aGPCR activation, termed the *tethered agonist model*, was proposed (Fig. 1; see also [82]). In this model, GAIN domain autoproteolysis (and/or conformational change) reveals a tethered cryptic agonist sequence contained within the NT region between the site of cleavage and the first transmembrane domain (i.e., the stachel or stalk region).

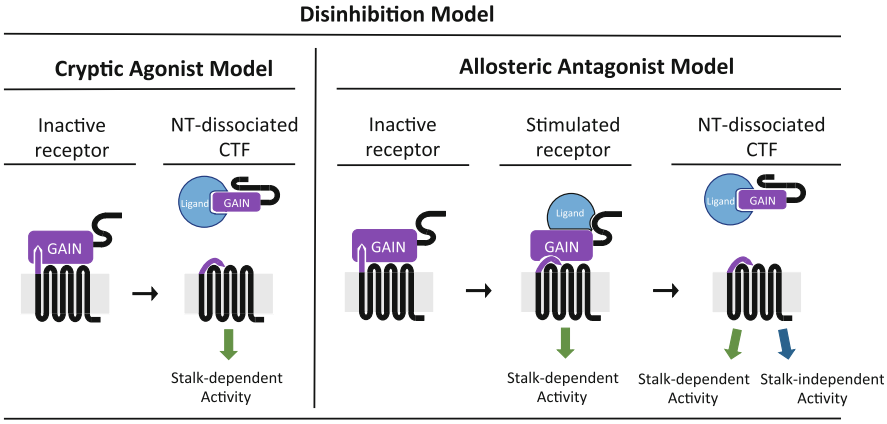


Fig. 1 Models of adhesion GPCR activation. Cryptic agonist model—*Inactive receptor*: The GAIN domain antagonizes receptor activity by concealing a cryptic agonist found in the N-terminal stalk region between the site of autoproteolysis and the first transmembrane domain. *NTF-dissociated CTF*: Following ligation of the N-terminal fragment (NTF) with an extracellular ligand and subsequent removal from the plasma membrane, the cryptic agonist sequence (the stachel) is unveiled and stimulates activity through interactions with the remaining C-terminal fragment (CTF). Allosteric antagonist model—*Inactive receptor*: In the absence of ligand engagement, the GAIN domain can inhibit receptor activity in two distinct ways: by concealing a cryptic agonist on the N-terminal stalk and also by dampening the inherent constitutive activity of the CTF. *Stimulated receptor*: Ligation of the NTF with an extracellular ligand induces a conformational change to allow for stimulation by the cryptic agonist within the stalk, even though the NTF may stay associated with the CTF for some time. *NTF-dissociated CTF*: If and when ligand binding induces NTF dissociation from the CTF, another wave of receptor activity may be unleashed, with the inherent, stalk-independent activity of the CTF being stimulated. In this stage, the receptor may achieve its maximal activity due to the summation of signals from both stalk-dependent and stalk-independent mechanisms

This mechanism of activation is conceptually similar to that of the protease-activated receptors, for which proteolysis of the N-terminal domain by an extracellular protease unveils an agonist in the remaining NT [83]. Evidence in favor of the cryptic agonist model was provided by two independent groups: Liebscher et al. and Stoveken et al. First, Liebscher et al. showed that deletion of the remaining NT (i.e., the stachel or stalk region) from constitutively active NTF-lacking versions of ADGRG6 and ADGRD1 ablated activity of both receptors in cAMP accumulation assays [37]. Moreover, synthetic peptides corresponding to the stalk regions of each receptor were able to restore activity of the stalkless mutants with varying degrees of efficacy. The most potent peptides displayed half-maximal effective concentrations in the high micromolar range. Further studies from Liebscher et al. along similar lines provided evidence for tethered agonist-mediated activation of ADGRG2 [31] and ADGRG5 [33]. Additionally, Stoveken et al. showed that stalkless versions of ADGRG1 and ADGRF1 lacked activity in reconstitution assays examining GTP binding to purified $G\alpha_{13}$ and $G\alpha_{q}$,

respectively [24]. Synthetic peptides fashioned after the stalk of each receptor were shown to resuscitate their cognate stalkless receptors in a dose-dependent manner, with the most potent peptides displaying submicromolar half-maximal effective concentrations. Moreover, the most potent stalk peptide of ADGRG1 was shown to stimulate receptor-mediated activity in cellular SRE luciferase assays in addition to the $G\alpha_{13}$ reconstitution studies.

The finding from Stoveken et al. that stalk-deficient ADGRG1 is unable to activate SRE luciferase was confirmed in recent studies using a similar readout, SRF luciferase [26]. However, the stalkless ADGRG1 was found in these studies to be functional in other readouts of receptor signaling activity including TGFα shedding, NFAT luciferase, beta-arrestin recruitment, and receptor ubiquitination [26]. In parallel, a stalkless truncated version of ADGRB1 was examined in the same battery of assays and found to have nearly identical activity to the constitutively active truncated version of ADGRB1 that retained the stalk. A conclusion from this work was that aGPCRs are capable of both stalk-dependent and stalk-independent signaling, with the relative contribution of the stalk varying between different receptors and even between different readouts for the same receptor. These findings led to the proposal of the *allosteric antagonist model* of aGPCR activation (Fig. 1), in which aGPCR NT regions can dampen receptor activity in at least two distinct ways: (1) by masking the stalk region to prevent stalk-dependent signaling and (2) by allosterically antagonizing the inherent, stalk-independent activity of the 7TM region.

Further evidence that a proteolytically liberated agonist in the stalk region may not be required for all aspects of aGPCR signaling comes from studies on non-cleavable aGPCR mutants. The GAIN domain crystal structures from Arac et al. revealed how mutation of a key catalytic threonine in the GPS motif could block GAIN domain cleavage but allow for normal GAIN domain folding [10]. Such non-cleaving mutants of ADGRD1 [49], ADGRG1 [26], and ADGRG2 [30] have been studied and found to be capable of robust constitutive signaling, although in the case of ADGRG2 the non-cleavable mutant receptor exhibited signaling comparable to the wild-type receptor in one pathway but reduced signaling when a distinct pathway was measured. There is also evidence that certain aGPCRs may not undergo GAIN cleavage at all [84]. ADGRG5 and ADGRB1 are examples of aGPCRs that are naturally cleavage deficient (at least in some cellular contexts) and yet retain signaling ability [33, 45]. Moreover, in vivo studies on lat-1, the *C. elegans* ortholog of ADGRL1, revealed that wild-type and mutant non-cleavable versions of the receptor performed just as well in the transgenic rescue of deficits resulting from receptor knockout [85]. The requirement of the stalk region for aGPCR signaling is also uncertain due to observations that individual aGPCRs, such as ADGRL1, undergo additional proteolytic processing wherein GAIN autoproteolysis is followed by one or more additional cleavage events that remove the stalk region [86] (see also [9] for an in-depth discussion on the relationship between proteolytic processing and aGPCR activity). These findings taken together suggest that neither GAIN domain autoproteolysis nor the

presence of the stalk region are absolutely required for aGPCR signaling activity but rather may be important for some receptors and certain downstream pathways.

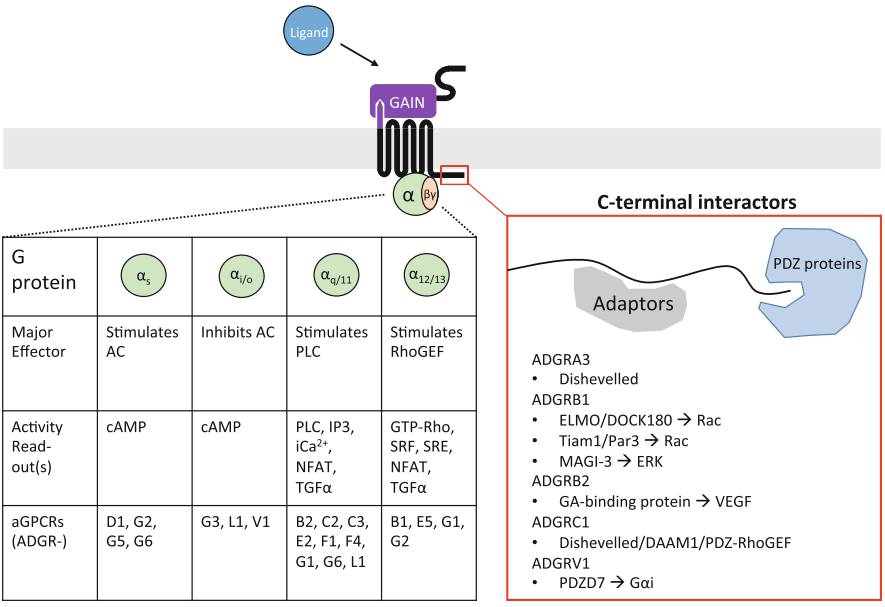
5 Adhesion GPCR N-Termini as Sensors of Mechanical Force

There is emerging evidence that aGPCRs may be involved in sensing mechanical forces. For example, it was shown that the ADGRE5 NTF is released from the CTF after engagement with the ligand CD55, but only under mechanical shaking conditions that are meant to recapitulate the shear stress associated with circulating blood [56]. In a similar vein, laminin-211, a ligand of ADGRG6 as mentioned above, was found to only stimulate the receptor under shaking conditions and actually antagonized receptor activity under static conditions [62]. In these studies, the mechanical forces may have helped laminin-211 to disengage the NTF from its CTF, whereas without shaking, the ligand binding may have actually stabilized the inhibitory NTF-CTF interaction. These examples support the idea that, for at least some ligand-receptor pairs, mechanical force may be a key determinant of the signaling output that results from the interaction. In a key in vivo study on aGPCR-mediated mechanosensation, Scholz et al. recently demonstrated that *Drosophila* larvae lacking the ADGRL1 ortholog CIRL exhibited diminished sensitivity to mechanical stimuli [87]. The role of aGPCRs in sensing mechanical force is likely to be an active area of research in the coming years and discussed in detail in [88].

6 Associations of aGPCRs with Signaling Proteins Other Than G Proteins

In addition to the aforementioned examples of aGPCR coupling to G proteins, there have also been a number of cytoplasmic proteins other than G proteins that have been found to interact with aGPCRs (see [89] for more on this topic). In some cases, these interactions appear to modulate G protein-mediated signaling, while in other cases these associations appear to mediate G protein-independent signaling (Fig. 2). One example of the regulation of G protein signaling comes from work on ADGRV1, which was found to interact with the PDZ domain-containing protein PDZD7, a key scaffold protein in the USH2 protein complex that is known to be pivotal for stereocilial development and function [48]. Association with PDZD7 was found to antagonize ADGRV1 activity, likely by competitively disrupting receptor association with $G\alpha_i$ [48, 90]. ADGRB1 is another aGPCR that has been found to associate with PDZ scaffold proteins. One such PDZ protein, MAGI-3, was found to potentiate receptor-mediated ERK signaling, possibly by recruiting positive regulators of the pathway [45].

In terms of G protein-independent signaling by aGPCRs, ADGRB1 and ADGRB3 have both been shown to bind to the intracellular adaptor protein ELMO1 [38, 91]. For ADGRB1, this interaction has been demonstrated to result in the formation of a complex at the plasma membrane capable of activating the



G protein	α_s	$\alpha_{i/o}$	$\alpha_{q/11}$	$\alpha_{12/13}$
Major Effector	Stimulates AC	Inhibits AC	Stimulates PLC	Stimulates RhoGEF
Activity Read-out(s)	cAMP	cAMP	PLC, IP3, iCa^{2+}, NFAT, TGFα	GTP-Rho, SRF, SRE, NFAT, TGFα
aGPCRs (ADGR-)	D1, G2, G5, G6	G3, L1, V1	B2, C2, C3, E2, F1, F4, G1, G6, L1	B1, E5, G1, G2

Fig. 2 G protein-dependent and G protein-independent signaling by adhesion GPCRs. The *left panel* shows the various G protein-dependent pathways that can be activated by aGPCRs. Also shown are the probable G protein-coupling preferences for selected members of the aGPCR family. The *right panel* displays various aGPCR C-terminal binding partners and briefly describes their influence on aGPCR signaling pathways (both G protein dependent and G protein independent)

small GTPase Rac1 in a G protein-independent manner [38]. ADGRB1-mediated activation of Rac1 has been implicated in phagocytosis and myoblast fusion [38, 42]. Intriguingly, ADGRB1 can also activate Rac in a distinct G protein-independent manner through association with the RacGEF Tiam1 [43]. Other examples of G protein-independent signaling by aGPCRs include ADGRB2 interaction with GA-binding protein (GABP) gamma to regulate VEGF expression [92]; ADGRC1 association with dishevelled, DAAM1, and PDZ-RhoGEF to regulate neural tube closure [93]; and ADGRA3 (GPR125) interaction with dishevelled to mediate the recruitment of planar cell polarity components [94].

7 Concluding Remarks

The versatility of aGPCR signaling described here highlights the need to comprehensively study the members of this family on a receptor-by-receptor basis in order to delineate the diversity of metabotropic pathways they serve. Further insights gained into the mechanisms of aGPCR activation will have important implications for drug development efforts aimed at these receptors. Given the number of human diseases linked to aGPCR mutations and the intriguing phenotypes observed upon

genetic deletion of aGPCRs [4], there are compelling reasons to believe that elucidation of the activation mechanisms and downstream pathways of aGPCRs will allow for an enhanced understanding of human disease and promote the development of novel classes of therapeutics.

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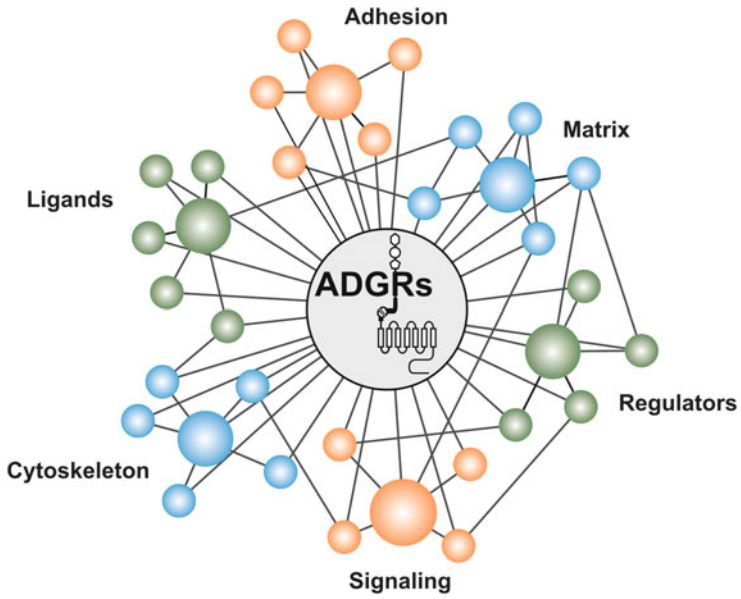
Adhesion GPCR-Related Protein Networks

Barbara Knapp and Uwe Wolfrum

Graphical Abstract



Contents

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Abstract

Adhesion G protein-coupled receptors (aGPCRs/ADGRs) are unique receptors that combine cell adhesion and signaling functions. Protein networks related to ADGRs exert diverse functions, e.g., in tissue polarity, cell migration, nerve cell function, or immune response, and are regulated via different mechanisms. The large extracellular domain of ADGRs is capable of mediating cell–cell or cell–matrix protein interactions. Their intracellular surface and domains are coupled to downstream signaling pathways and often bind to scaffold proteins, organizing membrane-associated protein complexes. The cohesive interplay between ADGR-related network components is essential to prevent severe disease-causing damage in numerous cell types. Consequently, in recent years, attention has focused on the decipherment of the precise molecular composition of ADGR protein complexes and interactomes in various cellular modules. In this chapter, we discuss the affiliation of ADGR networks to cellular modules and how they can be regulated, pinpointing common features in the networks related to the diverse ADGRs. Detailed decipherment of the composition of protein networks should provide novel targets for the development of novel therapies with the aim to cure human diseases related to ADGRs.

Keywords

Protein networks • Adhesion complexes • Adhesion GPCR • Affinity proteomics • Brain-specific angiogenesis inhibitor • Latrophillin • VLGR1 • GPR98 • ADGR • Signaling pathways

1 Introduction

Over the last years, the prominent impact of adhesion G protein-coupled receptors (aGPCRs/ADGRs) on diverse cellular functions, e.g., tissue polarity, cell migration, nerve cell function, and immune response, has been evident. The interaction of ADGRs with a myriad of other proteins and the assembly and maintenance of their cellular modules is essential for correct function and regulation of these diverse processes. The aim of the present book chapter is to outline the current knowledge

of supramolecular protein complexes and protein networks related to ADGRs. Although we consequently apply the nomenclature of the ADGRs which has been recently harmonized [1], we add historic and alternative names found in the literature, where necessary.

2 The ADGR Protein Structure Reveals Multiple Protein-Binding Domains

Proteins of the ADGR family possess a unique molecular structure (Fig. 1) [1] (see also [2]). They usually display an extraordinary large N-terminal extracellular domain (ECD) featuring various types of subdomains that are generally thought to communicate with the extracellular milieu and mediate their characteristic adhesive functions. The ECD of each receptor subfamily contains a specific combination of domains. Receptors from the same subfamily mainly differ in the number of domain repeats, which results in varying sizes of their ECDs. An important common feature of ADGRs is the G protein proteolytic site (GPS). It is an integral part of the much larger GPCR autoproteolysis-inducing (GAIN) domain [3]. Here, most ADGRs undergo autocleavage into an extracellular N-terminal fragment (NTF) and a C-terminal fragment (CTF). Both fragments usually remain non-covalently associated at the cell surface (see also [4, 5]). Similar to other extracellular protein domains, NTFs are highly glycosylated and their functional roles resemble those of adhesion proteins. The CTF, which is comprised of a canonical seven transmembrane domain (7TM) and the intracellular domain (ICD), mediates the activation of intracellular signaling cascades. At the very C-terminal end of the ICD, almost half of the ADGRs exhibit a PDZ-binding

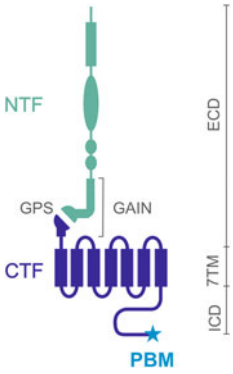


Fig. 1 Schematic representation of the ADGR protein structure. ADGRs are subdivided in an extracellular domain (ECD), a seven-transmembrane domain (7TM), and an intracellular domain (ICD), based on their topology. Autocleavage of ADGRs results in two protein fragments termed N-terminal fragment (NTF) and C-terminal fragment (CTF), respectively. The GPCR autoproteolysis-inducing (GAIN) domain includes the G protein proteolytic site (GPS); *PBM* PDZ-binding motif

motif (PBM) that allows the binding to PDZ domains. The latter domains are named after three scaffold proteins, the postsynaptic density protein PSD95/DLG4; the discs large (Dlg) protein, present in the septate junctions of arthropods; and ZO-1, a protein of the *zonula occludens* adhesion complexes. PDZ domains are characteristic for an abundant group of scaffold proteins, which organize supramolecular protein complexes and networks [6, 7]. In general, PDZ domain-mediated binding plays an important role in regulating receptor and channel protein localization within membrane domains at synapses and in other cell–cell adhesion complexes and function to scaffold intracellular signaling protein complexes.

3 Unraveling Protein–Protein Interactions and Protein Complexes Related to ADGR by Screening Methods and Affinity Proteomics

Table 1 represents a comprehensive overview of proteins, which have been reported to interact with the 33 ADGRs. Since only a few binding proteins have been identified in the zebra fish *Danio rerio* or the frog *Xenopus laevis*, respectively, most of the binding partners listed in Table 1 originate from studies on mammalian ADGRs, namely, human and rodent ADGRs. Only for single ADGRs additional data are available from orthologues in *Drosophila melanogaster*, as, e.g., for Flamingo (CELSR) [65, 66] or *C. elegans* [67]. The overall protein network of vertebrate ADGRs and their interaction partners are shown in Fig. 2. It is obvious that the number of identified interaction partners largely varies between the ADGRs (Table 1, Fig. 2). Due to the lack of studies, no information about the binding partners for several ADGR proteins, e.g., ADGR3 (EMR3) or ADGRD2 (GPR144), has yet been reported, so far.

The intracellular binding partners of ADGRs listed in Table 1 have mainly been identified via yeast two-hybrid screens [8, 9], protein-peptide assays [29], and affinity proteomics methods, e.g., co-immunoprecipitation. Extracellular binding partners of ADGRs have been identified via cell adhesion assays [14–16], cell aggregation assays [10, 11], or affinity chromatography followed by mass spectrometry [10].

A large number of confirmed intracellular interacting proteins of ADGRs comprise scaffold proteins (Table 1). Only a few signaling molecules and putative components of downstream signaling pathways have been identified as binding proteins so far. The lack of identified downstream signaling molecules might be due to the screening approaches and assays implemented. These have in common that they require relatively high binding affinities and long periods of protein retention within assembled protein complexes. Such interaction characteristics are only fulfilled by binding of scaffold proteins to their interaction partners. In contrast, the interaction between components of signaling cascades are often low affine and transient. Nevertheless, screening approaches are valuable in order to get an impression of the general picture of ADGR networks. In this chapter, we only discuss interactions that have been reported in the literature. However, data mining in databases, e.g., *IntAct* (http://www.ebi.ac.uk/intact/), *BioGRID* (http://thebiogrid.

Table 1 Extracellular and intracellular ADGR interaction partners. (**a**) Comprehensive list of experimentally confirmed ADGR interaction partners of ADGR subfamily members. The protein nomenclature follows the HGNC guideline, and protein designations of human orthologues are used for all interaction partners. *Black lettering*: direct or indirect physical interaction. *Grey lettering*: evidence for indirect interaction. (**b**) Classification of ADGR binding protein types and ADGR binding regions. *Ptd-L-Ser* phosphatidylserine, *LPS* lipopolysaccharide

A

	single TM receptors NRXN1α,1β,2β,3β TENM2				
extra	FLRT1/3	FLTR3	FLTR1/3		
I	**ADGRL1 (LPHN1)**	**ADGRL2 (LPHN2)**	**ADGRL3 (LPHN3)**		**ADGRL4 (ELTD1)**
intra	*PDZ scaffold proteins* SHANK1/2/3 DLG3 DLG4 SCRIB MAGI1/2 *scaffold proteins w/o PDZ* ANKS1A *kinases* MAST2 ROS1 *Ion-channels* CACNA1A *G proteins* Gαo Gαs	*PDZ scaffold proteins* SHANK1/2 *others* CLN3			*single TM receptors* ITGA5/B1 ITGAV/B3 *ECM proteins* Chrondroitin sulfate B *others* CD55 THY1
extra		*ECM proteins* Chrondroitin sulfate B			
II	**ADGRE1 (EMR1)**	**ADGRE2 (EMR2)**	**ADGRE3 (EMR3)**	**ADGRE4 (EMR4)**	**ADGRE5 (CD97)**
intra		*G proteins* Gα15			*kinases* SRC *7TM-receptors* LPAR1 *small GTPases* RHOA *G proteins* Gα12/13
extra					
III	**ADGRA1 (GPR123)**		**ADGRA2 (GPR124)**		**ADGRA3 (GPR125)**
intra	*PDZ scaffold proteins* DLG4		*PDZ scaffold proteins* DLG1 *single TM kinases* *RYK*		*PDZ scaffold proteins* DLG1 DVL1 *single TM kinases* *RYK*
extra					
IV	**ADGRC1 (CELSR1)**		**ADGRC2 (CELSR2)**		**ADGRC3 (CELSR3)**
intra	*7TM-receptors* FZD6 *PDZ scaffold proteins* DVL2 *scaffold proteins w/o PDZ* DAAM1 *GEF s* ARHGEF11 *kinases* RYK *others* VANGL2 ATP6AP2		*single TM kinases* RYK *others* ATP6AP2		*single TM kinases* RYK *GEFs* SWAP70 *others* DST
extra					
V	**ADGRD1 (GPR133)**		**ADGRD2 (GPR144)**		
intra	*G proteins* Gαs Gαi				
extra					*others* SFTPD

(continued)

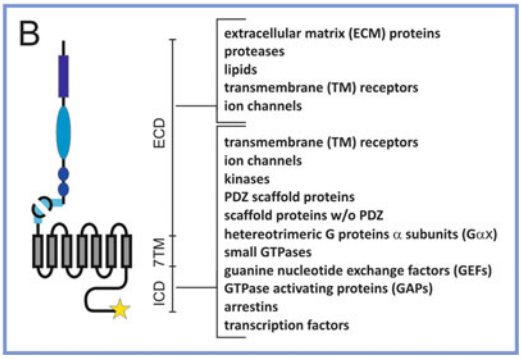
Table 1 (continued)

VI	ADGRF1 (GPR110)	ADGRF2 (GPR111)	ADGRF3 (GPR113)	ADGRF4 (GPR115)	ADGRF5 (GPR116)
intra	*G proteins* Gαq			*G proteins* Gα15	*G proteins* Gαq/11 *small GTPases* RHOA RAC1 *GEFs* ARHGEF25
extra	*others* CD36 *single TM receptors* ITGAV/B3	*proteases* MMP-14 *lipids* Ptd-L-Ser LPS	*proteases* FURIN		*others* C1QL1-4

VII	ADGRB1 (BAI1)		ADGRB2 (BAI2)	ADGRB3 (BAI3)
intra	*PDZ scaffold proteins* MAGI1/2/3 DLG 1/2/3/4 INADL LIN7A LRRC7 SNT1A,1B,2B,2G PDZD2 *GEF s* PARD3/ TIAM1 ELMO1/ DOCK1 *G proteins* Gα12/13 Gα16	*scaffold proteins w/o PDZ* BAIAP2 *7TM receptors* BAIAP3 *kinases* NEK4 MAPK1 *small GTPases* RAC1 RHOA *arrestins* ARRB2 *others* UBC PHYHIP	*PDZ scaffold proteins* SHANK1 *transcription factors* GABPB1/2 GABPA LRP2BP *arrestins* ARRB2 *GAPs* STARD13	*PDZ scaffold proteins* ELMO1/2/3 DOCK1 *arrestins* ARRB2
extra	*ECM proteins* COL3A1 *others* TGM2 CD9 CD81			*ECM proteins* COL4A4 LAMA2

VIII	ADGRG1 (GPR56)	ADGRG2 (GPR64)	ADGRG3 (GPR97)	ADGRG4 (GPR112)	ADGRG5 (GPR114)	ADGRG6 (GPR126)	ADGRG7 (GPR128)
intra	*7TM receptors* ADRB2 *arrestins* ARRB2 *small GTPases* RHOA *G proteins* Gαq/11 Gα11/13	*transcription factors* SRF NFKB1 *small GTPases* RHOA *G proteins* Gα12/13 Gαq	*arrestins* ARRB2 *small GTPases* RHOA CDC42 *G proteins* Gαo		*G proteins* Gαs	*G proteins* Gαs Gαi	

extra IX	ADGRV1 (VLGR1)
intra	*scaffold proteins with PDZ* USH1C DFNB31 PDZD7 *single TM protein* VEZT *others* SNAP25 MYO7A ADCY6 *G proteins* Gαs Gαq Gαi



References: ADGRL1 [8–12], ADGRL2 [8, 10], ADGRL3 [10], ADGRE2 [13], ADGRE5 [14–16], ADGRA1 [17], ADGRA2 [18, 19], ADGRA3 [18, 20], ADGRC1 [21], ADGRC2 [22, 23], ADGRC3 [22, 24], ADGRD1 [25], ADGRF1 [26], ADGRF4 [26], ADGRF5 [27, 28], ADGRB1 [29–42], ADGRB2 [8, 38, 43, 44], ADGRB3 [45, 46], ADGRG1 [47–51], ADGRG2 [52], ADGRG3 [53], ADGRG5 [26], ADGRG6 [54–57], ADGRV1 [58–64].

org/), or *MINT* (http://mint.bio.uniroma2.it), provides additional information on putative complex partners for ADGRs, which have mainly been acquired by proteomic or genetic screens.

4 ADGR Protein Networks Define Functional Modules

Combining data on protein–protein interactions from experiments and literature and database searches for all ADGRs results in a complex overall protein network (Fig. 2). This has revealed interactions of ADGR members across different subfamilies with similar proteins and even with the same protein. These data indicate common mechanisms underlying the function of the diverse ADGRs and their integration in similar cellular contexts and molecular modules (see below Sect. 6). However, the overall ADGR interactome is still far from completion.

To highlight features of protein networks related to ADGRs, we have chosen three subfamilies of ADGRs for which reliable protein–protein interaction data and information on protein networks exist. We chose the following: (A) ADGRLs (LPHNs), also known as latrophilins; (B) ADGRBs, better known as brain-specific angiogenesis inhibitors (BAIs); and (C) the ADGR goliath ADGRV1, also known as very large G protein-coupled receptor (VLGR1), GPR98, or MASS1. In the following parts of this chapter, we will review these protein networks and discuss the impact of their function in cellular modules.

4.1 ADGRLs (LPHNs, Latrophilins) Are Organized by Scaffold Proteins at Synaptic Junctions

The group of ADGRLs is one of the most intensely studied subfamilies of ADGRs. Over the last two decades, several labs have analyzed ADGRLs in different organisms. Their traditional name "latrophilins" originates from their ligand α-latrotoxin, a component of the black widow spider venom. The binding of this poison activates ADGRLs and results in massive Ca^{2+}-independent exocytosis of neurotransmitters into the synaptic cleft [68, 69]. In vertebrates, the ADGRL subfamily is comprised of three paralogous genes (*ADGRL1* to *ADGRL3*) which each can be alternatively spliced. ADGRL1 and ADGRL3 are mainly expressed in the central nervous system, whereas ADGRL2 is expressed more ubiquitously [70]. Besides the typical secretin-like 7TM and the GAIN domain, all ADGRLs contain a characteristic intracellular domain with a C-terminal class I PBM (Fig. 3a). Their ECD displays a hormone receptor motif (HRM), an olfactomedin-like domain, and a rhamnose-binding lectin (RBL) motif. For both the ICD and ECD, several binding proteins have been identified which are part of protein networks related to cell–cell adhesion complexes predominantly found at synapses.

In the ECD of ADGRLs, two different protein-binding sites, namely, the olfactomedin-like domain and the RBL motif, have been described in vertebrates, so far (Fig. 3a). The olfactomedin-like domain of ADGRL1 is capable of binding

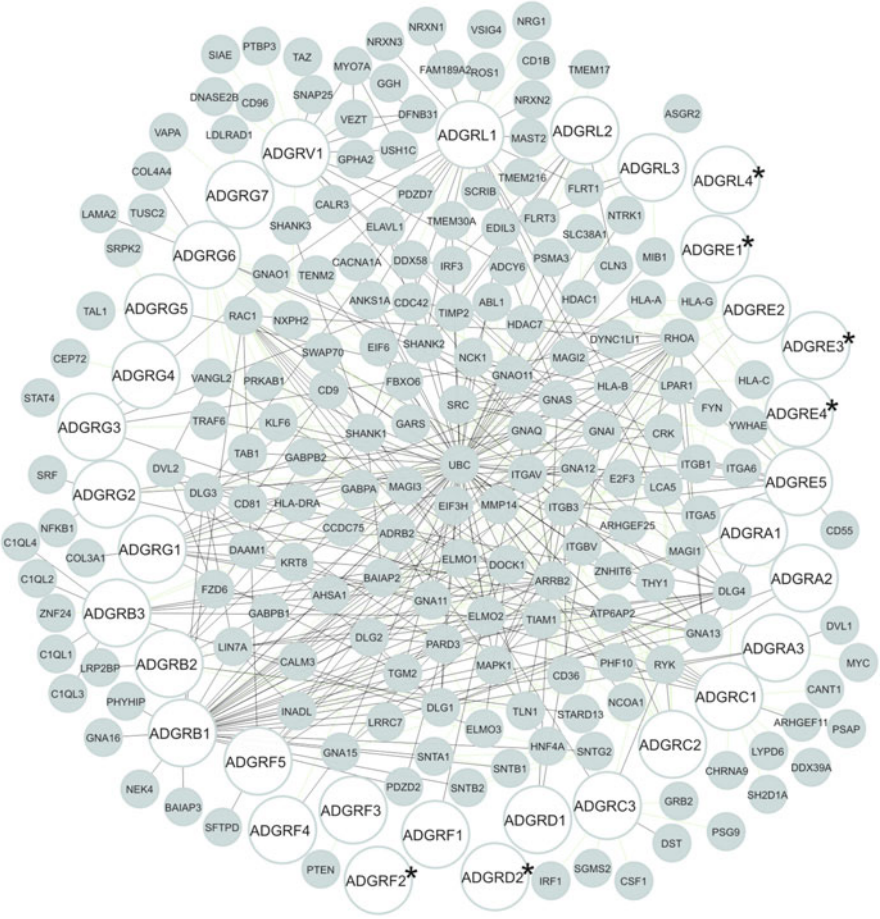


Fig. 2 The ADGR protein network. ADGRs are connected via their interaction partners and form complex networks. The present network is based on data from *STRING* (*Search Tool for the Retrieval of Interacting Genes/Proteins*, http://string-db.org/) and Cytoscape database searches (http://www.cytoscape.org/). The protein nomenclature follows the HGNC guideline, and protein designations of human orthologues are used for all interaction partners. *Black lines*, published experimental data; *green lines*, data from protein databases. Asterisk indicates ADGRs for which no known interaction partners are known so far

neurexins (NRXNs) (neurexins 1α, 1β, 2β, and 3β) [11]. Neurexins are single-pass transmembrane proteins and facilitate the formation of heterotypic intercellular transsynaptic junctions. Due to their frequent alternative splicing, a remarkable number of neurexin splice variants (~4000) is expressed [71]. The inclusion of a single exon at the splice site SS4 interrupts the sixth laminin/neurexin/sex hormone-binding globulin (LNS/LamG) domain of all neurexins [11]. This splicing event disrupts the neurexin–ADGRL1 interaction, indicating that the neurexin binding to ADGRL1 is mediated by this LNS domain. In neurons, neurexins are

found at the pre-synapse and interact with neuroligin 1 (NLGN1), a neural cell adhesion molecule of the post-synapse. In this heterotypic transsynaptic junction, ADGRL1 competes with neuroligin in the binding of neurexins [11].

The RBL binding motif of ADGRL1 mediates binding of the single-pass transmembrane receptor teneurin-2 (TENM2), also known as Lasso [12]. Like ADGRLs, teneurins are highly enriched in the central nervous system where they localize to pre- and post-synapses. Apart from the full-length transmembrane protein, shorter isoforms of teneurins are expressed, so-called teneurin C-terminal-associated peptides (TCAPs) which lack the transmembrane domain. TCAPs are involved in paracrine signaling and have high structural homology with peptide ligands that activate GPCRs of the secretin family [72]. The Ushkaryov lab showed that the C-terminal fragment of teneurin-2 that corresponds mainly to the TCAP2 region is sufficient to activate ADGRL1 and ADGRL2, which results in presynaptic calcium release in hippocampal neuron cultures [12]. The ICD of teneurins contains a "teneurin"-like domain characterized by two Ca^{2+}-binding EF-hands and two Cap/ponsin sites that provide a link to the actin cytoskeleton [72]. Both, neurexins and teneurins, link ADGRLs to the dystroglycan complex (DGC) by binding to the α-subunit of dystroglycan (DAG1) [73–75]. The dystroglycan β-subunit is associated with dystrophin (DMD) and utrophin, which connect the DGC to the actin cytoskeleton. The DGC provides a crucial connection between the extracellular matrix and the cytoskeleton in muscle cells and is of importance in other cell types, namely, neurons. Thus, it is present at the neuromuscular junction [76] and participates in the maintenance of Schwann cell myelination and axon guidance [77].

A crucial role in axon guidance was also shown for the fibronectin leucine-rich repeat (LRR) transmembrane proteins (FLRTs) [10]. FLRT3 expression is essential for the chemotactic response of rostral thalamocortical neurons to the guidance cues Slit1 and Netrin1 in primary neuronal cultures [78]. Further, FLRTs have been demonstrated to bind to the RBL motif and the HRM domain of ADGRLs via their LRRs. Knockdown of FLRT3 and competitive binding of ADGRL3 extracellular domain fragments in hippocampal neuron cultures both reduce the density of glutamatergic synapses [10]. This suggests that ADGRLs and FLRTs act in concert to regulate the number of excitatory synapses.

The ICD of ADGRL1 binds via its PBM to a group of PDZ domain-containing scaffold proteins (Fig. 3a) which are key components of the postsynaptic density (PSD), including SH3 and multiple ankyrin repeat domains protein 1 (SHANK1), DLG4 (previously known as PSD95), and MAGUK protein membrane-associated guanylate kinase inverted-2 (MAGI2) [79, 80]. Although these scaffold proteins are characteristic for the PSD, they can also be found in the presynaptic compartment of some synapses (e.g., inhibitory synapses, photoreceptor synapses). Therefore, interactions of PSD scaffold proteins with ADGRLs do not strictly define a post-synaptic localization of ADGRLs.

At the synaptic membrane, scaffold proteins organize supramolecular adhesion complexes, which include, besides ADGRLs, some of their identified interaction partners, e.g., neurexins (Fig. 3a). In these complexes, ADGRLs are connected to

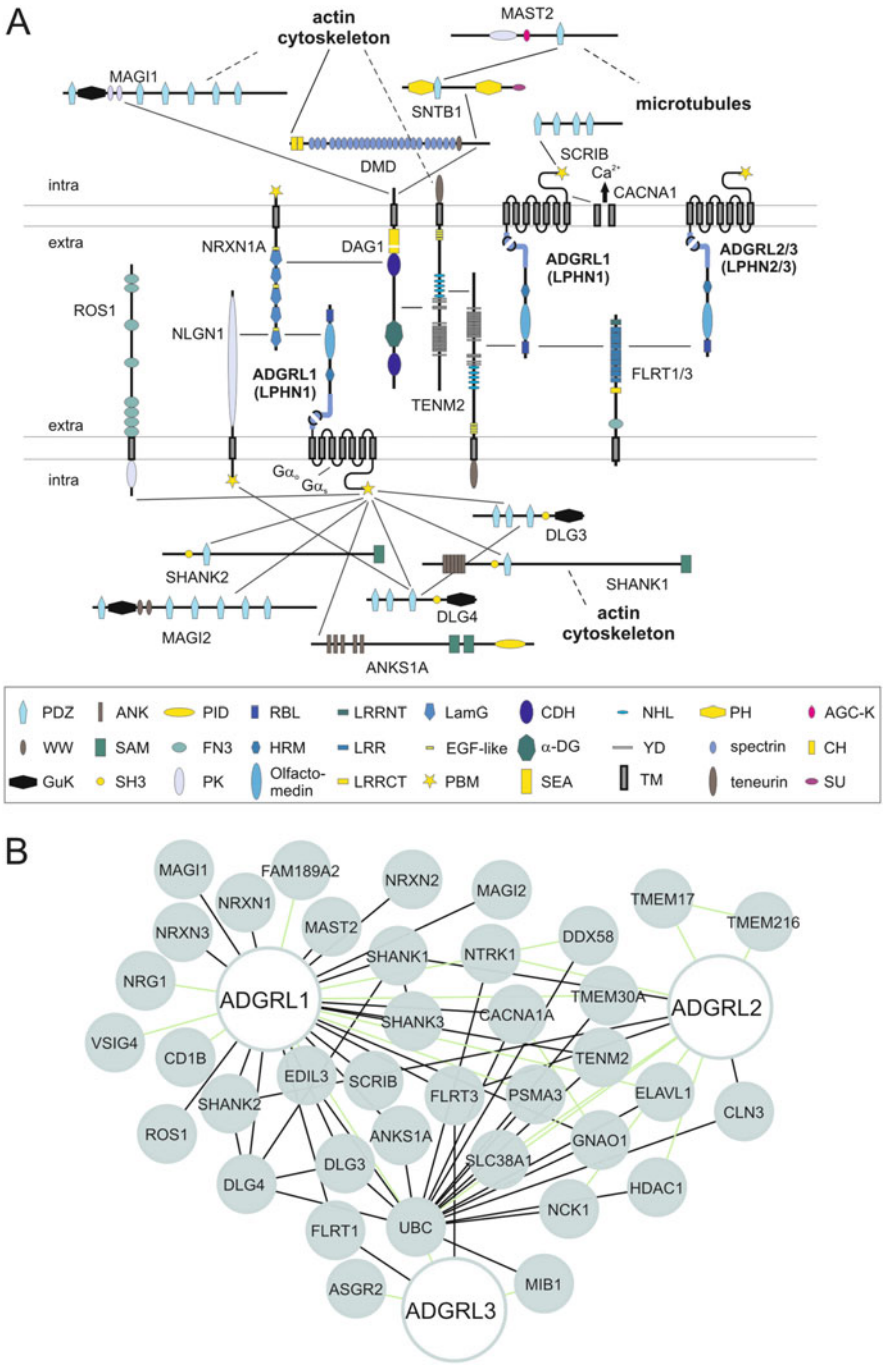


Fig. 3 The ADGRL (LPHN) network. (**a**) ADGRLs and their intracellular/extracellular interaction partners form a transmembrane-spanning protein network. *Black lines* depict experimental confirmed interactions. *Dotted lines* illustrate indirect connections. The protein nomenclature

actin filaments and the microtubule cytoskeleton via their interaction partners MAGI1/MAGI2 and the microtubule-associated serine/threonine 2 kinase (MAST2) [9]. Further, ADGRL1 binds to the planar cell protein (PCP) protein SCRIB [9], which is involved in cell migration, cell polarity, and cell proliferation [10, 81].

Insights into ADGRL signaling through heterotrimeric G proteins have been provided by two studies, so far. ADGRL1 interacts with Gαo, as demonstrated by affinity chromatography. In the same study an increase of cAMP and IP3 production in ADGRL1-transfected cells, but not in control cells, was observed [69]. A study in *C. elegans* on the ADGRL1 homologue LAT-1 showed coupling of LAT-1 to Gαs [67]. This Gαs-mediated cascade was essential for proper spindle orientation during cell division [67].

In summary, ADGRLs are mainly involved in synaptic protein networks, where they exert a role in axon guidance, synapse formation, the regulation of synaptic plasticity, and/or the control of the ratio between excitatory and inhibitory synapses. These functions are closely related to remodeling of the cytoskeleton and can be triggered by the reception of signaling cues from the extracellular space.

4.2 ADGRB (Brain-Specific Angiogenesis Inhibitor, BAI) Protein Networks Regulate Dendritic Spinogenesis, Synaptic Plasticity, and Phagocytosis

Intense studies conducted in the last decade have revealed a large number of molecules interacting with the three members of the ADGRB or BAI (brain-specific angiogenesis inhibitor) subfamily (Table 1, Fig. 4) [30]. ADGRB1–ADGRB3 are strongly expressed in neurons, astrocytes, and macrophages of the nervous system [31, 82, 83] but also found in non-neural tissues at lower levels of expression [32]. Their molecular structure is characterized by a variety of

Fig. 3 (continued) follows the HGNC guideline, and protein designations of human orthologues are used for all interaction partners. (**b**) Network of ADGRLs and their interactors. The visualized network is based on data from *STRING* (*Search Tool for the Retrieval of Interacting Genes/Proteins*, http://string-db.org/) and Cytoscape database search (http://www.cytoscape.org/). *Black lines*, published experimental data; *green lines*, data from protein databases. Abbreviations: *PDZ* (PSD95, Dlg, and ZO-1/ZO-2) domain; *WW* WW repeat; *GuK* guanylate kinase-like; *ANK* ankyrin repeats; *SAM* sterile alpha motif; *SH3* Src homology domain; *PID* phosphotyrosine interaction domain; *FN3* fibronectin-III type domain; *PK* protein kinase domain; *RBL* rhamnose-binding lectin-like domain; *HRM* hormone receptor motif; *Olfactomedin* olfactomedin domain; *LRRNT* leucine-rich repeat N-terminal; *LRR* leucine-rich repeat; *LRRCT* leucine-rich repeat C-terminal; *LamG* laminin G domain; *EGF* epidermal growth factor-like domain; *PBM* PDZ-binding motif; *CDH* cadherin-like repeat; *α-DG* α-dystroglycan domain; *SEA* sea urchin sperm protein, enterokinase and agrin domain; *NHL* NCL-1, HT2A, and Lin41 repeat; *YD* YD repeat; *TM* transmembrane domain; *PH* pleckstrin homology; *spectrin* spectrin repeats; *teneurin* teneurin intracellular domain; *AGC-K* AGC-kinase C-terminal domain; *CH* calponin homology domain; *SU* syntrophin unique domain

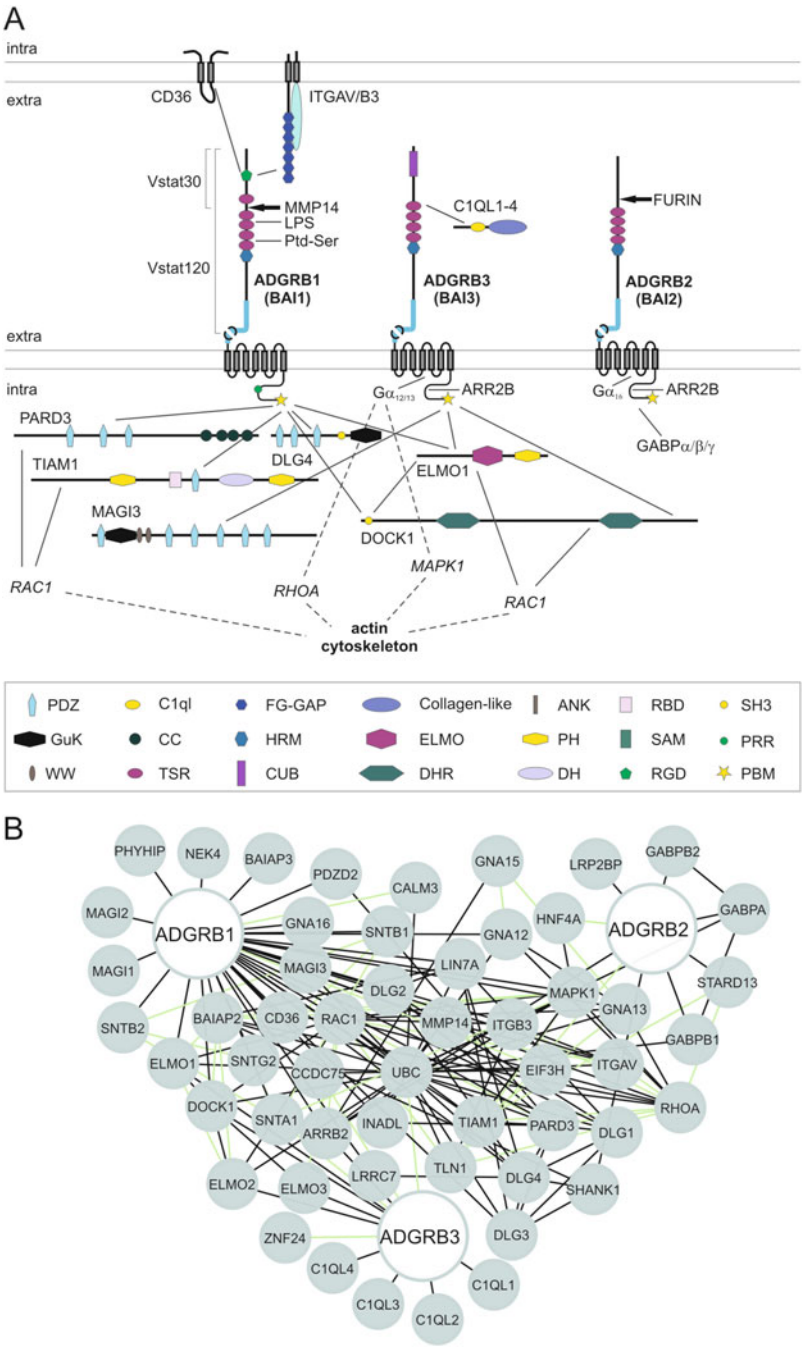


Fig. 4 The ADGRB (BAI) network. (**a**) ADGRBs form complex networks involved in actin cytoskeleton remodeling via activation of small RHO GTPases. The ECD of ADGRBs can be processed by proteases and interact with a heterogenous group of ligands. *Black lines* depict

conserved domains on both their ECD and ICD regions [30]. The ECDs of ADGRBs contain multiple thrombospondin type 1 repeats (TSRs) and a single hormone-binding domain (HBD). ADGRB1 additionally displays an Arg-Gly-Arg integrin-binding motif (RGD), whereas ADGRB3 possesses a (Cs1 and Csr/Uegf/BMP1) (CUB) domain on the ECD. Like most ADGRs, ADGRBs can be autoproteolytically cleaved at the GPS [3, 29, 84]. All ADGRBs possess a class I PBM at the very end of the C-terminus. The ICD of ADGRB1 also features a proline-rich region (PRR) suitable for binding to Src homology 3 (SH3) and WW domains.

The NTF of ADGRB1 was originally identified to regulate angiogenesis and thereby prevent tumorigenesis [30, 85]. The soluble N-terminal 120 kDa fragment, termed vasculostatin 120 (Vstat120), has a CTF-independent function [85]. Vstat120 inhibits endothelial cell migration in vitro and in vivo, and its antiangiogenic effect depends on its binding to CD36 which is expressed on the surface of endothelial cells [33]. The CLESH domain of CD36 and the antiangiogenesis TSR module of the ECD of ADGRB1 specifically mediate this in trans interaction [33]. TSRs are characteristic features of thrombospondins, proteins that are generally involved not only in angiogenesis, but also in the regulation of synaptogenesis [86].

In the CNS, ADGRB1 is highly enriched at the postsynaptic density (PSD) of dendrites [29, 34]. Similar to ADGRLs, ADGRB1 binds to various scaffold proteins that assemble complex networks at excitatory synapses as, e.g., MAGI1–MAGI3, DLG4 (PSD95), and DLG1 [29, 30, 35]. These protein–protein interactions are mediated by the binding of the C-terminal PBM of ADGRB1 to PDZ domains of the scaffold proteins [29].

ADGRB1 activates the RHO pathway via coupling to $G\alpha_{12/13}$, which concurrently leads to ERK (MAPK1) phosphorylation, which affects the actin cytoskeleton and thereby propagates synaptogenesis and dendritic spine formation [87]. Interestingly, the CTF alone activates the RHO pathway stronger than the full-length protein. Binding of the MAGUK family protein MAGI3 modulates the

Fig. 4 (continued) experimental confirmed interactions. *Dotted lines* illustrate indirect connections. The protein nomenclature follows the HGNC guideline, and protein designations of human orthologues are used for all interaction partners. (**b**) Network of ADGRBs and their interactors. The visualized network is based on data from *STRING* (*Search Tool for the Retrieval of Interacting Genes/Proteins*, http://string-db.org/) and Cytoscape database search (http://www.cytoscape.org/). *Black lines*, published experimental data; *green lines*, data from protein databases. Abbreviations: *PDZ* (PSD95, Dlg, and ZO-1/ZO-2) domain; *WW* WW repeat; *GuK* guanylate kinase-like; *C1ql* C1q-like domain; *CC* coiled-coiled domain; *TSR* thrombospondin type 1 repeats; *FG-GAP* FG-GAP repeat; *HRM* hormone receptor motif; *CUB* (C1r and C1s, uEGF, and bone morphogenetic protein) domain; *Collagen-like* collagen-like domain; *ELMO* ELMO domain; *DHR* dock homology region; *ANK* ankyrin repeats; *PH* pleckstrin homology domain; *DH* Dbl homology domain; *RBD* Raf-like Ras-binding domain; *SAM* sterile alpha motif; *RGD* Arg-Gly-Asp integrin-binding motif; *SH3* Src homology domain; *PRR* proline-rich region; *PBM* PDZ-binding motif

activation of the ERK pathway [29]. For this, scaffold proteins may negatively regulate ß-arrestin 2 (ARRB2)-mediated receptor deactivation.

Further, a G protein-independent pathway of ADGRB1 signaling has been described in dendritic spines [34]. Here, ADGRB1 activates RAC1-mediated modulation of actin filament dynamics by the interaction of the two PDZ domain-containing proteins PARD3 and TIAM1 with the C-terminal PBM of ADGRB1. The induced PARD3/TIAM1 complex functions as a guanine exchange factor (GEF) for RAC1. Its recruitment to specific dendritic sites by ADGRB1 is crucial for proper spine and synapse formation [34]. Knockdown of ADGRB1 causes mislocalization of PARD3/TIAM1 and consequently loss of RAC1 and actin filaments from the spines [34]. Interestingly, the recruitment of the GEF by ADGRB1 seems to be regulated by a previously identified extracellular interaction partner of ADGRB1, integrin $\alpha v\beta 5$, that binds in trans to the RGD motif of ADGRB1 [34, 36].

Apart from its synaptic function, ADGRB1 also plays an important role in phagocytosis [31, 88] and myoblast fusion [89]. The engulfment of bacteria or apoptotic cells is induced by binding of lipopolysaccharides (LPS), endotoxins found in the outer membrane of Gram-negative bacteria, or phosphatidylserine (Ptd-L-Ser), a phospholipid of the plasma membrane enriched in neurons, respectively. For this, lipid compounds bind to the TSR domains of ADGRB1 and subsequently trigger a G protein-independent signaling pathway. Direct binding of ELMO1/DOCK1 to the ADGRB1 ICD activates the small GTPase RAC1 [31, 88]. Strikingly, the same signaling pathway is involved in the fusion of myoblasts forming syncytial muscle cells. During this process, a small subset of myoblasts undergo apoptosis, which then promotes the fusion of healthy myoblasts via the abovementioned ADGRB1-mediated RAC1 activation [89]. More recently, a comparable role in myoblast fusion has been demonstrated for ADGRB3 [45].

Secreted C1QL proteins, which bind to the TSR module of ADGRB3, can activate ADGRB3 [46]. C1QLs are unique among the C1Q/TNF superfamily of proteins [90], which are almost exclusively expressed in the brain during synaptogenesis [91, 92]. Together with ADGRB3 they regulate the density of excitatory synapses and thereby play an important role in synaptic plasticity [46]. During vertebrate brain development, C1QL1 expression controls climbing fiber synaptogenesis and the territory on Purkinje cells, thereby modulating Purkinje cell spinogenesis by activation of ADGRB3 [93].

Similar to the other two members of the ADGRB subfamily, ADGRB2 is also essential for proper neuronal cell development. Studies on ADGRB2-deficient mice reveal increased neurogenesis in hippocampal neurons [94]. The mechanism underlying the ADGRB2-dependent regulation of neurogenesis might be based on the binding of the transcription regulator GA-binding proteins α/γ and β/γ (GABPA, GABPB1, and GABPB2) to the ICD of ADGRB2. This association leads to the suppression of vascular endothelial growth factor (VEGF) expression, which is a stimulator of neurogenesis in the hippocampus [43].

In summary, ADGRBs regulate muscle cell development, neuronal function, and synaptogenesis through several different signaling pathways. This functional

diversity is obtained via the variety of binding partners present in the interaction modules of the three ADGRBs (Fig. 4). First, the large number of PDZ domain-containing scaffold proteins provides multiple ways, which enable ADGRBs to integrate into membrane-associated protein networks. These modules are mostly associated with defined cell–cell contacts, in particular with synaptic junctions. Second, ADGRBs activate RAC1 via G protein-independent pathways by recruitment of the GEFs ELMO1/DOCK1 and TIAM1/PARD3, respectively. Third, ADGRBs can induce actin filament remodeling via a $G\alpha_{12/13}$-coupled pathway resulting in RHO activation. ADGRB signaling can be modulated by PDZ-proteins, β-arrestin 2 and potentially also via kinases such as NEK4 [37]. An additional mode of regulation of ADGRBs is provided by the NTF and CTF. Both fragments of ADGRBs can act independently or as a functional unit. Furthermore, the cleavage of the NTF by matrix metalloprotease-14 (MMP14) and furin can modulate ADGRB function [38, 95].

Interestingly, a couple of binding partners that are unusual for integral membrane proteins have been identified as ADGRB interactors, e.g., the GABP transcription factors [43] and PHYHIP [32], a component of peroxisomes. Together, this suggests a complex interplay between ADGRB regulation and its cellular function, depending on the cellular context.

4.3 ADGRV1 (VLGR1, GPR98), the Gigantic Receptor of Membrane Adhesion Networks in Sensory Cells and Synapses

ADGRV1, also known as very large G protein-coupled receptor (VLGR1), GPR98, or MASS1, is the largest member of the ADGR family, with a molecular weight of more than 700 kDa for the full-length protein [96]. To date, at least nine splice variants are known in vertebrates [97–100], but most probably many more splice variants remain uncharacterized [58]. All ADGRV1 splice variants contain varying numbers of Ca^{2+}-binding CalX-β repeats that resemble the aggregation factors of marine sponges [97]. ADGRV1b (VLGR1b), the largest isoform, contains 35 CalX-β domains, one laminin G/pentraxin domain (LamG/PTX), and seven epitempin/epilepsy-associated repeats (EPTP/EAR) on its NTF [96]. In contrast ADGRV1a (VLGR1a) exhibits a much shorter extracellular domain composed of only six CalX-β domains. Both ADGRV1a and ADGRV1b possess a CTF comprising the GAIN domain with the integral GPS, the 7TM domain, and an ICD with a class I PBM at the C-terminal end. However, similar to the vasculostatins of ADGRB1, most ADGRV1 isoforms lack the entire CTF. The function of these secreted soluble ECD polypeptides is unknown so far.

With the exception of Ca^{2+} ions there are no ligands known to bind to the ECD of ADGRV1 [59]. In contrast several proteins have been identified which bind to the ICD of ADGRV1 (Table 1, Fig. 5). The majority of these proteins are related to the human Usher syndrome (USH), the most common form of hereditary deaf-blindness [101, 102]. Mutations in the *ADGRV1* gene lead to the USH type 2C

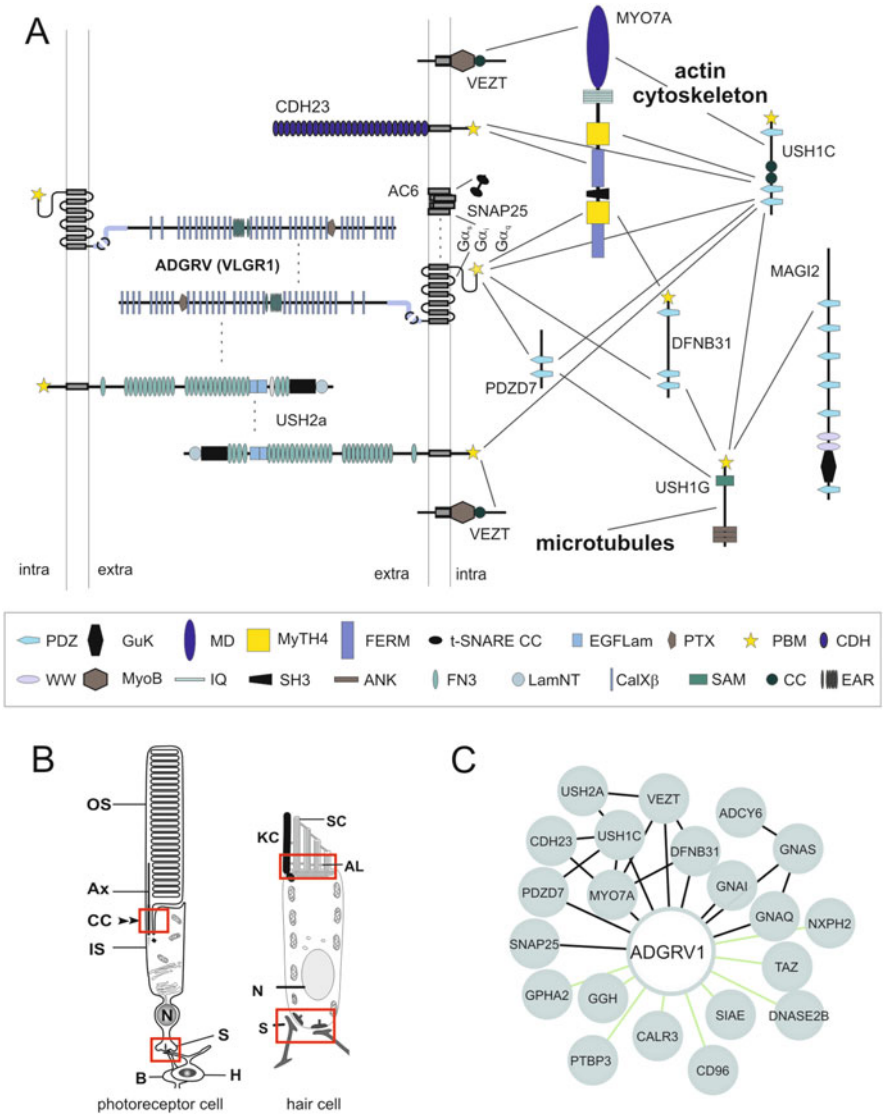


Fig. 5 The ADGRV1 (VLGR1) network. (**a**) ADGRV1 forms protein networks involving diverse PDZ scaffold proteins. The extracellular domain of ADGRV1 spans between membranes, potentially via homophilic interaction or by heterophilic interactions with USH2a (*dotted lines*). *Black lines* depict experimental confirmed interactions. *Dotted lines* illustrate indirect connections. The protein nomenclature follows the HGNC guideline, and protein designations of human orthologues are used for all interaction partners. (**b**) ADGRV1 networks are localized at the apical inner segment and the synapse of photoreceptor cells and at ankle-links between stereocilia and synapses of hair cells. (**c**) Network of ADGRV1 and its interactors. The visualized network is based on data from *STRING* (*Search Tool for the Retrieval of Interacting Genes/Proteins*, http://string-db.org/) and Cytoscape database search (http://www.cytoscape.org/). *Black lines*, published experimental data; *green lines*, data from protein databases. Abbreviations: *PDZ* (PSD95, Dlg, and ZO-1/ZO-2) domain; *WW* WW repeat; *GuK* guanylate kinase-like; *MyoB* myosin-binding domain; *MD* myosin

(USH2C), characterized by profound deafness and *retinitis pigmentosa* (retinal degeneration) (see below). ADGRV1 variants are mainly expressed in the affected neurons of the retina and the inner ear [96, 101], but they are also found in olfactory cells of the olfactory epithelium [103] and the brain [96].

Together with other USH proteins, ADGRV1 is localized at synaptic junctions, preferentially at the post-synapse of neurons (Fig. 5b) [60, 104], where they are thought to act in concert in the regulation of synaptic function [105]. The disruption of ADGRV1 function at CNS synapses most probably underlies the phenotype associated with audiogenic seizures and epilepsy in the *Frings* mouse [98]. However, in the sensory cells of the inner ear, the eye, and the nose, ADGRV1 is an essential component of membrane-membrane adhesion complexes at the proximal pole of the sensory neurons (Fig. 5b) [60, 61, 103].

In the mechanosensitive hair cells of the inner ear, the ECD of ADGRV1 is a core component of the ankle-links, fibers which span the membranes of neighboring stereocilia in the developing hair bundles (Fig. 5b) [62, 106, 107]. ADGRV1 deficient mice show splayed stereocilia which result in a auditory phenotype [62]. In these mice the absence of ADGRV1 leads to mislocalization or disappearance of other components of the Usher protein complex from hair bundles, e.g., USH2A, whirlin (DFNB31, USH2D), myosin VIIa (MYO7A, USH1B), and vezatin (VEZT) or specific isoforms of cadherin 23 (CDH23, USH1D) [62, 108, 109]. Furthermore, the adenylate cyclase 6 (ADCY6) is not only mislocalized, but its expression is also increased in the absence of ADGRV1 [62] arguing for a coupling of ADGRV1 to the Gαs/AC/cAMP pathway (see below). These findings support a signaling role of ADGRV1 in the ankle-links during hair bundle differentiation. More recently, PDZD7 has been identified as a binding partner of ADGRV1 [63, 110]. PDZD7 was previously identified as a deafness gene [110] and later as a genetic modifier of USH2 [63]. The PDZD7 protein co-localizes with whirlin and ADGRV1 and has been suggested to be a further component of the ankle-link complex [109].

Similar to the stereocilia of hair cells, whirlin (USH2D) and harmonin (USH1C), the scaffold proteins of the USH protein network, anchor and define the position of ADGRV1 in the photoreceptor cell membrane (Fig. 5a) [101]. In rod and cone photoreceptor cells, ADGRV1 is integrated in an USH protein network found at the periciliary ridge complex at the base of the photoreceptor cilium (Fig. 5b) [111]. Here, the ECD of ADGRV1 is essential for the formation of fibers linking the periciliary membrane of the inner segment with the membrane of the connecting

Fig. 5 (continued) motor domain; *IQ* IQ motif; *MyTH4* myosin tail homology 4 domain; *SH3* Src homology domain; *FERM*, 4.1 protein, ezrin, radixin moesin domain; *ANK* ankyrin repeats; *t-SNARE CC* t-SNARE coiled-coil homology; *FN3* fibronectin-III type domain; *EGFLam* laminin EGF-like 1; *LamNT* laminin N-terminal; *PTX* laminin G/pentraxin domain; *CalX-β* CalX-beta domain; *PBM* PDZ-binding motif; *SAM* sterile alpha motif; *CDH* cadherin-like repeat; *CC* coiled-coil; *EAR* epitempin/epilepsy-associated repeats; *OS* outer segment; *Ax* axoneme; *CC* connecting cilium; *IS* inner segment; *B* bipolar cell; *H* horizontal cell; *KC* kinocilium; *SC* stereocilia; *AL* ankle-links; nucleus

cilium bridging the so-called ciliary pocket. We believe that this membrane-membrane adhesion complex and its intrinsic mechanical stabilization defines the target membrane for the vesicular transport and the import into photoreceptor cilia [111–113]. The relationship between ADGRV1 with intracellular vesicular transport is supported by more recent findings which indicate the presence of diverse ADGRV1 isoforms on transport vesicles and their association with the v-SNARE protein SNAP25 [58].

To date, only little is known about signaling pathways coupled to ADGRV1. Interestingly the two available studies on ADGRV1 signaling led to controversial results [59, 64]. First, Shin and coworkers (2013) reported the activation of PKC and PKA via $G\alpha_s$ and $G\alpha_q$, respectively, triggered by Ca^{2+}-binding to the ECD. This pathway seems to be involved in the stabilization of the myelin-associated glycoprotein, which is strongly expressed in oligodendrocytes. However, ADGRV1 mutant mice show no diminishment of myelination [59]. In contrast, Hu and coworkers (2014) demonstrated the coupling of ADGRV1 to a $G\alpha_i$-mediated pathway. Importantly both studies used different ADGRV1 fragments in their experiments. [59] created a "mini-ADGRV1" that contained an NTF composed of the first five CalX-β domains, the PTX domain and the EAR domain, which was fused to the CTF with an intact GAIN domain. In contrast, [64] applied only the CTF of ADGRV1. As shown similarly for other ADGRs, the absence of the NTF might abrogate its inhibiting effect on the activation of a $G\alpha_i$-mediated pathway [1]. However, the NTF might also provide a switch between the two different signaling pathways.

In any case, it remains elusive how ADGRV1 is activated, since no extracellular interaction partners or ligands have been identified so far. Furthermore, the role of the different ADGRV1 splice variants is unclear. Evidently, the elucidation of the ADGRV1 signaling network is still far from complete and seems to display a high degree of complexity, due to the large number of isoforms and potential modes of regulation.

4.4 Protein Networks Related to the Other Six ADGR Subfamilies

In comparison to the protein networks related to ADGRBs, ADGRLs, and ADGRV1 discussed above, the other six ADGR subfamilies have been less well studied so far. Nevertheless, at least for some of them, binding partners have been identified which are summarized in Table 1 and included in the overall network in Fig. 2.

For ADGRE5 (CD97) several ligands are known which activate the receptor via binding to its ECD [1]. Among these ligands is CD55, which is an inhibitor of the complement system and therefore of high clinical relevance [14]. In addition, THY1, an antigen present on the surface of immune cells, binds to the GAIN domain of ADGRE5 [14, 15]. ADGRE5 can also be activated by chondroitin sulfate B, a polysaccharide of the extracellular matrix, which can bind to the ADGRE5 paralogue ADGRE2 (EMR2) as well [13]. Further, the Arg-Gly-Asp

(RGD) motif downstream of the EGF-like domains in the ECD of ADGRE5 is recognized by integrins α5β1 and αvβ5, mediating cell attachment [16]. ADGRE5 further dimerizes with the LPA receptor (LPAR1), which results in activation of RHO signaling [114].

All three members of ADGR subgroup III, ADGRA1–3 (GPR123–125), bind via their PBM to synaptic scaffold proteins of the MAGUK family, DLG4 and DLG1, respectively [17, 18]. ADGRA3 (GPR125) and ADGRA2 (GPR124) are additionally connected to Wnt signaling via their interaction with Dishevelled 1 (DVL1) [20] and the receptor tyrosine kinase RYK, respectively [22]. RYK is a receptor for Wnt molecules and involved in neuron differentiation and axon guidance [115]. Additionally, ADGRA2 functions as a Wnt7a-/Wnt7b-specific co-stimulator of β-catenin signaling [116].

Like ADGRA2 and ADGRA3, ADGRCs (CELSR1–3) are coupled to the Wnt signaling pathway. All three receptors interact with RYK [22]. ADGRC1 is additionally connected to Wnt signaling by further binding partners. Together with the Wnt receptor Frizzled 6 (FZD6), it forms a network that involves Dishevelled 2 (DVL2), DAAM1, and the GEF ARHGEF11. This network is localized at adherents junctions and is involved in RHO activation, which results in actomyosin-dependent contractions [21]. ADGRC1, FZD6, and DVL2 are key proteins of the planar cell polarity (PCP) pathway [117], and a further key component of the PCP pathway, VANGL2, has also been identified to bind ADGRC1. Both proteins work in concert with FZD6 and are indispensable for proper hair follicle orientation [118]. ADGRC3, however, is connected to the cytoskeleton by the linker protein DST and the GEF SWAP70, which both bind to F-actin [119, 120].

ADGRF5 (GPR116) is involved in lamellipodia and actin stress fiber formation, by $G\alpha_q$-ARHGEF25-mediated activation of RHOA and RAC1 [27]. For ADGRF5 the extracellular matrix surfactant protein D (SFTPD) has been described as the only ECD ligand so far [28]. It is a collagen-containing C-type lectin secreted from alveolar type 2 epithelial cells in the lung. ADGRF5 monitors the level of SFTPD and its deletion results in an emphysema-like pathology.

Two members of the VIII subfamily, ADGRG1 (GPR56) and ADGRG6 (GPR126), bind to fiber-forming collagen subtype 3 (COL3A1) and collagen subtype 4 (COL4A4) which does not assemble into fibers, respectively [47, 48]. COL4A4 interacts with the ECD of ADGRG6 that contains the CUB and PTX domains [54]. Furthermore, the interaction of ADGRG6 with a second ECM protein, laminin-211 (LAMA2), has been demonstrated. Both, COL4A4 and LAMA2, activate $G\alpha_s$-mediated signaling, which is important for the myelination of Schwann cells [54]. ADGRG1, however, does not contain such domains and therefore interacts via a different binding mode.

ADGRG1 (GPR56) and ADGRG3 (GPR97) can be regulated by β-arrestin 2 [48, 121] and have been shown to activate small RHO GTPases [47, 53], leading to remodeling of the actin cytoskeleton. ADGRG3 knockdown results in a change of the ratio of activated CDC42 vs. activated RHO leading to the redistribution of F-actin and paxillin. Binding of collagen subtype 3 (COL3A1) to ADGRG1

introduces the activation of the RHO pathway, which plays a crucial role in the regulation of proper lamination in the cerebral cortex [47].

Other ADGR groups are not well studied, although a growing set of data from public databases are available, e.g., for ADGRE1 (EMR1) and ADGRE2 (EMR2), which originate from proteomic screens but have not yet been validated [122, 123]. Pharmacological analyses have provided additional information on the heterotrimeric G proteins coupled to ADGR, which are described in [124, 125]. In conclusion, to date only a limited number of binding partners for ADGRs are known, and in most cases these interactions are poorly validated or uncharacterized. The number of valid binding partners of a specific ADGR is certainly highly dependent on the experimental efforts, which have been focused on that particular protein network.

5 Diseases Associated with ADGR Network Disturbances

Coherent protein networks require the smooth interplay of all network partners to provide proper cellular function. There are several examples in the ADGR field that the disruption of a single network component can have severe consequences for cell function and in turn for the affected organism. Malfunction of compounds within the same network often results in identical or similar phenotypes. The integration of molecules in the same protein network or pathway may provide potential common targets for pharmacological therapeutic interventions.

Mutations in *ADGRV1* (VLGR1) cause sensory neuronal degeneration, namely, the human Usher syndrome (USH) [102, 127] (see above Sect. 4.3). USH is a complex autosomal recessive disorder and the most common form of hereditary deaf-blindness. Based on the age of onset and the progression of the symptoms, three USH types (USH1–USH3) can be clinically differentiated. So far, ten different USH-causing genes and three genetic modifiers have been identified, and recent research revealed that all proteins related to USH are integrated into common protein networks [101, 102]. It is thought that defects in one USH protein can cause the disruption of the entire USH protein complexes in inner ear hair cells and retinal photoreceptor cells in the eye, leading to the sensory neuronal degeneration, which manifests in deaf-blindness. Furthermore, defects in the murine *Adgrv1* (*Vlgr1*) are associated with audiogenic seizures and epilepsy [98], which has also been discussed for human patients [24, 128].

Another severe disorder associated with an ADGR is bilateral frontoparietal polymicrogyria (BFPP), which is caused by mutations in ADGRG1 (GPR56) [129, 130]. Like USH, BFPP is an autosomal recessive disorder [131, 132]. Patients with BFPP suffer from cognitive interference, a delay in development of motor neurons, susceptibility to seizures, and ataxia. The most striking phenotype of BFPP-affected brains is the cobblestone-like cortical malformation [133, 134]. Interestingly, defects in collagen III (COL3A1), a validated ligand of ADGRG1 (see above, Sect. 5), develop an equivalent phenotype in mice [135] indicating that they interact in the same functional module.

A similar connection has been observed between ADGRB1 and its interaction partner BAIAP2 (IRS53). Both participate in the regulation of dendritic spine formation [34, 136], and their defects are suggested to be related to autism disorders [137, 138]. Interestingly, the expression of another interactor of ADGRB1, DLG4 (PSD95) [39], is increased in ADGRB1-deficient mice [34], and there are hints that the regulation of DLG4 is a general feature for genes involved in autism [139].

SNPs in *ADGRB3* lead to development of schizophrenia and probably participate in susceptibility to addiction [140, 141]. Further, ADGRB3 deficient mice show a depression-resistant phenotype [94].

For mutations in *ADGRL1* and *ADGRL3*, a connection to the attention deficit hyperactivity disorder (ADHD) [142, 143], hearing defects, brain malformations, and retardation has been reported [142]. One group of ADGRL1's extracellular interaction partners, neurexins, are associated with mental retardation and autism as well [144, 145].

ADGRCs are also mandatory for proper brain development. In humans, mutations in the corresponding genes cause neural tube defects and are associated with caudal agenesis [146, 147]. In addition, ADGRCs are involved in the proper development of ependymal cilia and disruptions of the network result in hydrocephalus. These effects occur due to disturbances of PCP and Wnt signaling pathways that are connected to ADGRCs [148].

Moreover, some ADGRs act as tumor suppressors and therefore gene defects in ADGRs can cause cancer and tumorigenesis. One of these receptors is ADGRE5 (CD97), which was found to be significantly upregulated in diverse cancer cells [149–151]. Others are ADGRG1 (GPR56), which is up- or downregulated, respectively, in a cell-specific manner in cancer cells [49, 152]. Specifically ADGRF5 is associated with breast cancer [27], and ADGRB1, is absent or downregulated in various cancer types [153–155]. The relation between cancer genesis and defects in ADGR networks might be due their role in cytoskeleton regulation via small GTPases.

Several additional ADGRs have been associated with severe human diseases but no proteomics and interactomics data are available for these so far. Further decipherment of protein networks related to diseases associated with ADGRs would not only enlighten the pathomechanisms leading to the disease but also elucidate potential targets for treatment and cure.

6 Common Features of ADGR-Related Protein Networks and Signaling Pathways

The data acquired for ADGRs so far indicate a high degree of similarity within the protein family. Since similar or identical interactions have been reported for ADGRs of various subgroups, it is probable that ADGRs are integrated in common networks and may even act in concert. ADGRs are authentic G protein-coupled receptors, and for several receptors heterotrimeric G proteins have been identified [156] (Fig. 6). Furthermore, the canonical desensitizing mechanism of GPCRs

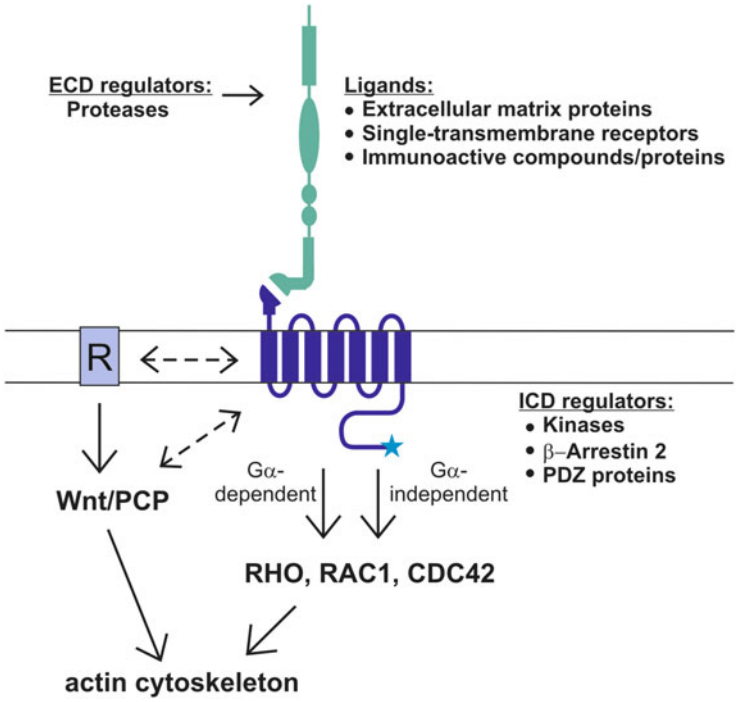


Fig. 6 Common features of ADGRs. ADGRs show similarities concerning ligand binding, signaling, and regulation. Many ADGRs activate RHO/RAC1/CDC42-mediated pathways. Some ADGRs may cross talk to Wnt/PCP signaling through other transmembrane receptors (R) or other components. Extracellular matrix proteins, single-transmembrane receptors, and immunoactive proteins/compounds mainly activate ADGRs. Regulation of ADGRs has been demonstrated for β-arrestin 2, PDZ scaffold proteins, proteases, and kinases

through β-arrestin 2 binding has also been identified in some ADGRs (Fig. 6), namely, ADGRBs (BAIs), ADGRG1 (GPR56), and ADGRG3 (GPR97) [48, 121].

Although ADGRs signal through diverse Gα-subunits to a variety of the downstream signaling pathways [1], the activation of small GTPases (RHO, RAC1, or CDC42) is a common theme (see above, Fig. 6). However, some ADGRs can also activate small GTPases independent from heterotrimeric G proteins as, e.g., ADGRB1 through PARD3/TIAM1 or ELMO/DOCK1, respectively [31, 34]. The downstream target of the small GTPases pathways is the actin cytoskeleton, and remodeling the cytoskeleton is a common process during neurite outgrowth or dendritic spine formation, which are known to be regulated by several ADGRs (see above). For at least five of the ADGRs, namely, the three ADGRCs (CELSRs) and ADGRAs (GPR125, GPR124), there is evidence for cross talk with the Wnt signaling pathway [20, 21]. They either are coupled to the receptor tyrosine kinase RYK, the Wnt receptor Frizzled 6 (FZD6) or interact with the FZD mediator Dishevelled (DVL) [22]. Interestingly, DVL can mediate the activation of the RHO pathway regulating the actin cytoskeleton [21].

Besides their obvious links to signaling networks, almost all ADGRs examined thus far are integrated in membrane-associated protein networks organized by scaffold proteins. This is usually mediated by the interaction of the C-terminal PDZ-binding motif (PBM) often found at the C-terminal end of ICD of ADGRs and PDZ domains of the diverse scaffold proteins. Most of these scaffold proteins belong to the membrane-associated guanylate kinase (MAGUK) protein family, e.g., DLG1–4 and MAGI1–MAGI3 (Figs. 2–4). These scaffold proteins facilitate the organization of ADGR-related networks at specific domains of the cell membrane, but may additionally regulate the signaling of the CTF of ADGRs. Strikingly, numerous ADGRs are integral components of adhesion complexes at synapses and dendritic spines showing a highly regulated expression profile during development (e.g., ADGRBs, ADGRLs, ADGRCs, and ADGRV1) [30, 96, 157, 158].

In cell adhesion complexes, the long ECD of ADGRs mediates cell–cell and cell–matrix interactions, which have been shown to promote ADGR signaling. Collagens or collagen-like proteins were identified as common interaction components from the extracellular matrix binding to different adhesion domains of the ECD of ADGRs [46, 47, 55]. Beside homophilic trans interactions, various adhesion domains in the ECDs of ADGRs are capable to interact with the ECDs of single-span transmembrane proteins or immunoactive proteins and compounds [10–12, 14–16].

Analysis of the protein interactome related to ADGRs presented in Fig. 2 shows that ten ADGRs interact with ubiquitin C (UBC). These interactions have been recently identified in proteomic screens targeting ubiquitinated proteins [159, 160]. So far the ubiquitination of ADGRs was not systematically studied, but it may have functional implications. As frequently reported for other signaling, ubiquitination of cell surface receptors may regulate the availability of ADGRs to interact with their extracellular ligands [161, 162].

Although ADGR families differ mainly in their ECD structure, several ADGRs from different families share the same domains, e.g., ADGRC1–ADGRC3 (CELSR1–CELSR3), ADGRD1 (GPR133), ADGRD2 (GPR144), ADGRG6 (GPR126), ADGRG4 (GPR112), and ADGRV1 (Vlgr1) share a LamG/PTX domain, whereas ADGRLs (LPHNs), ADGRBs (BAIs), ADGRA2 (GPR124), ADGRA3 (GPR125), and ADGRF3 (GPR113) have hormone receptor motifs (HRMs) [1]. The common ECD features of these ADGRs indicate that they may also be targets for similar or identical binding proteins and ligands, which are not described so far. Nevertheless, the available data indicate common mechanisms for ADGRs (see Fig. 6) in their integration and function in cellular contexts and molecular modules. Since similar or identical interactions have been reported for ADGRs of various subgroups, ADGRs seem to be integrated in common networks and may even act in concert.

7 Conclusions

To date, comprehensive approaches toward the systematic identification of valid interaction partners for ADGRs are still lacking. From future (larger) interactome data sets and systematic data mining, we expect to gain further insights into ADGR networks and a more holistic understanding of ADGR functional roles in cellular adhesion and signaling. The application of proteomic screening methods provides potential to learn more about these unique types of receptors. However, these approaches have to be complemented by functional assays and studies in animal models. Understanding the molecular mechanisms behind ADGR function will provide novel strategies for the cure of diseases that have been linked to malfunction of ADGRs, particularly various types of cancer, BFPP, and the human Usher syndrome.

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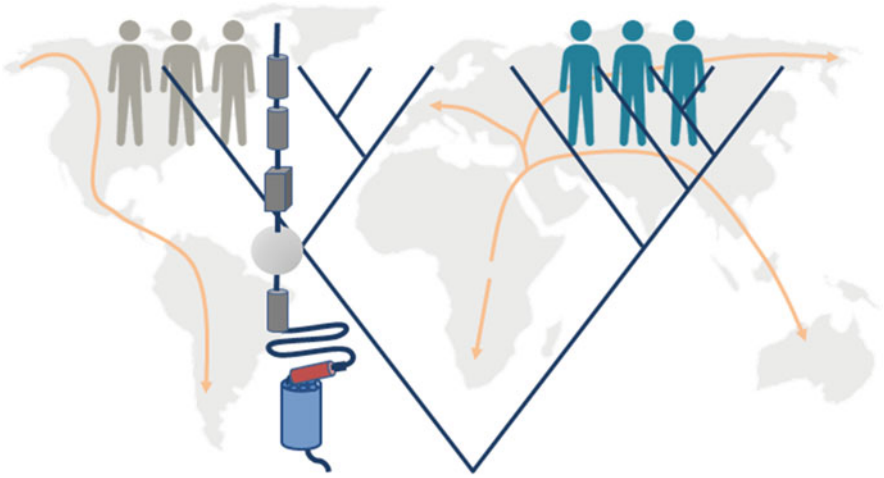
The Relevance of Genomic Signatures at Adhesion GPCR Loci in Humans

Peter Kovacs and Torsten Schöneberg

Graphical Abstract



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Contents

Abstract

Adhesion G protein-coupled receptors (aGPCRs) have a long evolutionary history dating back to very basal unicellular eukaryotes. Almost every vertebrate is equipped with a set of different aGPCRs. Genomic sequence data of several hundred extinct and extant species allows for reconstruction of aGPCR phylogeny in vertebrates and non-vertebrates in general but also provides a detailed view into the recent evolutionary history of human aGPCRs. Mining these sequence sources with bioinformatic tools can unveil many facets of formerly unappreciated aGPCR functions. In this review, we extracted such information from the literature and open public sources and provide insights into the history of aGPCR in humans. This includes comprehensive analyses of signatures of selection, variability of human aGPCR genes, and quantitative traits at human aGPCR loci. As indicated by a large number of genome-wide genotype-phenotype association studies, variations in aGPCR contribute to specific human phenotypes. Our survey demonstrates that aGPCRs are significantly involved in adaptation processes, phenotype variations, and diseases in humans.

Keywords

Adhesion G protein-coupled receptors • Population genetics • Natural selection • Genome-wide association studies • Mutation • Disease

1 Introduction

Since their first availability in the mid-2000s, the next-generation sequencing (NGS) technologies have been proven to be powerful tools in many fields of genetics, evolutionary biology, and biomedicine. Whole-genome sequencing and whole-transcriptome sequencing are efficient to provide sequence data not only for ortho- and paralog genes and transcripts of gene families but also for variants of genes involved in adaptation processes and responsible for diseases. The trove of genetic data yielded by NGS has made a significant impact on understanding the origin of gene families, their evolutionary expansion and contraction, and their structural variability. By identifying rare variants in known genes, NGS contributes to today's clinical diagnoses and helps in the discovery of molecular

pathomechanisms underlying human diseases. In parallel to this explosion in sequence information, an armada of bioinformatics tools and pipelines were developed to handle and meaningfully explore the complexity of these data. Since sequencing has become faster and more affordable, the number of available complete genomic sequences is exponentially increasing. As a result, the cost to process, analyze, transfer, and store the data is becoming the bottleneck for research and medical applications.

The wealth of sequence information comes from large sequencing consortia. For example, the whole genomes of over 2500 human individuals of 26 populations have been sequenced with low-coverage yielding 86 million variants [1]. A program has been launched in 2009 sequencing the whole genomes of 10,000 vertebrate species suitable for comparative genomic analyses [2]. Since then over 200 vertebrate species have been sequenced or completed [3]. Data of those multiple species and population genome ventures are used to construct the evolutionary tree of vertebrate clades, e.g., birds [4], and to reconstruct of migration waves of humans [5], respectively.

The advent of such large and diverse genomic data provides not only a valuable source to study the evolutionary history of species but also the evolution of specific gene families. This usually requires high-quality genomes of individual species and previously unavailable or unappreciated bioinformatics methods. Thus, the history of a protein family, the numeric gain and loss of family members, and their structural evolution by comparing orthologs and paralogs can be studied in the light of evolution. The comparison of orthologous sequences between species can shed light on evolutionary constrains and inventions which are of advantage in a given ecologic niche [6].

Evolutionary methods based on NGS data have already found numerous applications in biomedical research. Sequence differences in human populations or between extinct and recent hominoids are analyzed for signatures of selection providing clues for adaptation processes to specific environmental factors during human evolution. However, those differences may also influence or even cause human phenotypes and diseases. A great number of genome-wide genotype-phenotype association studies (GWAS) revealed previously unknown traits and pathways involving individual genes or gene families.

In this review we summarize applications of evolutionary methods specifically on the family of aGPCRs. Information that can be drawn from the currently available sequence data on the evolutionary history, selective forces acting on aGPCRs, and human phenotypes associated with aGPCR variants are summarized in this review.

2 The Evolutionary Origin and Development of aGPCRs

It has been estimated that GPCR prototypes were present already in the last common ancestor of eukaryotes [7], and most probably glutamate and cAMP receptors evolved in eukaryotic evolution about 1400 million years ago [8]. aGPCR

apparently appeared later in eukaryotic life (1275 million years ago) since aGPCR-specific sequence signatures in 7TM segments were found in the genomes of *Amoebozoa* (*D. discoideum*) and *Alveolata* (*P. tetraurelia*) [8]. In these basal eukaryotes, aGPCRs present with short N termini. The lack of a large ectodomain is apparent also in fungi aGPCRs where this receptor family started to expand. The unicellular non-metazoa choanoflagellate *Monosiga brevicollis* and filasterea *Capsaspora owczarzaki* contain several genes for aGPCRs already equipped with GPS site [8]. Further structural diversification of the large ectodomain occurred in pre-bilaterian species [9]. One can speculate that structural diversification and numeric expansion of aGPCRs soon after the origin of metazoans were driven by cell-cell and cell-matrix contacts which are major factors of multicellularity. Being present in metazoan genomes, aGPCRs became apparently very important as suggested by the structural conservation and numerical expansion of aGPCR genes between invertebrate and vertebrate species [10]. However, the number of aGPCRs varies between vertebrate species [11]. In the human genome, there are 34 genes encoding aGPCRs. Out of these genes, 33 aGPCR genes contain an open reading frame, and one, *EMR4*/*ADGRE4,* is a pseudogene presenting a deletion-caused disruption of the open reading frame. The deletion is not present in great apes suggesting that EMR4 became nonfunctional only after human speciation [12]. Interestingly, available databases do not contain mRNA sequence of the human GPR144/ADGRD2 and only rare transcripts in other vertebrate species (own observations). In mouse GPR144 is a pseudogene. It is therefore unclear whether GPR144/ADGRD2 is functional in humans. There are several clusters where aGPCRs are genomically organized in a tandem-like structure. One clusters at chromosome X containing *GPR110*/*ADGRF1, GPR111*/*ADGRF2, GPR115*/*ADGRF4,* and *GPR116*/*ADGRF5,* and another group of aGPCRs (EMRs/ADGRE1–4, CD97/ADGRE5) clusters in two sections on human chromosome 19p13, suggesting common evolutionary origins, respectively [12, 13].

3 Signature of Selections in aGPCR Genes

The recent increase in human sequence and polymorphism data, together with the availability of genome sequences from several closely related primate species, provides an unprecedented opportunity to investigate how natural selection has shaped the human genome. A central aim in evolutionary biology is to understand the genetic mechanisms of how organisms adapt to their ecological niche. Typically, adaptation is viewed as a process where beneficial alleles are driven from low to high frequency in a population, reducing the variability in a genomic segment—so-called selective sweeps. On a genome-wide scale, immune-related genes and genes associated with fitness, reproduction, and fertility appear to be major targets of positive selection [14]. Lactase persistence is one of the classical examples where a line of genetic evidences suggests recent selection within the past 5000–10,000 years at the lactase locus in European-derived populations [15].

Positive selection can yield an excess of non-synonymous fixed differences (mainly between species), extended haplotype homozygosity (EHH), modified allele frequency distributions, and long-branch length in evolutionary trees. A comprehensive overview on the methods used and their limitations is given elsewhere [16, 17]. In brief, faster evolving genes can be detected between species by comparing the number of non-synonymous substitutions (dN) to the number of synonymous substitutions (dS). A likelihood method considering the dN/dS ratios across sites is implemented in phylogenetic analysis using maximum likelihood (PAML) [18, 19]. The McDonald-Kreitman (MK) test uses polymorphism data from a single species and fixed differences between multiple species to detect regions under selection [20]. The often used Tajima's *D* test compares the number of variants in a genomic region with the average number of pairwise differences [21]. The first scans for selection between populations that took advantage of differentiation across populations focused on single-marker F_{ST} [22]. F_{ST} statistic is useful to identify genomic regions that exhibit high variation in allelic frequency between groups, which is a characteristic of genomic regions that have gone through differential selection. Haplotype statistics, such as long-range haplotype (LRH) test [23], EHH [24], and integrated haplotype score (iHS) [25], use data from linked sites to identify past targets of natural selection. These methods are based on the expectation that recent positive sweeps yield extended haplotypes that have not had enough time to break down by recombination. An additional approach to identifying signals of selection through population comparison is the cross population extended haplotype heterozygosity (XP-EHH) test, which was designed to detect ongoing or nearly fixed selective sweeps by comparing haplotypes from two populations [26, 27].

Numerous studies have used the different methods to identify signature of selection and of convergent evolution between species and between or within populations of a single species producing long lists of genomic loci and candidate genes. Screening of those lists frequently reveals aGPCR loci with significant signatures of selection. For example, *GPR110* has been identified with such signatures linked to convergent evolution in echolocating mammals [28]. However, most studies focus on signals of selection during human evolution. Thus, sequence differences between primates, as closest relatives of humans, are used to identify obvious lineage-specific genomic events that are significantly different from random changes between species. Again, differences at aGPCR loci are frequently extracted from whole-genome comparison approaches (Table 1). For example, *GPR111* and *GPR123*/*ADGRA1* were found with signatures of selection in the human lineage in several studies. However, all these studies can neither provide the driving force of selection nor the selected phenotype but provide a rational to dig deeper into the physiology of the respective aGPCR also considering cross species differences in phenotypes. Significant cross species differences in the mRNA expression profile and level can also be helpful to elucidate the physiological significance of aGPCR. For example, a human-specific expression profile in brain prefrontal cortex and cerebellum compared to chimpanzees and rhesus macaques was found for *GPR116, CELSR2*/*ADGRC2*, *LPHN2*/*ADGRL2*, *BAI2*/*ADGRB2*, and

Table 1 Signatures of selection at genomic aGPCR loci in the human lineage

Gene name	New name	Human vs. species	Methods	Reference
LPHN1	*ADGRL1*	Chimpanzee	MK	[29]
LPHN3	*ADGRL3*	Chimpanzee	MK	[29]
EMR1	*ADGRE1*	Chimpanzee	MK	[29]
GPR123	*ADGRA1*	Chimpanzee	MK	[29]
		Primates	MK	[30]
CELSR1	*ADGRC1*	Chimpanzee	MK	[29]
GPR133	*ADGRD1*	Chimpanzee	MK	[29]
GPR110	*ADGRF1*	Chimpanzee	MK	[29]
GPR111	*ADGRF2*	Mammals	PAML	[31]
		Chimpanzee	PAML	[32]
BAI1	*ADGRB1*	Chimpanzee	MK	[29]
GPR56	*ADGRG1*	Chimpanzee	MK	[29]
GPR64	*ADGRG2*	Chimpanzee	MK	[29]
GPR126	*ADGRG6*	Chimpanzee	MK	[29]

BAI3/*ADGRB3*. Together with numerous other differentially expressed genes, they are considered to contribute to human-specific brain functions [33].

Recent advances in DNA extraction and amplification from fossil remains [34] and the new sequencing technologies for short DNA fragments have made it possible to retrieve substantial amounts of ancient DNA sequences and even almost complete genomes dating back close to 1 million years ago [35, 36]. Genome sequences of extinct organisms contain valuable information about past functions and gene evolution also of GPCRs [37, 38]. In a selective sweep analysis of the Neanderthal genome, a genomic locus containing *CELSR3*/*ADGRC3* was identified presenting signatures of selection in the Neanderthal lineage [39]. However, there are no specific genomic signals that aGPCRs may be involved in evolution of modern humans when compared to ancient hominids [40–44]. In contrast, analyses between different populations yielded a number of aGPCR loci with significant signatures of selection. Here, the clustered aGPCRs *GPR110*, *GPR111*, *GPR115*, and *GPR116* showed multiple signatures of recent selection among different populations (Table 2). Comparison of populations which majorly differ in respect to their environment revealed, for example, that the *GPR111* locus harbors significant signatures of selection in populations adapted to high altitude levels as found in Tibet, Mongolia, and Ethiopia, suggesting convergent evolution [45, 54]. The impact of different *GPR111* variants on this physiological trait is unsolved yet. Although analyses fall short in providing explanations for their unique genomic features and most probably many signals found at those loci are not related to the function of the specific gene product, they are indicative for a significant genomic dynamics at those loci in the recent human history.

Table 2 Signatures of selection at aGPCR loci in human genomes

Gene name	New name	Population	Methods	Reference
LPHN2	*ADGRL2*	Tibetan and Mongolian	iHS	[45]
LPHN3	*ADGRL3*	Oceanian vs. European	F_{ST}	[46]
EMR1	*ADGRE1*	South Asian vs. European	F_{ST}	[46]
EMR3	*ADGRE3*	African vs. Oceanian	F_{ST}	[46]
GPR123	*ADGRA1*	European vs. African	F_{ST}	[47]
GPR124	*ADGRA2*	Siberian	XP-EHH	[48]
CELSR1	*ADGRC1*	Yoruba, Japan	LRH, iHS, XP-EHH	[27]
		HapMap	HH	[49]
		Middle East, South Asian	iHS, XP-EHH	[50]
		Asian vs. African	SNP frequency	[51]
GPR133	*ADGRD1*	HapMap	Entropy of LD	[52]
		South Asian vs. European	F_{ST}	[46]
GPR110	*ADGRF1*	American, East Asian	iHS, XP-EHH	[50]
		East Asian vs. European	F_{ST}	[46]
GPR111	*ADGRF2*	Tibetan, Mongolian	iHS	[45]
		Asian	LRH	[53]
		Ethiopians	F_{ST}	[54]
		European	Neutrality test	[55]
		American, East Asian	iHS, XP-EHH	[50]
GPR113	*ADGRF3*	Americans	iHS	[50]
GPR115	*ADGRF4*	Tibetan, Mongolian	iHS	[45]
		Asian	LRH	[53]
		American, East Asian	iHS, XP-EHH	[50]
		South Asian vs. European	F_{ST}	[46]
GPR116	*ADGRF5*	American, East Asian	iHS, XP-EHH	[50]
		East Asian vs. South Asian	F_{ST}	[46]
BAI3	*ADGRB3*	African	LRH tests	[53]
		HapMap	EHH	[26, 56]
		African vs. European vs. East Asian	F_{ST}	[46]
GPR64	*ADGRG2*	Middle East	iHS	[50]
		European	iHS	[53]
GPR126	*ADGRG6*	European vs. Asian, Bantu vs. Pygmy	F_{ST}	[50]
GPR98	*ADGRV1*	HapMap	HH	[49]
		European, Middle East, South Asia	XP-EHH	[50]

4 Genetic Variability in the Coding Sequence of Human aGPCRs

Deep sequencing data, e.g., from population genetic studies revealed an unexpected variability of genes in humans. Most variants are single nucleotide polymorphisms (SNPs) without obvious impact on gene function. However, some SNP can have

functional relevance and can even reduce the repertoire of genes by inactivation when gene function no longer provides a selective advantage. This process is called pseudogenization. Pseudogenes are inheritable and characterized by homology to a known gene and lack of functionality [57]. As a result of their nonfunctionality, most pseudogenes are released from selective pressure and accumulate missense, frameshifting, and nonsense mutations. Pseudogenization by mutations is considered for odorant and taste receptors [58] because of the loss of environmental constraints or the gain of other senses, such as vision. However, loss-of-function mutations, as observed in chemokine receptors, may have also some advantage in respect to pathogens [59, 60]. As listed in Table 3, the gene for the chemokine receptor *CCR5* showed a significant higher number of missense, nonsense, and frameshifting variants in human populations compared to other GPCR genes. Similarly, a great number of variants are found in the melanocortin type 1 receptor (*MC1R*) gene (Table 3), a major regulator of pigmentation in humans and other mammals [61]. Depending on the exposition to sunlight, full function of the MC1R (equatorial region) but also loss-of-function (northern hemisphere) intact can have an advantage, although the MC1R is not essentially required for life. Analysis of the non-synonymous SNP number in aGPCR revealed some receptors with similar variability as *CCR5* and *MC1R*. For example, a relative high number of inactivating mutations are naturally found in *EMR1*/*ADGRE1* and *EMR2*/*ADGRE2* (Table 3). In line with these findings, *Emr1*-deficient mice were healthy and fertile, indicating that EMR1 deficiency is most probably compatible with life also in humans [62]. Similarly, the relative high number of nonsense and frameshifting mutations found in *GPR110* and *GPR111* suggests vitality in humans even without these aGPCRs. Indeed, mice lacking *GPR110* or *GPR111* are obviously healthy and fertile [13]. This, however, does not exclude phenotypical differences between receptor-positive and receptor-negative individuals. Quantitative trait locus (QTL) analyses and GWAS may help to identify the physiological impact of the presence, modification, and absence of individual aGPCRs.

5 QTL and GWAS Linking aGPCR to Human Phenotypes

Identification of genetic determinants contributing to susceptibility to diseases is not only crucial for understanding the complexity of human health and disease but can also play an important role in designing novel diagnostic and treatment strategies. The ultimate goal would be the so-called personalized medicine, which tailors treatment to individual patients according to their genetic background. Along with candidate gene strategies focusing on biologically plausible genes, hypothesis-free genome-wide studies are the driving force in identification of genetic variants contributing to the complex etiology of human diseases. Until 2005, most genome-wide studies relied on the technique of genetic linkage. For decades, this approach has been the dominant way in investigating the genetic basis of inherited diseases. Its design is based on searching for genomic regions which carry alleles shared by the affected family members, i.e., identifying those regions

Table 3 Natural mutations in coding regions of human aGPCR

Gene name	New name	Missense	Nonsense	Frameshift	Length (codons)	Sum	Mutations/codon	Inactive/codon
LPHN1	ADGRL1	176	1	1	1475	178	0.121	0.001
LPHN2	ADGRL2	135	1	2	1474	138	0.094	0.002
LPHN3	ADGRL3	158	0	0	1500	158	0.105	0.000
ELTD1	ADGRL4	84	3	0	739	87	0.118	0.004
EMR1	ADGRE1	175	7	9	886	191	**0.216**	**0.018**
EMR2	ADGRE2	175	12	9	823	196	**0.238**	**0.026**
EMR3	ADGRE3	81	4	3	652	88	0.135	**0.011**
CD97	ADGRE5	159	4	2	835	165	0.198	0.007
GPR123	ADGRA1	276	11	7	1280	294	**0.230**	**0.014**
GPR124	ADGRA2	187	3	4	1338	194	0.145	0.005
GPR125	ADGRA3	184	2	2	1321	188	0.142	0.003
CELSR1	ADGRC1	552	2	1	3019	555	0.184	0.001
CELSR2	ADGRC2	395	2	0	2924	397	0.136	0.001
CELSR3	ADGRC3	402	4	7	3312	413	0.125	0.003
GPR133	ADGRD1	170	1	1	874	172	0.197	0.002
GPR144	ADGRD2	95	5	5	963	105	0.109	**0.010**
GPR110	ADGRF1	138	4	6	910	148	0.163	**0.011**
GPR111	ADGRF2	117	8	6	708	131	0.185	**0.020**
GPR113	ADGRF3	156	8	2	1079	166	0.154	0.009
GPR115	ADGRF4	103	2	3	752	108	0.144	0.007
GPR116	ADGRF5	163	1	0	1346	164	0.122	0.001
BAI1	ADGRB1	158	0	1	1584	159	0.100	0.001
BAI2	ADGRB2	170	1	2	1585	173	0.109	0.002
BAI3	ADGRB3	135	2	0	1522	137	0.090	0.001

(continued)

Table 3 (continued)

Gene name	New name	Missense	Nonsense	Frameshift	Length (codons)	Sum	Mutations/codon	Inactive/codon
GPR56	ADGRG1	90	4	1	693	95	0.137	0.007
GPR64	ADGRG2	111	0	1	1017	112	0.110	0.001
GPR97	ADGRG3	101	3	3	549	107	0.195	**0.011**
GPR112	ADGRG4	446	15	11	3080	472	0.153	0.008
GPR114	ADGRG5	124	1	1	528	126	**0.239**	0.004
GPR126	ADGRG6	145	2	1	1250	148	0.118	0.002
GPR128	ADGRG7	126	4	3	797	133	0.167	0.009
GPR98	ADGRV1	860	20	7	6306	887	0.141	0.004
TSHR		104	4	3	764	111	0.145	0.009
CaSR		102	1	0	1078	103	0.096	0.001
V2R		70	1	0	371	71	0.191	0.003
GPR34		31	0	1	381	32	0.084	0.003
GPR82		33	1	4	336	38	0.113	**0.015**
LHCGR		77	1	0	699	78	0.112	0.001
MC4R		44	2	1	332	47	0.142	0.009
MC1R		123	6	6	317	135	**0.426**	**0.038**
HTR1A		44	0	0	422	44	0.104	0.000
CCR5		69	5	8	352	82	**0.233**	**0.037**
GRM1		101	2	0	1194	103	**0.086**	**0.002**
GABBR1		109	3	0	961	112	**0.117**	**0.003**

The numbers of missense, nonsense, and frameshifting mutations in the coding regions of human aGPCRs were taken from dbSNP (www.ncbi.nlm.nih.gov/snp/) (last check: 2015/12/18). Only those variants were counted which were validated by frequency. The total number was referred to the maximum length of the proteins (www.ncbi.nlm.nih.gov/protein/). There was no significant difference ($P = 0.4$) in the variant/length ratio between aGPCR and non-aGPCR (in italics). Genes, one standard deviation above the mean (0.151 ± 0.060), are in bold. The ratio of obviously inactivating mutations (nonsense, frameshift) to the receptor length is given in the right row (although missense mutations can also inactivate a GPCR). All ratios >0.010 are in bold

which segregate with the disease. Linkage studies turned out to be most powerful in identification of high-risk disease variants, such as those in *CFTR* (Cystic Fibrosis Transmembrane Conductance Regulator) gene responsible for cystic fibrosis [63]. Although genome-wide linkage analyses were successfully applied for a number of other single-gene Mendelian diseases (e.g., Huntington disease) [64], they did not appear pivotal for common diseases such as type 2 diabetes, cardiovascular diseases, or various cancer forms. However, with advances in high-throughput genotyping and sequencing technologies, GWAS became feasible and made a major contribution toward better understanding the genetics of complex traits and diseases. Based on the common disease-common variant hypothesis, GWAS allows detection of common disease variants with modest disease risks. In contrast to linkage studies, modest-risk allele sharing among unrelated affected patients is more striking than between affected subjects within families [65]. One of the major advantages of GWAS is, therefore, the relatively easy recruitment of unrelated affected individuals and healthy controls. However, since the region flanking the genetic marker in GWAS is much smaller than shared regions in related individuals in families, thousands of markers are required for a sufficient coverage of the genome and, so, successful identification of a potentially interesting genetic variant. With the availability of high-throughput array-based genotyping technologies, millions of SNPs can be determined easily and taken forward for association analyses with the clinical outcome of interest. Indeed, GWAS became the major tool in identification of genetic factors for common diseases such as obesity and type 2 diabetes in the last decade [66, 67]. GWAS did not only provide a proof of principle for many plausible candidate genes but also highlighted novel pathways and mechanisms underlying complex pathophysiologies of various diseases (e.g., *CDKN2A* (cyclin-dependent kinase inhibitor 2A) and *CDKAL1* (CDK5 regulatory subunit associated protein 1-like 1) in regeneration of the beta cell in type 2 diabetes) [66].

In respect to aGPCRs, numerous loci carrying the respective aGPCR genes have been shown to co-segregate with complex traits and diseases. In various family-based analyses, QTLs harboring aGPCRs have been identified for phenotypes of the immune system, skeleton (joint/bone inflammation) [68], renal/urinary system [69], and respiratory system (chronic and reversible airflow obstruction) [70, 71]. Most strikingly, majority of the aGPCR loci have been shown to be linked to traits related to homeostasis/metabolism (body weight/BMI, body fat, lipid levels, glucose levels, leptin) [72–74] and tumorigenesis (mammary and prostate tumor susceptibility) [75–78] (Table 4). Yet, like for most of the linkage studies, the genomic intervals with QTLs are very large to track down the respective causal genes. In this respect, numerous GWAS helped to refine data from linkage analyses and pointed to several interesting aGPCR candidates. For instance, the *LPHN1*/*ADGRL1* locus on chromosome 19 has previously been linked to obesity, lipid, and glucose levels [72–74] (Table 4). Consistently, recent GWAS identified rs4528684 within the locus to be associated with increased mortality in heart failure [189] (Table 5). Also for the *CELSR2* locus, consistent data have been obtained from both linkage and association studies. Whereas previous linkage analyses pointed to the

Table 4 Human aGPCR genes within QTLs

Receptor		QTL		
Gene name	New name	Chr.	Trait	Reference
LPHN1	*ADGRL1*	19	Urinary albumin excretion	[69]
			Chronic airflow obstruction	[70, 71]
			Multiple sclerosis susceptibility	[79]
			Lipid level	[73, 80–82]
			Body weight	[72, 83]
			Body fat	[84]
			Prostate tumor susceptibility	[76–78, 85]
			Glucose level	[74, 86]
LPHN2 *ELTD1*	*ADGRL2* *ADGRL4*	1	Joint/bone inflammation	[68]
			Chronic airflow obstruction	[87]
			Reversible airflow obstruction	[88]
			Leptin level	[89]
			Apolipoprotein B level	[90]
			Blood pressure	[91]
LPHN3	*ADGRL3*	4	Joint/bone inflammation	[92, 93]
			Chronic airflow obstruction	[70, 71, 94]
			Body fat	[84]
			Body weight	[95, 96]
			Mammary tumor susceptibility	[75]
			Glucose level	[74, 97]
EMR1 *EMR4*	*ADGRE1* *ADGRE4*	19	Urinary albumin excretion	[69]
			Blood pressure	[98]
			Chronic airflow obstruction	[71, 99]
			Multiple sclerosis susceptibility	[79]
			Lipid level	[73, 80–82]
			Birth weight	[83]
			Prostate tumor susceptibility	[76–78, 85]
			Glucose level	[74, 86]
EMR2 *EMR3* *CD97*	*ADGRE2* *ADGRE3* *ADGRE5*	19	Urinary albumin excretion	[69]
			Chronic airflow obstruction	[70, 71]
			Multiple sclerosis susceptibility	[79]
			Lipid level	[73, 80–82]
			Body weight	[72, 83]
			Body fat	[84]
			Prostate tumor susceptibility	[76–78, 85]
			Glucose level	[74, 86, 100]
GPR123	*ADGRA1*	10	Body weight	[101, 102]
			Body fat	[103]
			Prostate tumor susceptibility	[78, 104, 105]
			Glucose level	[74, 86, 106]

(continued)

Table 4 (continued)

Receptor		QTL		
Gene name	New name	Chr.	Trait	Reference
GPR124	*ADGRA2*	8	Blood pressure	[98]
			Body weight	[107]
			Multiple sclerosis susceptibility	[79]
			STNFR hormone level	[89]
			Lipid level	[108]
			Waist to hip ratio	[109]
			Adiponectin level	[110]
			Prostate tumor susceptibility	[111–113]
			Glucose level	[114]
GPR125	*ADGRA3*	4	Blood pressure	[115]
			Body weight/BMI	[116, 117]
			Serum IgE titer	[118]
			LDL particle size	[119]
			Body fat	[84]
			Prostate tumor susceptibility	[112, 120]
			Glucose level	[74, 100]
CELSR1	*ADGRC1*	22	Blood pressure	[98, 121]
			Chronic airflow obstruction	[71, 99]
			Body weight	[109]
			Prostate tumor susceptibility	[78, 105, 112, 113, 122, 123]
			Mammary tumor susceptibility	[124, 125]
CELSR2	*ADGRC2*	1	Blood pressure	[91, 98]
			Chronic airflow obstruction	[70, 71, 87]
			Body weight/BMI	[126, 127]
			Lipid level	[128]
			Prostate tumor susceptibility	[76, 129, 130]
			Glucose level	[86]
CELSR3	*ADGRC3*	3	Body fluid distribution	[131]
			Joint/bone inflammation	[132]
			Body weight/BMI	[133]
			Chronic airflow obstruction	[70, 71]
			Glucose level	[74, 134]
GPR133	*ADGRD1*	12	Joint/bone inflammation	[68]
			Reversible airflow obstruction	[135]
			Apolipoprotein A1 level	[136]
			Myocardial infarction	[137]
			Body weight/BMI	[95, 101]
			Body fat (abdominal)	[109, 138]
			Lipid level	[139]
			Leptin level	[140]
			Prostate tumor susceptibility	[104, 141]
			Glucose level	[142, 143]

(continued)

Table 4 (continued)

Receptor		QTL		
Gene name	New name	Chr.	Trait	Reference
GPR144	*ADGRD2*	9	Joint/bone inflammation	[132, 144]
			Multiple sclerosis	[145]
			Body weight/BMI	[101, 103, 126]
			Lipid level	[73]
			Glucose level	[74, 86, 146, 147]
			Prostate tumor susceptibility	[78, 129, 148]
			Insulin level	[149]
GPR110 *GPR111* *GPR115* *GPR116*	*ADGRF1* *ADGRF2* *ADGRF4* *ADGRF5*	6	Joint/bone inflammation	[93, 132]
			Blood pressure	[115]
			Reversible airflow obstruction	[150]
			Lipid level	[151, 152]
			LDL particle size	[153]
			Body fat	[109, 154]
			Prostate tumor susceptibility	[155]
GPR113	*ADGRF3*	2	Body weight/BMI	[107, 156, 157]
			Leptin level	[158–161]
			Lipid level	[73, 80]
			Glucose level	[86, 142, 146]
			Insulin level	[157]
			Body fat	[157]
			Prostate tumor susceptibility	[123]
BAI1	*ADGRB1*	8	Joint/bone inflammation	[132]
			Body weight/BMI	[131]
			Blood pressure	[162]
			Chronic airflow obstruction	[70]
			Body fat	[96]
			Waist to hip ratio	[163]
			Prostate tumor susceptibility	[78, 123]
			Glucose level	[164]
BAI2	*ADGRB2*	1	Body weight/BMI	[165]
			Multiple sclerosis	[145]
			Myocardial infarction	[137]
			Lipid level	[81]
			Blood pressure	[91]
			Body fat (abdominal)	[109]
			Ghrelin level	[166]
			Prostate tumor susceptibility	[78, 105, 123, 129, 141, 148, 155]
			Glucose level	[142, 167]

(continued)

Table 4 (continued)

Receptor		QTL		
Gene name	New name	Chr.	Trait	Reference
BAI3	*ADGRB3*	6	Joint/bone inflammation	[93, 168]
			Blood pressure	[115]
			Lipid level	[151]
			Body fat	[109]
			Body weight	[83]
			Glucose level	[74]
GPR56 *GPR97* *GPR114*	*ADGRG1* *ADGRG3* *ADGRG5*	16	Body weight/BMI	[72, 101, 140, 169]
			Lipid level	[170, 171]
			Leptin level	[140]
			Body fat	[140]
			Prostate tumor susceptibility	[78, 105, 112]
			Glucose level	[172]
GPR64	*ADGRG2*	X	Body weight	[117]
			Prostate tumor susceptibility	[78, 104, 105]
GPR112	*ADGRG4*	X	Prostate tumor susceptibility	[77, 111, 112, 129, 173, 174]
			Glucose level	[175]
GPR126	*ADGRG6*	6	LDL particle size	[176]
			Lipid level	[177–179]
			Body weight/BMI	[72, 179, 180]
			Waist	[140]
			Waist to hip ratio	[109]
			Glucose level	[146, 181–184]
			Leptin level	[140]
			Insulin level	[182]
GPR128	*ADGRG7*	3	Blood pressure	[98]
			Chronic airflow obstruction	[70, 71]
			Coronary artery disease	[185]
			Lipid level	[170]
			Adiponectin level	[110]
			Body weight/BMI	[186]
			Prostate tumor susceptibility	[141]
GPR98	*ADGRV1*	5	Joint/bone inflammation	[132]
			Reversible airflow obstruction	[88]
			Body weight/BMI	[84, 101, 126, 180, 187, 188]
			Apolipoprotein A-II level	[136]
			LDL particle size	[119]
			Prostate tumor susceptibility	[76, 123]
			Myocardial infarction	[137]

Table 5 Association of genetic variants in GPCRs identified in GWAS

Receptor		Association of genetic variants in GWAS			
Gene name	New name	SNP	Associated trait	*P*-value	Reference
LPHN1	*ADGRL1*	rs4528684-T	Mortality in heart failure	1×10^{-6}	[189]
LPHN2	*ADGRL2*	rs17498581-T	Toenail selenium levels	9×10^{-6}	[191]
		rs11163372-T	Temperament	3×10^{-6}	[192]
		rs6681544-C	Metabolite levels	6×10^{-6}	[193]
		rs1770678-G	TGF-β1	7×10^{-6}	[194]
		rs480745	Preterm birth	7×10^{-7}	[195]
		–	Climate	<0.05	[196]
LPHN3	*ADGRL3*	rs6856328	Response to antipsychotic treatment in schizophrenia	8×10^{-7}	[197]
			Partial epilepsies	3×10^{-6}	[198]
		rs2172802	HDL cholesterol	2×10^{-6}	[199]
		rs4599440-A	Subsistence	<0.05	[196]
		–	Climate	<0.05	[196]
ELTD1	*ADGRL4*	rs11162963-T	Aging (time to event)	4×10^{-6}	[200]
		rs10873998-T	Autism spectrum disorder, attention deficit-hyperactivity disorder, bipolar disorder, major depressive disorder, and schizophrenia (combined)	4×10^{-6}	[201]
EMR1	*ADGRE1*	rs3826782	Periodontitis	8×10^{-7}	[202]
EMR2	*ADGRE2*	–	Subsistence	<0.05	[196]
CD97	*ADGRE5*	rs4528684-T	Mortality in heart failure	1×10^{-6}	[189]
GPR124	*ADGRA2*	rs12676965-T	Axial length	3×10^{-3}	[203]
GPR125	*ADGRA3*	rs4697263 rs2141322	Age-related hearing impairment	9×10^{-9}	[204]
		rs6448119	Rheumatoid arthritis	7×10^{-6}	[205]
CELSR1	*ADGRC1*	rs5767218-C	Classic bladder exstrophy	3×10^{-6}	[206]
		rs9615362	Ischemic stroke	<0.05	[207]
CELSR2	*ADGRC2*	rs646776-G	Progranulin levels	2×10^{-30}	[208]
		rs646776-C	Response to statin therapy	4×10^{-6}	[209]
		rs629301-G	Total cholesterol	2×10^{-170}	[210, 211]
		rs646776-G	Total cholesterol	9×10^{-22}	[212]
		rs3902354-A	Total cholesterol	1×10^{-8}	[213]
		rs646776-C	LDL cholesterol	3×10^{-29}	[214]
		rs660240-A	LDL cholesterol	1×10^{-26}	[215]
		rs646776-G	LDL cholesterol	8×10^{-23}	[212, 216]
		rs12740374-T	LDL cholesterol	2×10^{-42}	[217]
		rs629301-G	LDL cholesterol	1×10^{-170}	[211]

(continued)

Table 5 (continued)

Receptor		Association of genetic variants in GWAS			
Gene name	New name	SNP	Associated trait	*P*-value	Reference
		rs646776	LDL cholesterol (fasting/whole)	2×10^{-20}	[218]
		rs660240-T	LDL cholesterol	2×10^{-22}	[219]
		rs12740374-T	LDL cholesterol	9×10^{-29}	[220]
		rs599839-G	LDL cholesterol	1×10^{-33}	[221]
		rs629301-G	LDL cholesterol	5×10^{-241}	[210]
		rs599839-A	LDL cholesterol	6×10^{-33}	[222]
		rs599839-G	LDL cholesterol	1×10^{-7}	[223, 224]
		rs602633-T	Coronary artery disease	1×10^{-8}	[225]
		rs646776-T	Coronary artery disease	8×10^{-11}	[226, 227]
		rs646776-T	Myocardial infarction (early onset)	8×10^{-12}	[228]
		rs7528419-A	Lipoprotein-associated phospholipase A2 activity and mass	1×10^{-17}	[229]
		rs12740374-T	Lipoprotein-associated phospholipase A2 activity and mass	2×10^{-22}	[230]
		rs646776	APOB (whole/fasting)	2×10^{-53}	[218]
GPR133	*ADGRD1*	rs1569019	Height	5×10^{-8}	[231]
		rs3847687	Longevity	$\leq 1 \times 10^{-6}$	[232]
		rs10466868	Erythropoietin	1×10^{-6}	[233]
		rs885389-A	RR interval (heart rate)	4×10^{-8}	[234]
		rs11061269	Amyotrophic lateral sclerosis (sporadic)	8×10^{-10}	[235]
		–	Pathogens	<0.05	[196]
		–	Subsistence	<0.05	[196]
		–	Climate	<0.05	[196]
GPR110	*ADGRF1*	–	Climate	<0.05	-
GPR111	*ADGRF2*	–	Subsistence	<0.05	[196]
		–	Climate	<0.05	[196]
GPR113	*ADGRF3*	rs6753473-G	Non-small cell lung cancer	4×10^{-6}	[236]
GPR116	*ADGRF5*	–	Climate	<0.05	[196]
BAI3	*ADGRB3*	rs2585617-A	Phospholipid levels (EPA)	6×10^{-7}	[237]
		rs3757057-T	Response to statin therapy (triglyceride sum)	4×10^{-6}	[209]
		rs9351730	Prostate cancer	5×10^{-6}	[238]
		rs9364554			
		rs9363918-A	Pancreatic cancer	1×10^{-6}	[239]
		rs78536982-T	Triglycerides	7×10^{-9}	[213]
		–	Pathogens	<0.05	[196]

(continued)

Table 5 (continued)

Receptor		Association of genetic variants in GWAS			
Gene name	New name	SNP	Associated trait	*P*-value	Reference
GPR126	*ADGRG6*	rs6570507	Scoliosis	1×10^{-14}	[240]
		rs6570507-A	Height	2×10^{-11}	[241]
		rs3748069-A	Height	5×10^{-14}	[242]
		rs4896582-A	Height	2×10^{-18}	[243]
		rs6570507-G	Height	2×10^{-7}	[244]
		rs6570507	Height	4×10^{-11}	[245]
		rs7763064-A	Height	1×10^{-33}	[246]
		rs225694	Height	1×10^{-7}	[246]
		rs7741741-T	Height	1×10^{-20}	[247]
		rs4896582-A	Height	3×10^{-55}	[248]
		rs3817928-A	Pulmonary function	1×10^{-9}	[249]
		rs3817928	Pulmonary function	3×10^{-12}	[250]
		rs1329705	Airflow obstruction	3×10^{-6}	[251]
		rs225675-G	Thiazide-induced adverse metabolic effects in hypertensive patients	1×10^{-7}	[252]
		rs7763064-A	Birth length	6×10^{-6}	[253]
GPR128	*ADGRG7*	–	Subsistence	<0.05	[196]
		–	Climate	<0.05	[196]
GPR98	*ADGRV1*	rs10514345	Hip geometry (Neck Z1)	2×10^{-7}	[254]
		rs1967256	Response to antipsychotic treatment	3×10^{-8}	[255]
		rs10074525-G	Obesity-related traits (weight z-score)	9×10^{-6}	[194]
		–	Pathogens	<0.05	[196]
		–	Climate	<0.05	[196]

GWAS signals according to the GWAS Catalog [190]

segregation of this locus with lipid levels and blood pressure- and obesity-related traits [98, 126, 128], it was the GWAS which ultimately identified polymorphisms within the *CELSR2* gene which were significantly associated with total and LDL cholesterol, clinical outcomes of coronary artery disease, and myocardial infarction but also response to statin therapy [209, 225, 228] (Table 5). Worth mentioning are also further traits shown to be associated with SNPs in aGPCR genes. In particular, genetic variants in *GPR123* and *GPR133*/*ADGRD1* have been shown to be associated with height [231, 246] and SNPs in *GPR125*/*ADGRA3* with rheumatoid arthritis and age-related impaired hearing [204, 205] (Table 5).

Despite the rapid and tremendous progress in the field of genetics of complex diseases by recovering hundreds of genetic variants in GWAS for numerous traits and diseases, the biological impact of these associated variants remains largely unknown. As mentioned above, common genetic variants such as SNPs are in

strong contrast to rare high-risk alleles responsible for Mendelian genetic disorders such as cystic fibrosis [256]. Most of the identified SNPs associated with the trait of interest map to inter- or intragenic regions, thus suggesting their regulatory role in gene transcription and function. Therefore, one of the crucial steps following GWAS is to identify and validate target genes potentially affected by the associated SNPs. eQTL (expression QTL) analyses allow testing for correlations between genotype and tissue-specific gene expression levels and, so, help to identify chromosomal regions that influence whether and, if so, how much a gene is expressed. Thus, integrative approach combining GWAS and eQTL studies allows clarifying whether the genotype-phenotype association uncovered in a GWAS might be mediated by the regulation of a gene transcription by the corresponding SNP. According to the GTEx (Genotype-Tissue Expression) portal, a unique resource to study human gene expression and its relation to genetic variation [257], SNPs within *CELSR2* locus (e.g., rs660240) associated with lipid traits in recent GWAS [215] (Table 5), has also been shown to correlate with *CELSR2* mRNA expression in liver, skeletal muscle, and esophagus mucosa. Using GTEx, *GPR126*/*ADGRG6* SNPs (e.g., rs6570507, rs4896582, rs7763064) related to height in GWAS [241, 243–246] (Table 5) are associated with *GPR126* mRNA expression in subcutaneous adipose tissue. These findings strongly support the GWAS data and the potential functional consequences of the associated variants.

6 Monogenic Diseases Caused by Mutations in aGPCR

As shown in Table 5, there are numerous pathological traits in humans associated to genomic variations in aGPCR loci. *GPR133* was among the first aGPCRs identified in GWAS where SNPs were associated with human phenotypes [231, 258]. Usually, the SNPs linked or associated with phenotypes are not causal for the phenotypical trait but rather genetically linked (linkage disequilibrium, LD) to additional, likely causal variants at such loci. Thus, locus sequencing is required to identify those variants which are in LD with the indicative SNP (tag SNP). Database analysis of more than 1000 sequenced human genomes revealed over 5300 SNPs in the human *GPR133* gene [259]. Out of those 5 % SNPs are located in exons leading to 170 amino acid changes (Table 3). Compared to other GPCR genes, the number of non-synonymous SNPs/1000 bp of the coding region is not significantly different in the *GPR133* gene [260]. Interestingly, some non-synonymous SNP showed a functional relevance including complete loss of function when experimentally tested [259]. However, a direct causal link between individual SNPs and differences in the phenotypes linked to the *GPR133* locus needs to be provided.

Sequence variants can modulate GPCR activity in two directions—activation (gain-of-function) and inactivation (loss-of-function). In humans activating and inactivating mutations in GPCR can cause diseases with contrary phenotypes but also convergent phenotypes. For example, the rhodopsin-like GPCR V2 vasopressin receptor (V2R) is a key component in regulating renal water reabsorption.

Inactivating mutations of the V2R cause reduced water reabsorption (congenital nephrogenic diabetes insipidus), whereas activating mutations cause increased water reabsorption with serum hyposmolarity and high urinary sodium levels (nephrogenic syndrome of inappropriate antidiuresis) [261]. In contrast activating and inactivating mutations of rhodopsin cause retinitis pigmentosa [262]. Disease-causing mutations are rather rare in humans, and only 30–40 monogenic diseases caused by mutations in GPCR have been described so far [263–266].

Database entries from large-scale sequencing programs show that functionally relevant aGPCR variants exist in recent human populations (Table 3), and some are responsible for human diseases. There are several inherited diseases caused by loss-of-function mutations in humans. Inactivating mutations in *CELSR1*/*ADGRC1* have been found responsible for craniorachischisis and further neural tube defects [267] and hereditary lymphedema [268]. One inherited form of bilateral frontoparietal polymicrogyria is caused by inactivating mutations in the *GPR56*/*ADGRG1* gene [269] (see [270]) and the Usher syndrome type IIC by mutation in the *GPR98*/*ADGRV1* gene [271] (see [272]). Recently, the lethal congenital contracture syndrome-9 and arthrogryposis multiplex congenital have been linked to inactivation mutation in *GPR126*; however, final confirmation is pending [273]. Vibratory urticaria has been linked to a missense mutation in EMR2 [274] (see [275–277]). No disease-causing activating mutations have been described for aGPCR yet, but this is most probably just a matter of time.

7 Summary and Future Perspectives

Large-scale sequencing projects have provided a wealth of information to the aGPCR field in respect to evolution and sequence variability in humans and another species. Now, this information can be mined for studying the impact of aGPCR function on speciation, phenotypes, and susceptibility to diseases. Phenotypic and environmental comparisons of species which differ in the number of aGPCRs may provide clues for the loss or gain of individual aGPCRs. We are just at the beginning of utilizing this source to generate hypotheses which then can be tested in suitable model systems. For example, QTL studies suggest the involvement of CELSR2 and CELSR3 in the regulation of glucose homeostasis (Table 4). Although both aGPCRs were initially identified as highly relevant for planar cell polarity signaling in ependymal cilia development [278], detailed analyses revealed that loss of function of *CELSR2* and *CELSR3* alters differentiation of endocrine cells from polarized pancreatic progenitors, with a prevalent effect on insulin-producing β-cells and, therefore, glucose tolerance [279]. Numerous GWAS suggest that CELSR2 function is required for cholesterol homeostasis (see Table 5). However, sortilin, encoded by the *SORT1* gene which is in close genomic proximity to *CELSR2*, is an intracellular sorting receptor for apolipoprotein B100 and regulates plasma low-density lipoprotein (LDL) cholesterol [280]. It is therefore likely that functionally relevant SNPs in *SORT1* but not in *CELSR2* account for the strong GWAS signals. This example illustrates the need for experimental testing

sequence data-derived hypothesis in the future. On the other hand, the field will majorly profit from an iterative process of linking-defined phenotypes to genomic variants and detailed characterizing gene-deficient animal models in order to unveil the functional relevance of the individual aGPCR in specific tissues. As an ultimate result, new human syndromes will be discovered taking advantage of well-defined sub-phenotypes identified in animal models. But not only inherited human diseases should be in the focus. There is mounting evidence that acquired diseases such as tumors harbor functionally relevant aGPCR variants. For example, melanomas and other skin cancers carry mutated aGPCR genes such as *GPR116*, *BAI3*, and *VLGR1*/*ADGRV1* [281, 282]. Also, changes in expression levels of different aGPCRs are highly associated with specific tumors and may serve as prognostic markers [283, 284]. It will be a future task to clarify whether such aGPCR variants promote cancer proliferation and invasion.

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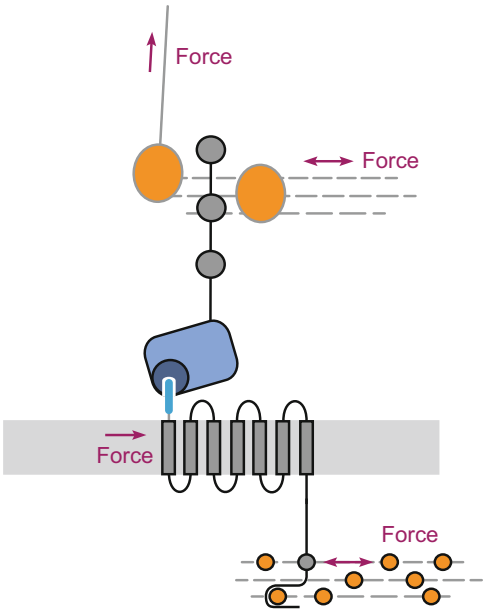
Part II

Adhesion GPCRs as Pharmakotargets in Organ Function and Development

Adhesion GPCRs as a Putative Class of Metabotropic Mechanosensors

Nicole Scholz, Kelly R. Monk, Robert J. Kittel, and Tobias Langenhan

Graphical Abstract



Adhesion GPCRs as mechanosensors. Different aGPCR homologs and their cognate ligands have been described in settings, which suggest that they function in a mechanosensory capacity. For details, see text

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Contents

Abstract

G protein-coupled receptors (GPCRs) constitute the most versatile superfamily of biosensors. This group of receptors is formed by hundreds of GPCRs, each of which is tuned to the perception of a specific set of stimuli a cell may encounter emanating from the outside world or from internal sources. Most GPCRs are receptive for chemical compounds such as peptides, proteins, lipids, nucleotides, sugars, and other organic compounds, and this capacity is utilized in several sensory organs to initiate visual, olfactory, gustatory, or endocrine signals. In contrast, GPCRs have only anecdotally been implicated in the perception of mechanical stimuli. Recent studies, however, show that the family of adhesion GPCRs (aGPCRs), which represents a large panel of over 30 homologs within the GPCR superfamily, displays molecular design and expression patterns that are compatible with receptivity toward mechanical cues (Fig. 1). Here, we review physiological and molecular principles of established mechanosensors, discuss their relevance for current research of the mechanosensory function of aGPCRs, and survey the current state of knowledge on aGPCRs as mechanosensing molecules.

Keywords

Adhesion GPCR • Mechanosensor • Mechanosensation

1 General Overview of Mechanosensation

Each cell in our body is constantly exposed to a multitude of mechanical cues emanating from cell movements, hydrostatic pressure, shear stress exerted by fluids, or compressive and tensile forces of various origins. In order to react to mechanical conditions, these need to be (1) conveyed to mechanosensitive

molecules (a process termed *mechanotransmission*; Fig. 2). (2) These molecular mechanosensors perceive force application through conformational changes (*mechanoreception* or *mechanosensing*). (3) Force-dependent changes in mechanosensor structure are finally handed over to intracellular signaling cascades that are not necessarily force dependent per se, including metabotropic effectors and transcriptional pathways, which finally shape the cellular response to the mechanical change (*mechanoresponse*) [1]. Here, we will concentrate on the role of aGPCRs in the process of mechanoreception.

Mechanosensors frequently constitute integral membrane proteins, and it is commonly accepted that mechanical forces that impinge onto the cell surface affect local and global membrane tension. Thus far, a number of molecules have been attributed with a function in mechanoreception, among them ion channels, cell surface receptors (e.g., integrins, cadherins/selectins), and, more recently, G protein-coupled receptors (GPCRs).

2 Established Mechanosensors

Mechanosensors evolved different receptive strategies to reliably perceive, integrate, and convey mechanical information into the cell. Even though detailed molecular knowledge of the mechanotransduction cascade is, for many mechanosensors, still unknown, an understanding of the receptive strategies of these molecules will help define to what extent aGPCRs serve as metabotropic mechanosensitive devices. Thus, the following section delineates potential mechanisms that underlie mechanotransduction and provides an overview of established mechanosensors.

2.1 Molecular Models of Mechanosensation

Two models currently encompass how mechanical strain is transmitted onto mechanosensors. The "tethered" model is based on the concept that surface molecules are anchored to intra- and/or extracellular components via molecular springs, which convey mechanical forces to membrane integral mechanosensors (e.g., TMC, extracellular; see Sect. 2.2.5 NOMPC, intracellular; see Sect. 2.2.3). Alternatively, in the "membrane" model, mechanosensitivity is shaped by the lateral pressure profile [2] at the receptor/bilayer interface promoting conformational changes of mechanoreceptive proteins. Given that a cell's response to force is signified by its mechanical context by means of the surrounding extracellular matrix (ECM), neighboring cells, and the mechanical properties of the cell itself, it is reasonable to assume that both mechanisms are employed in parallel to ensure reliable integration of physical forces.

2.2 Ionotropic Mechanosensors

Guharay and Sachs were the first to discover that skeletal muscles in chick express an ion channel that can be activated by membrane stretch [3, 4]. After that, several years passed until the first mechanosensitive channel genes, *mscL* and *mscS*, were identified and cloned from prokaryotes [5–7]. In *Bacteria* and *Archaea*, mechanosensitive channels are primarily involved in detecting osmotically induced cell swelling to regulate turgor [6, 8].

A large number of mechanosensitive channels were identified in eukaryotic cells [9] and, based on their ion permeability, can be divided into two classes: excitatory cation-selective ion channels and inhibitory depolarization-gated ion channels. We will briefly discuss examples for both groups below. Furthermore, a novel ion channel family—transmembrane channel-like 1 and 2 (TMC1/TMC2)—was recently identified.

2.2.1 Piezo

Vertebrate Piezo1 and Piezo2 (or Fam38A and Fam38B) channel subunits comprise large membrane proteins with 14 predicted membrane-spanning segments [10]. Heterologous expression of Piezo conveys mechanosensitivity to a mechanically insensitive cell [11]. Furthermore, functional ion channels could be reconstituted from Piezo proteins in liposomes [12]. Thus, ectopically expressed Piezo appears to form mechanosensitive ion channels independent from cytoskeletal elements implying that the channel activating tension originates from the membrane [11–13].

Mammalian *Piezo 1*/*Piezo 2* was detected in the lung, bladder, colon, and skin. *Piezo 2* was additionally enriched in dorsal root ganglia (DRGs) [11], where it acts as a mechanotransducer to regulate the perception of tactile stimuli [14, 15]. Similarly, the *piezo2b* homolog plays a role in zebrafish touch sensation [16]. Moreover, Piezo1 was assigned roles in the regulation of erythrocyte cell volume and vascular development in zebrafish and mouse, respectively [17–19]. The sole Piezo homolog in *Drosophila* (*dmpiezo*) produces, similar to its vertebrate counterpart, mechano-dependent ion currents and is required for the perception of noxious mechanical stimuli in vivo. Interestingly, Piezo appears to act in parallel to Pickpocket (PPK), which belongs to the (DEG)/ENaC family, to regulate mechanical nociception in *Drosophila* larvae [20].

2.2.2 DEG/ENaC Channel

The DEG/ENaC channel family was named after their first members, degenerins (*mec-4 and deg-1*) and epithelial sodium channel (ENaC) from *C. elegans* and mammals, respectively [21–24]. DEG/ENaC channels are expressed in different tissues across a large range of phyla, are usually Na^+-selective, and appear to be activated by a wide spectrum of stimuli including mechanical force [25]. Two transmembrane domains (N- and C-termini intracellular) connected by a large extracellular loop define the protomer architecture of DEG/ENaC proteins, which can form both homo- and heteromeric ion channels [26–28].

The first identified eukaryotic genes involved in touch sensation, *mec-4* and *mec-10* (*mec*hanosensory abnormal) [29], were uncovered through forward genetic screens in *C. elegans*, in which mutants were probed for their ability to respond to gentle body touch conveyed through a defined set of touch receptor neurons [29–31]. Genetic, biochemical, functional, and morphological analyses implicate four MEC proteins (MEC-4, MEC-10, MEC-2, MEC-6) to compose mechanosensory channel complexes [32–34]. The pore-forming subunits are presumably contributed by MEC-4 and MEC-10 [23, 32, 35, 36]. Interestingly, the local lipid milieu appears to influence the function of this mechanosensory complex [33, 37–39], suggestive of a membrane stretch-mediated gating mechanism.

2.2.3 TRP Channels

Transient receptor potential (TRP) channels are involved in recognizing a broad range of mechanical and chemical stimuli [40]. Each TRP channel gene belongs to one of seven subfamilies [TRPC (classical), TRPV (vanilloid), TRPM (melastatin), TRPN (NOMPC), TRPA (ANKTM1), TRPML (mucolipin), and TRPP (polycystin)] [41] and encodes a channel subunit with at least six transmembrane-spanning segments and intracellular N- and C-terminal regions with subfamily-specific domain layout [42]. Four subunits assemble into functional homo- or heteromeric ion channels, commonly nonselective for cations and permeable to Ca^{2+} [41, 43].

Mechanosensitivity has been reported for a number of TRP channel subunits; however, to what extent these proteins are directly involved in mechanotransduction remains to be resolved for many family members. Interestingly, TRP channels were also identified as partners of GPCRs and aGPCRs in this context.

For example, TRPC6 is a downstream target of a mechanosensitive GPCR responsible for mediating myogenic vasoconstriction in response to elevated intraluminal pressure [44]. Further, mouse sensory neurons express TRPC1, TRPC3, and TRPC6 [45]. *Trpc3*/*Trpc6* double knockout mice display altered responses to tactile stimuli. These mutants also exhibit pronounced hearing impairments and vestibular defects consistent with TRPC3 and TRPC6 expression in cochlear and vestibular hair cells [45]. Recent work indicated that TRPC1, TRPC3, TRPC6, and TRPC5 heteromultimerize to contribute to cutaneous and auditory mechanosensation. Moreover, these TRP channel subunits were proposed to contribute indirectly to cochlear mechanotransduction [46].

Further, members of the TRPN family have been associated with the perception of mechanical stimuli in *Drosophila* (no receptor potential; NOMPC) [47, 48], hearing in zebrafish (TRPN1) [49], and proprioception in *C. elegans* (TRP-4) [50]. NOMPC localizes to the ciliary tips of mechanosensory neurons of the Johnston's organ and chordotonal organs in *Drosophila* [51, 52]. NOMPC can be directly mechanically activated and confers mechanosensitivity to otherwise insensitive cells, rendering NOMPC a bona fide mechanotransduction channel [53, 54]. Recent data indicate that the 29 ankyrin repeats of NOMPC's N-terminus constitute a molecular spring that conveys forces generated by the

cytoskeleton to modulate ion channel gating [55, 56]. However, loss of *nompC* does not completely abolish fly hearing [47], suggesting the existence of additional transducer molecules [57]. Alternatively, NOMPC may sensitize or adjust the mechanosensory complex for mechanical input [58]. Interestingly, genetic analysis suggests that the aGPCR dCIRL/latrophilin modulates NOMPC activity in sensory chordotonal neurons [59] (see Sect. 3.2).

The *C. elegans* TRPV proteins OSM-9 and OCR-2 form heteromeric channel complexes in sensory neurons that respond to touch and hyperosmolarity [60–62]. Consequently, loss of function of *osm-9* results in worms that are less sensitive to osmotic and mechanical challenges [60, 62]. *Drosophila* encodes TRPV proteins, INACTIVE and NANCHUNG, that are mutually required for the assembly of heteromultimeric channels involved in fly hearing and possibly auditory mechanotransduction [57, 58, 63, 64]. Epistatic analysis implicated NANCHUNG function in a mechanosensory signaling pathway together with the aGPCR latrophilin/CIRL [59].

Finally, TRPP homologs TRPP2 (polycystin 2 or PC2) and TRPP3 (polycystic kidney disease 2-like 1, PKD2L2) have been associated with mechanosensation. TRPP2 forms a receptor-ion channel complex with polycystin 1 (PC1, TRPP1) [65, 66], which localizes to primary cilia of renal epithelial cells and endothelial cells [67–70]. PC1 does not belong to the TRP channel family; it contains 11 -transmembrane-spanning regions and a large extracellular N-terminal domain that promotes cell-cell and cell-matrix interactions [71, 72] (see also [73]). The TRPP2/PC1 complex mediates Ca^{2+} transients in response to ciliary deflections induced by luminal shear stress [67, 68, 74]. Intriguingly, filamin A cross-links TRPP2 and the actin cytoskeleton to regulate stretch-activated cation channels (SACs) involved in cardiovascular pressure sensing [70]. Furthermore, TRPP2 forms ciliary mechanosensitive sensors with TRPV4 to transduce mechanical stress [75].

2.2.4 K_{2P} Channels

K_{2P} channels are comprised of four transmembrane-passing segments, two pore-forming regions, and intracellular N- and C-terminal portions. They assemble into hetero- and homodimeric ion channels. TWIK-related K^+ channels (TREK) and TWIK-related arachidonic acid-stimulated K^+ channel (TRAAK) are membrane tension-gated channels characterized by low mechanical threshold and a broad range of tension activation [76].

TREK-1 and TREK-2 are widely expressed within the central and peripheral nervous systems as well as nonneuronal tissues such as the cardiovascular system, lung, colon, and kidney. By contrast, TRAAK expression appears to be confined to neuronal tissues [77]. In the nervous system, K_{2P} channels are physiologically relevant as they contribute considerably to cell hyperpolarization, which balances/counteracts depolarization-induced action potentials, thereby shaping the electrical response to mechanical force. For example, TRAAK activity counteracts activation of Piezo to curtail action current generation in cultured neuroblastoma cells [78]. Further, TRAAK/TREK and mechanosensitive cation channels are coexpressed in sensory neurons of DRGs [79]. Deletion of *Traak*, *Trek-1*, or

Trek-2 genes in mice renders them hypersensitive to mechanical challenge [80, 81], which is in line with the notion that K_{2P} channels set the mechanical threshold for action potential generation [76]. Evidence for the physiological relevance of TREK-1 in mechanosensation outside the nervous system has emerged as well. Polycystins play a role in force-dependent apoptosis of renal epithelial cells, a function that was shown to rely on the opening of TREK-1 [82]. TREK-1 is enriched in the bladder, uterus, and colon where it regulates stretch-induced contraction of smooth muscle cells [83].

2.2.5 Transmembrane Channel-Like 1 and 2 Proteins

Recently, the transmembrane channel-like 1 and 2 (TMC1 and TMC2) proteins emerged as novel components required for auditory and vestibular mechanosensation in mammals [84–86]. The *Tmc* gene family encodes proteins with at least six transmembrane regions flanked by intracellular N- and C-terminal portions [87] reminiscent of TRP channel proteins. However, TMC proteins do not share sequence homologies with known ion channels [85, 88], even though recent work indicated that *C. elegans tmc-1* assembles into nonselective cation channels when heterologously expressed [89]. Several lines of evidence implicate TMC proteins as key components of the hair cell transduction complex. First, consistent with the mRNA expression pattern [90], TMC1 and TMC2 fusion proteins localize to stereocilia of the inner ear hair cells [86]. Second, mutations of the human *Tmc1* gene have been associated with dominant and recessive nonsyndromic sensorineural hearing deficits [84]. Similarly, dominant and recessive mutations of mouse *Tmc1* result in animals that exhibit defective hearing (*Beethoven*) and deafness (*dn*), respectively [84, 91]. Third, in mice, loss of function of *Tmc1* or *Tmc2* deprives hair cells of mechanosensory responses [90, 92]. Fourth, robust apical FM1-43 dye or gentamicin uptake reported for wild-type hair cells was abolished in the absence of TMC1 and TMC2 [90, 93, 94]. Fifth, expression of either TMC1 or TMC2 rescues loss of hair cell mechanosensitivity displayed by homozygous null mutants [90]. Sixth, protocadherin-15 (PCDH15) together with cadherin-23 forms extracellular tethers (called tip links), which connect adjacent pairs of stereocilia and transmit hair cell bundle deflection-based mechanical forces to mechanosensors [95, 96]. Strikingly, recent work uncovered a direct interaction between PCDH15 and TMC1 as well as TMC2 in fish and mouse [97, 98].

Taken together, these results suggest that TMC proteins are strong candidates for the long sought-after mechanosensitive channel required for mechanotransduction in mammalian hair cells [85].

2.3 Integrins and Cadherins

Cell adhesion enables the generation of mechanical tissue cohesion as well as cell-cell and cell-matrix communication vital for a myriad of physiological processes. But how do cells sense the mechanical context of their microenvironment (e.g., extracellular matrix or adjacent cells), and how are these signals translated into

cellular responses? Based on structural and functional properties, integrins and cadherins have been associated with mechanosensation in this vein.

2.3.1 Integrins

Integrins constitute a large family of glycoprotein receptors that bridge cell-cell contacts (cell adhesion) and interconnect intra- and extracellular matrices (ECMs) in metazoans. Integrins exist as heterodimers comprised of non-covalently bound α- and β-subunits, which are combined to form more than 20 distinct, functionally nonredundant receptor variants [99, 100].

Integrin heterodimers are inserted into the plasma membrane through a single transmembrane domain per protomer. The large extracellular domain (ECD) mediates interactions with ECM components (e.g., collagens, fibronectins, or laminins) and adjacent receptors [e.g., vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule (ICAM)] [100, 101]. The short intracellular domain (ICD) interacts, via cytoplasmic adaptors, with cytoskeletal elements to modulate local actin polymerization and global cytoskeletal dynamics [102, 103]. This molecular layout allows integrins to mechanically interlink the cell's exterior and interior providing the backbone for mechanical stress-induced assembly of focal adhesions (FA) [104–107], stimulation of signaling pathways, and gene transcription [108–110].

Importantly, integrins possess the capacity to confer signals in an "outside-in" or "inside-out" fashion associated with different biological ramifications. "Outside-in" signaling is based on the interaction of integrin ECDs with ECM molecules or opposing cellular receptors. These engagements provide information about ECM rigidity as well as adjacent cell/tissue geometry, which in turn regulate cellular growth, differentiation and apoptosis, cell polarity, and formation of focal adhesion complexes [105, 111–114]. During "inside-out" signaling, intracellular activators such as kindlins or talins associate with integrin ICDs to induce conformational changes that alter the affinity to extracellular ligands ("integrin activation"). Hence, this signaling mode affects cell adhesion and migration as well as ECM assembly [102]. Interestingly, the ionotropic Piezo1/Fam38A channel subunit was shown to induce integrin activation through an R-Ras-dependent mechanism [115].

In sum, integrin-based adhesion complexes are fit to perceive and integrate external forces into the preexisting cellular mechanical context to balance the ECM resistance according to tension forces generated by the cytoskeleton.

2.3.2 Cadherins

Cadherins are a large group of cell surface receptors that mediate Ca^{2+}-dependent cell adhesion [116]. The cadherin family includes a vast number of cell surface molecules; however, classical cadherins (E-, N-, P-cadherins) and the related desmosomal cadherins are, thus far, the best characterized. They feature cadherin repeat-containing ECDs, a single membrane-spanning region, and ICDs which interact with β-catenin (classical cadherins), γ-catenin (desmosomal cadherins), and components of the cytoskeleton [117].

Cadherins engage in ECD-dependent homo- and heterophilic interactions that may mediate adhesive and selectivity functions, respectively [118]. For example, force measurements uncovered that cadherins establish three spatially distinct bonds at different ECD regions that possess varying kinetic and mechanical properties [119, 120]. In contrast, ICDs associate with F-actin via catenin/vinculin interactions [121–124]. This structural setup allows cadherin complexes to act as mechanosensors that actively sense and transduce exogenous and endogenous mechanical fluctuations to trigger biochemical changes that direct cell responses [125]. Strikingly, the tensile force administered to an adhesion site is directly proportional to ECM stiffness [126].

Several lines of evidence support the role of cadherin-based adhesive junctions in mechanotransduction. First, cell-cell junctions undergo force-dependent remodeling not only in vitro but also in vivo [127, 128]. In *Drosophila* and *C. elegans*, cytoskeletal-provoked tension at cadherin junction coincides with a decline in junction expansion in various developmental processes. For example, the association of the actomyosin apparatus with cadherin junctions is a prerequisite for contraction-driven apical constriction during *C. elegans* gastrulation. A similar mechanism has been reported for ventral furrow formation in *Drosophila* [129]. Intriguingly, recent work demonstrated that during germ band extension in *Drosophila*, junction shrinkage appeared to result from tension-generated cadherin accumulation, which was counteracted by cadherin endocytosis [130]. Second, the mechanics of intercellular junctions (e.g., cell traction, adhesion strength, and junction rigidity) change according to the mechanical environment [122, 131–134].

In *Drosophila*, junctional mechanics are regulated in a forward loop that controls cadherin levels with respect to actomyosin density to enable adequate tissue morphogenesis [130, 135].

Taken together, cadherin complexes probe their environment for tensional changes to preserve the mechanical integrity of cellular junctions and to regulate morphogenesis and homeostasis [136–139].

2.4 Metabotropic Mechanosensors

GPCRs are biosensors vital for the transduction of light, olfactory and gustatory stimuli, as well as neurotransmitters and hormones into biochemical signals and thus shape a multitude of physiological processes [140]. Based on this perception profile, GPCRs were classically considered chemoreceptors. However, in recent years, mounting evidence suggests that mechanical forces can also be perceived through GPCRs. Therefore, GPCRs most likely constitute polymodal sensors with both chemoreceptive and mechanoreceptive properties.

The first mechanosensitive GPCR identified was the angiotensin II type 1 receptor (AT_1R), which mediates functions in preload-induced cardiac hypertrophy in vitro and in vivo. In contrast to autocrine-mediated vasoconstriction, activity of AT_1R is brought about independently of angiotensin II and facilitates intracellular signaling through $G_{q/11}$ proteins [141, 142]. In addition to this

pathophysiological function, intraluminal pressure stimulates AT_1Rs located in the arterial blood vessels causing myogenic vasoconstriction, a mechanism referred to as the Bayliss effect [44]. Mounting evidence implicates PLC kinases in mechano-dependent signaling pathways, which may subsequently trigger activation of ionotropic TRPC channels (see Sect. 2.2.3) to set arterial myogenic tone [44, 141, 143, 144].

Strikingly, AT_1R undergoes mechanically induced conformational changes that stabilize the seventh transmembrane domain near the agonist-binding pocket, a molecular organization that is different from that induced by agonist and inverse agonist signaling [145–147], suggesting stimulus modality-dependent signaling modes of this GPCR.

Thus far, eight additional putative mechanosensitive $G_{q/11}$ protein-coupled receptors have been described [148]. For example, time-resolved fluorescence microscopy of endothelial cells unveiled that mechanical strain causes a ligand-independent rise in bradykinin 2 receptor (B_2R) activity [149]. The authors suggest that, similar to AT_1R, conformational alterations of B_2R are due to tension-based properties of the lipid bilayer. However, it remains unclear if mechanical stimulation of B_2R is causally linked to effective G protein coupling. Another interesting example of a potential mechanosensitive GPCR derives from type 1 parathyroid hormone receptor (PTH_1R) essential for Ca^{2+} homeostasis in osteoblasts [150, 151]. Bone formation and bone mass regulation rely on the availability of parathyroid hormone (PTH) and mechanical loading, signal modalities proposed to converge at PTH_1R [151]. Evidence for a role in mechanoreception was also reported for dopamine receptor type 5 (D_5R) and formyl peptide receptor (FPR_1), which are both, similar to PTH_1R, excitable through fluid flow-generated shear stress in endothelial cells and neutrophils, respectively [152, 153]. Interestingly, D_5R localizes to primary cilia in vitro and in vivo and regulates their length through cofilin and actin polymerization. In addition, the authors report that ciliary sensitivity to fluid shear stress can be modified through chemosensory properties of the receptor [152]. Furthermore, electrophysiological recordings unveiled hypotonicity-dependent responses of heterologously expressed vasopressor receptors, ET_A endothelin receptor (ET_AR) and V_{1A} receptor ($V_{1A}R$), as well as H_1 histamine receptor (H_1R) and M_5 muscarinic acetylcholine receptor (M_5R) [44].

3 Adhesion GPCRs in Mechanosensation

A novel intriguing line of evidence for the mechanoreceptive role of GPCRs derives from the large family of aGPCRs, which stand out from canonical GPCRs because of their structural and functional profile as well as the conspicuous lack of soluble ligands [154]. Thus, are aGPCRs particularly prone to sense mechanical cues?

Three members of the aGPCR class, GPR56/ADGRG1, GPR126/ADGRG6, and latrophilin/CIRL/ADGRL, have been recently suggested to be involved in mechanosensation (Fig. 1) [59, 155, 156]. Like canonical GPCRs, aGPCRs possess a seven-transmembrane helix region (7TM) that can intracellularly couple to

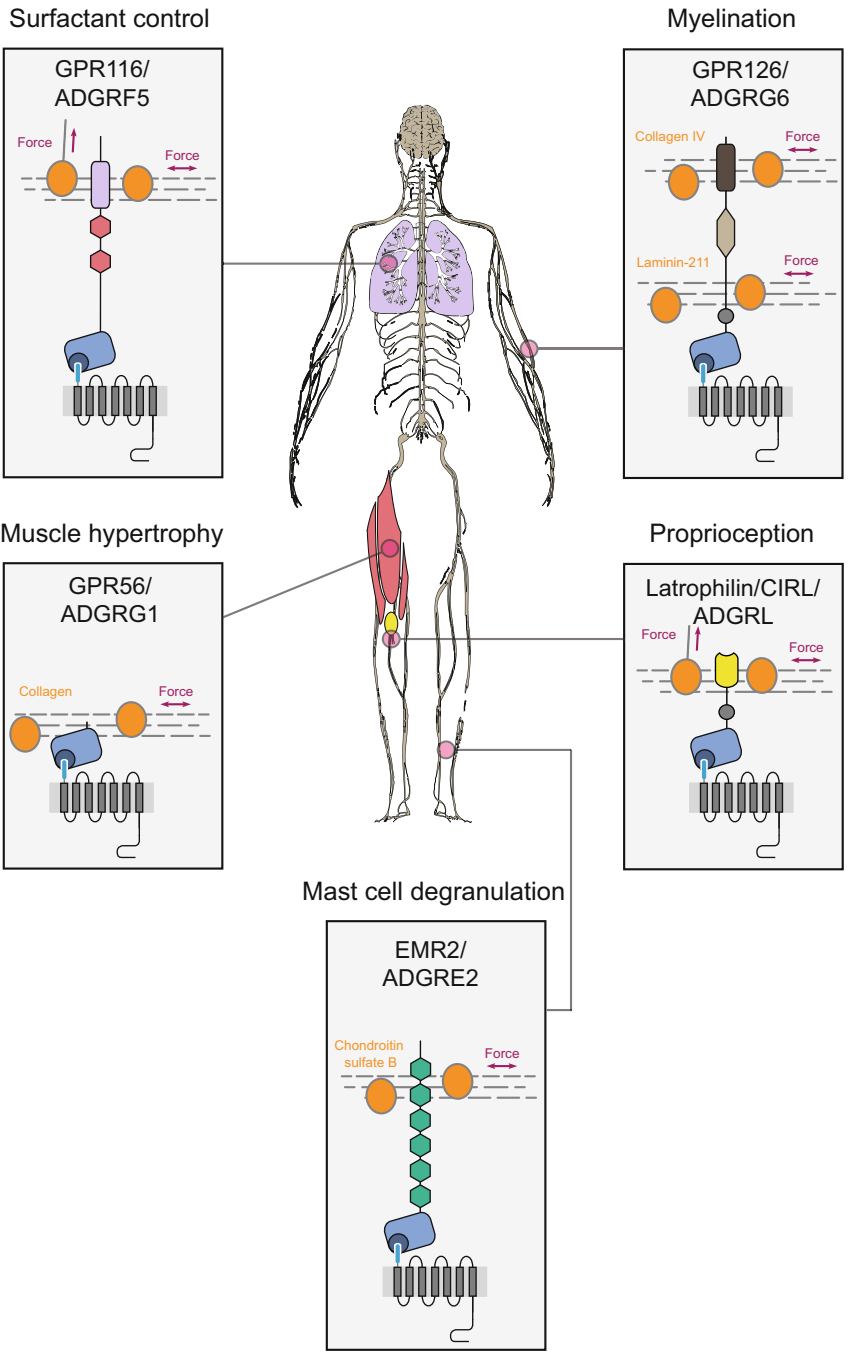

Fig. 1 Adhesion GPCRs as mechanosensors. Different aGPCR homologs and their cognate ligands have been described in settings, which suggest that they function in a mechanosensory capacity. For details, see text

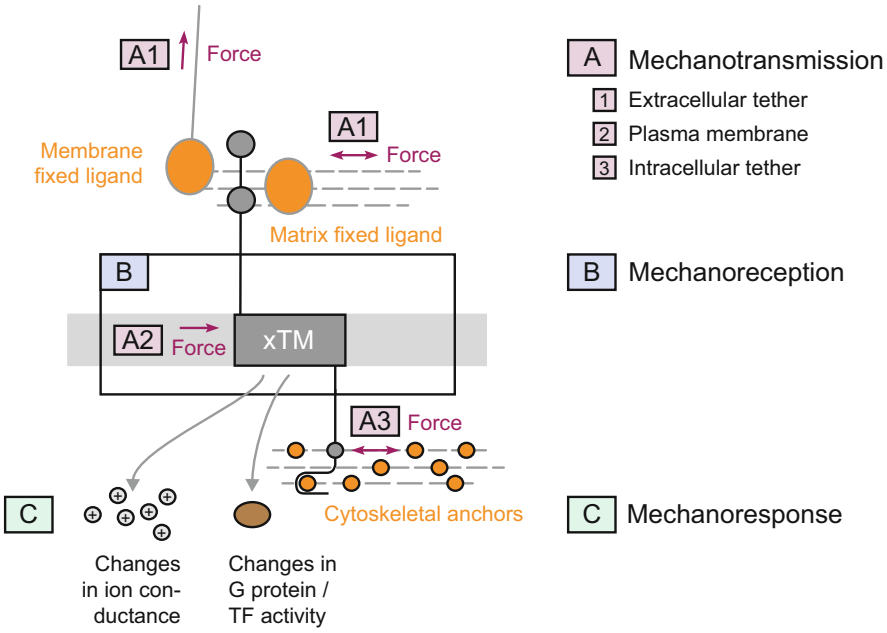

Fig. 2 Principal elements of mechanotransduction. The process of mechanotransduction can be grossly divided into three steps. (**a**) Mechanotransmission (indicated in *light pink*) that, according to current models, can be conveyed through extracellular or intracellular tethers and/or the plasma membrane. (**b**) Mechanosensation (*blue*), perceived through force-dependent conformational changes of membrane integral sensor proteins (*gray*). (**c**) Mechanoresponse (*green*), which induces changes in cell physiology (e.g., ion channel conductances, metabolic states, or modulation of transcriptional activity). xTM = variable number of transmembrane helices depending on the mechanosensor protein architecture (TRP channels, 6TM; aGPCR, 7TM; polycystin 1, 11TM; Piezo, 14TM)

heterotrimeric G proteins capable of triggering a multitude of signaling cascades that determine cellular responses (Fig. 2; see [157, 158]).

Further, aGPCRs possess large extracellular N-termini, which for many aGPCRs contain domains that are involved in cell-cell or cell-matrix adhesion [154] (see also [159]). Uniquely, the N-termini of many aGPCRs can be separated from the 7TM through an autoproteolytic event catalyzed by the GPCR proteolytic site (GPS) motif [160, 161], which is part of a much larger protein fold (GPCR autoproteolysis-inducing (GAIN) domain) [162]. Receptor cleavage is thought to occur early in the secretory pathway during protein maturation and generates an N-terminal fragment (NTF) and C-terminal fragment (CTF), which are predicted to traffic together to the cell surface where they are located as non-covalently bound heterodimers (Fig. 3; see also [73, 159]).

For several aGPCRs, a cryptic tethered ligand, termed the *Stachel* sequence, has been described, which acts as a potent agonist for downstream signaling [163–166] (Fig. 3; see also [157, 158]). Yet, crystal structures of aGPCR GAIN domains

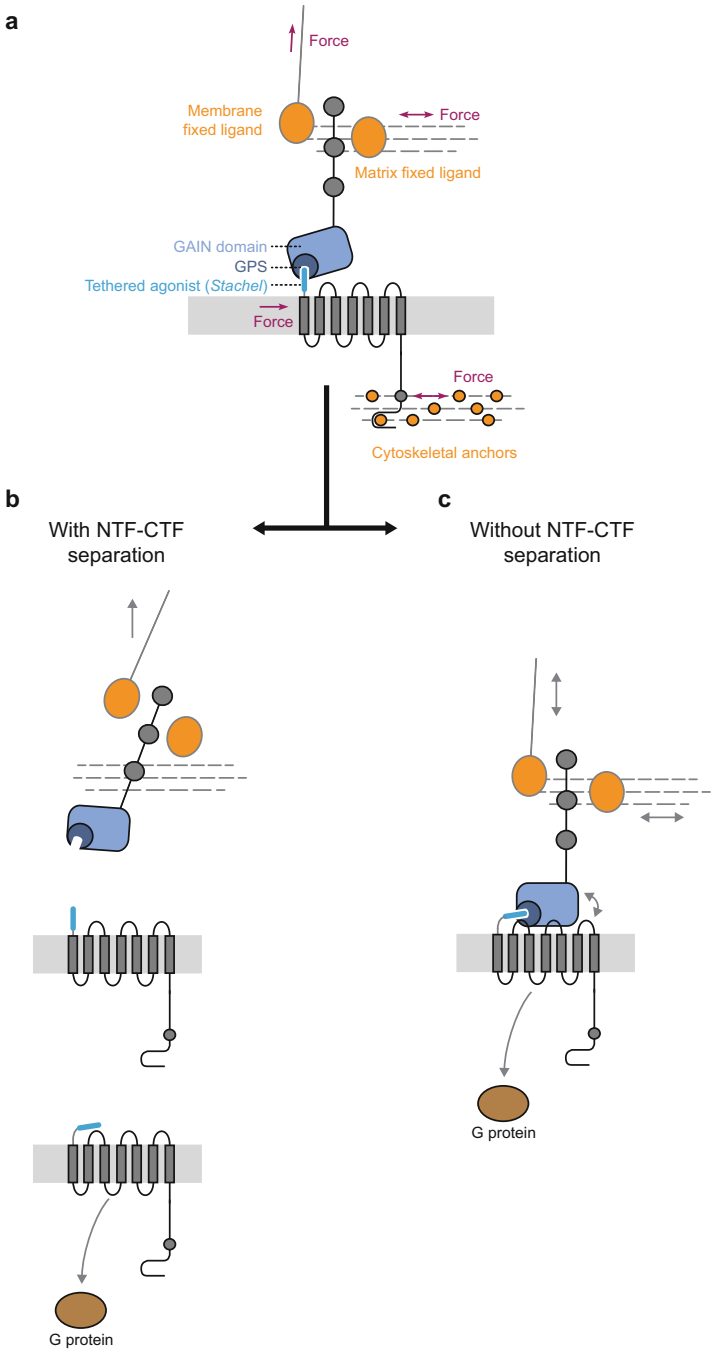

Fig. 3 Sequence of putative events during aGPCR-mediated mechanotransduction. (**a**) aGPCRs are subjected to mechanical force through their adhesive ECDs, at the level of the membrane, or through intracellular anchors. (**b**) Depending on whether the receptor is autoproteolyzed and

demonstrate that the *Stachel* sequence is deeply buried between two β-sheets [162], suggesting that the tethered agonist, at least for some aGPCRs, may not be readily exposed. How, then, might the *Stachel* sequence "escape" the ensconcement of the GAIN domain β-sheets in order to confer a signal to the receptor's 7TM stretch? Recent studies suggest that mechanical activation may be at play, which could induce conformational changes within the GAIN domain or complementary portions of the receptor to liberate the *Stachel* sequence and to allow receptor activation (Fig. 3).

3.1 GPR126/ADGRG6

As described in [167], myelin is the multilamellar sheath generated by specialized glial cells called Schwann cells in the peripheral nervous system and oligodendrocytes in the central nervous system. In the peripheral nervous system, GPR126 couples to $G\alpha_s$ and elevates cAMP in Schwann cells to initiate myelination (Fig. 1) [155, 163, 168–171]. The *Stachel* sequence of GPR126 is a potent agonist to trigger cAMP elevation, and analysis of zebrafish mutants has recently provided evidence that *Stachel*-mediated signaling is critically important in vivo. Liebscher and colleagues generated a new mutant allele of *gpr126*, $gpr126^{stl215}$, in which two amino acids essential for signaling were precisely deleted from the *Stachel* sequence. In vitro analysis demonstrated that this mutant receptor could be cleaved and that it trafficked appropriately to the cell surface, although it was incapable of signaling via cAMP. Accordingly, in vivo, $gpr126^{stl215}$ mutant Schwann cells could not initiate myelination [163]. This work suggested a model in which *Stachel*-mediated activation of Gpr126 is required for cAMP elevation and subsequent myelin initiation.

To determine how the *Stachel* sequence of GPR126 might be exposed during Schwann cell myelination, Petersen and colleagues investigated the relationship between one GPR126 binding partner, laminin-211, and GPR126 signaling [155] (discussed further in [167]). Laminin-211 is a heterotrimeric protein encoded by *Lama2*, *Lamb1*, and *Lamc1* genes [172], and like GPR126, laminin-211 is required for Schwann cell development and myelination [173]. Overexpression of *lama2* in zebrafish rescues myelination in *gpr126* hypomorphic mutants in a cAMP-dependent manner, suggesting that laminin-211 activates $G\alpha_s$ signaling.

Fig. 3 (continued) present as an NTF/CTF heterodimer at the cell surface, force transmission may separate the NTF from the CTF, thereby exposing the cryptic tethered agonist that interacts with the 7TM domain and triggers intracellular responses. (**c**) Several aGPCRs cannot be autoproteolytically cleaved, yet they appear to harbor a *Stachel* sequence that can stimulate receptor activity. This indicates that the tethered agonist can interact with the 7TM domain even if it remains associated with GAIN domain, possibly through conformational changes of the GAIN domain that render the agonist accessible. Note that this scheme illustrates putative intramolecular interactions between GAIN/*Stachel* and the 7TM region of aGPCRs; however, intermolecular interactions are conceivable as well

Intriguingly, addition of laminin-211 to GPR126 in heterologous cell systems suppressed cAMP elevation under standard culture conditions and elevated cAMP levels under dynamic conditions (vibration or shaking) [155]. These data can be reconciled by observations from related aGPCRs, noted above, that the *Stachel* sequence is buried, not exposed, between two β-sheets of the GAIN domain [162]. The *Stachel* sequence is so deeply buried that significant structural changes might be required for *Stachel*-mediated receptor activation to occur (Fig. 3). In vitro analyses of mutant receptors demonstrated that laminin-211 addition facilitates greater GPR126 activation than dynamic forces alone and that dynamic force signaling requires the *Stachel* sequence to activate GPR126.

These in vitro findings suggested that laminin-211 enhances GPR126 structural changes to allow for *Stachel*-mediated signaling, and the overexpression studies in zebrafish demonstrated that increased *lama2* levels can drive Gpr126-dependent myelination. What physical forces might laminin-211 impart upon GPR126 in vivo? Interestingly, laminin-211 polymerization is known to be essential for Schwann cell development; $Lama2^{dy2j}$ mouse mutants have a spontaneous mutation that leads to aberrant splicing and deletion of the laminin α2 polymerization domain, and these mice have defects in Schwann cell development and myelination [174–176]. To test if laminin α2 polymerization could be an activating force on Gpr126, reminiscent of physical forces in vitro, Petersen and colleagues engineered a non-polymerizable Lama2 overexpression construct to mimic the $Lama2^{dy2j}$ mouse mutation. Unlike wild-type *lama2*, the polymerization-defective *lama2 (dy2j)* was not sufficient to rescue myelination defects in hypomorphic *gpr126* zebrafish mutants [155].

In summary, the in vitro studies suggested the possibility that GPR126 might be sensitive to mechanical force, while the in vivo studies implicated laminin-211 polymerization as a potential source to transmit forces and stimulate mechanosensation in vivo.

3.2 Latrophilins/ADGRL1–ADGRL3

Contemporaneous work in *Drosophila* has even more strongly implicated an aGPCR in mechanosensation, as latrophilin/CIRL was shown to shape the response of chordotonal sensory neurons by determining the sensitivity of certain mechanosensors in a cell-autonomous manner [59]. Chordotonal neurons are peripheral, compound mechanosensory neurons in *Drosophila*, which perceive mechanical signals such as sound, touch, and muscle stretch [47, 52, 177]. Latrophilin/CIRL is robustly expressed in these cells (unpublished data) suggestive of a function in this cell type. Indeed, analyses of a newly engineered *dCirl* null allele revealed many interesting phenotypes consistent with a role for latrophilin/CIRL in mechanosensation. *dCirl* mutant larvae exhibited aberrant crawling patterns, and although locomotion is a complex behavior controlled by both motor outputs and peripheral sensory inputs, this defect could be partially rescued by chordotonal neuron-specific expression of *dCirl*. Moreover, *dCirl*

mutant larvae were also less responsive to gentle touch (touch perception is a known function of the chordotonal organ), and this phenotype could only be suppressed by chordotonal neuron-specific re-expression of this aGPCR [59]. Interestingly, the morphology of chordotonal neurons is grossly normal in *dCirl* null mutants, suggesting that latrophilin/CIRL is required specifically for the function of these neurons and not for their development or morphology.

To elucidate the potential role of latrophilin/CIRL in mechanosensation, Scholz and colleagues directly applied mechanical stimulation (vibration stimuli) to the cap cells of the chordotonal organ and simultaneously recorded action currents from chordotonal neurons. While *dCirl* mutant neurons maintained spontaneous activity in the absence of vibration stimuli, the mutant neurons displayed significantly lower action current frequencies across the entire tested vibration spectrum. Compound mutant analyses also demonstrated that *dCirl* genetically interacts with TRP channels, known as mechanosensor molecules of *Drosophila* chordotonal neurons [59] (see also Sect. 2.2.3).

All in all, these studies establish that latrophilin/CIRL can modulate the sensitivity of neuronal mechanosensation (Fig. 1). Given that several cell-specific rescue experiments demonstrate that *dCirl* functions cell autonomously in chordotonal neurons, likely via CTF signaling, it will be interesting to determine if and how *Stachel* sequence signaling modulates these functions of latrophilin/CIRL in mechanosensation (Fig. 3).

3.3 GPR56/ADGRG1

The role of aGPCRs as mechanosensors also extends beyond the nervous system. Skeletal muscle can respond to multiple stimuli, including mechanical tension, which is a potent regulator of muscle mass. In myotubes, a splice isoform of the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) induces muscle hypertrophy. Interestingly, overexpression of PGC-1α increases *Gpr56* expression, and GPR56 overexpression also induces muscle hypertrophy dependent on $G\alpha_{12/13}$ signaling. Resistance exercise causes muscle hypertrophy, but this phenotype is attenuated in *Gpr56* mutant mice [156] (see also [178]). These results collectively highlight a potential role for Gpr56 in sensing muscle fiber size, which could be mediated by mechanical sensitivity to stretch (Fig. 1).

Furthermore, in the central nervous system, GPR56 is required for proper cortical development (i.e., cortical folding), which has been directly linked to mechanosensation [179]. GPR56 and other aGPCRs including GPR114/ADGRG5 and GPR97/ADGRG3 are also highly expressed on lymphocytes [180]; it is tempting to speculate that signaling via these aGPCRs might be affected during the rolling and extravasation behaviors characteristic of these cells.

3.4 Mechanosensation by Other Adhesion GPCRs

These recent studies implicating Gpr126, latrophilin/CIRL, and GPR56 as putative mechanosensitive receptors raise the interesting and obvious question as to whether other aGPCRs might share this mechanosensitive property.

Indeed, one splice variant of GPR114 is sensitive to mechanical stimulation [166], although it is not yet clear in what physiological context this receptor may partake in mechanoreception. In addition, recent reports implicate *EMR2*/*ADGRE2* in vibratory urticaria [181], an autosomal dominant disorder that is signified by hives and systemic manifestations in response to cutaneous vibration. Interestingly, mast cells express EMR2, and mechanosensing through this receptor appears to depend on the stability between the NTF and CTF heterodimer of EMR2. A human mutation in the GAIN domain destabilizes this interaction and conceivably results in increased receptor activity upon mechanical challenge, which ultimately leads to massive mast cell degranulation [181]. Moreover, observations from previous studies on several aGPCRs can be interpreted in the light of these new insights to support a unifying model of mechanical susceptibility of this receptor class.

In the murine lung, loss of *Gpr116*/*Adgrf5* causes reduced surfactant uptake, leading to massive and pathological accumulations of surfactant lipids and proteins in the alveolar space [182–184] (see also [185]). Given that surfactant is essential to temper lung surface tension to allow for effortless lung expansion during inhalation, it is enticing to speculate that GPR116 is involved in monitoring lung tension (Fig. 1).

Similar to the expression of dCIRL in mechanosensory cells, GPR98/VLGR/ADGRV1 localizes to inner ear hair cells [186], where it is involved in proper development of the cochlear organ of Corti [187]. In the future, it will be interesting to determine the exact role of GPR98 during auditory mechanotransduction in these cells.

4 Conclusions

In sum, ionotropic mechanosensors are distributed throughout various organ systems across different species where they assure mechanosensory responses with latencies on millisecond timescales, rather atypical for metabotropic mechanosensors. Interestingly, however, in some instances these responses appear to be adjusted through the activity of GPCRs, which begs the question if ionotropic and metabotropic mechanosensors form functional units to enable reliable mechanotransduction.

aGPCRs regulate a multitude of physiological processes signified by the capacity to sense mechanics in different cellular contexts, which could explain the participation of aGPCRs in various, seemingly unrelated, biological phenomena. The structural and functional layouts of aGPCRs potentially reflect their optimization for the perception of mechanical forces, rather than a general sensitivity to a range of multiple sensory stimuli known for other GPCR families. Another

interesting aspect will be to unveil the signaling cascades through which aGPCRs influence ionotropic mechanotransduction processes and whether ion channels and GPCRs form functional mechanosensory units. Further research efforts will clarify the physiological and pharmacological properties underlying the mechanobiological roles of aGPCRs.

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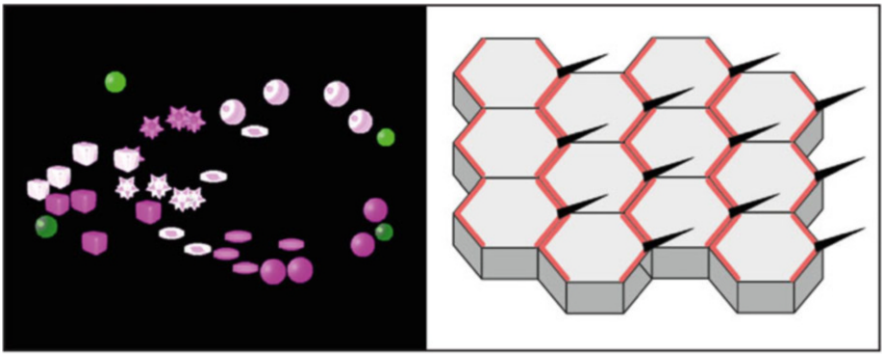
Adhesion GPCRs Govern Polarity of Epithelia and Cell Migration

David Strutt, Ralf Schnabel, Franziska Fiedler, and Simone Prömel

Graphical Abstract



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Contents

Abstract

In multicellular organisms cells spatially arrange in a highly coordinated manner to form tissues and organs, which is essential for the function of an organism. The component cells and resulting structures are often polarised in one or more axes, and how such polarity is established and maintained correctly has been one of the major biological questions for many decades. Research progress has shown that many adhesion GPCRs (aGPCRs) are involved in several types of polarity. Members of the two evolutionarily oldest groups, Flamingo/Celsr and Latrophilins, are key molecules in planar cell polarity of epithelia or the propagation of cellular polarity in the early embryo, respectively. Other adhesion GPCRs play essential roles in cell migration, indicating that this receptor class includes essential molecules for the control of various levels of cellular organisation.

1 Introduction

Life is order, life is counteracting entropy. It has been a very interesting question for a long time in biology how the linear information of the DNA codes for the three-dimensional topology of cells, tissues and ultimately the whole organism. The spatial arrangement of cells as well as their structure, in general referred to as polarity, is an indispensable prerequisite to order cells and tissues in a multicellular organism. Various types of polarity are distinguished. Even cell migration can be considered as a form of polarity in a broader sense: migrating cells are polarised as they have a clear front and back [1]. In most cells, asymmetric organisation of cellular components and structures form the basis for the establishment and maintenance of polarity.

Polarity not only defines cellular function, it is also an essential part of various biological processes such as embryogenesis or the formation of tissues. Cell

polarity is already evident very early in embryogenesis when cells divide asymmetrically or in an oriented manner, and it is a fundamental requirement for the organisation and patterning of the developing embryo, especially during gastrulation (reviewed in [2]). The formation of anterior-posterior, dorsal-ventral and left-right axes rely on polarisation. Later on in development, the collective alignment of cell polarity across a tissue plane mediates coordination and organisation of entire cell layers (reviewed in [3, 4]). This process is referred to as planar cell polarity (PCP) and is involved, for example, in the uniform orientation of hairs [5, 6] or branching morphogenesis in the lung [7].

Although various forms of polarity have been subject to intense study, and progress has been made in the past decades towards a detailed knowledge about the mechanisms underlying each type of polarity, our understanding on a molecular level of the cellular signals involved is still limited. Several major pathways have been identified to be essential for establishment and maintenance of polarity. Anterior-posterior tissue polarity cascades as well as the non-canonical Wnt/Frizzled (Fz) and Fat/Dachsous/Four-jointed (Fat/Ds/Fj) PCP pathways are involved in oriented cell division (reviewed in [8]), with PCP pathways also playing other major regulatory roles in animal development (reviewed in [3, 4]). However, these molecular cascades cover only certain aspects of polarity, and many components of the complex network of regulation remain elusive.

It has become increasingly clear that aGPCRs are not only involved in shaping polarity but are potentially major players in the coordination of various types of polarity such as oriented cell division, planar cell polarity and cell migration. Receptors of five of the nine subfamilies have been identified to play a role in these processes [9]. Besides the recently described Gpr125 the two most prominent and the best characterised ones are Latrophilins and Flamingo/Celsr (Fig. 1). This

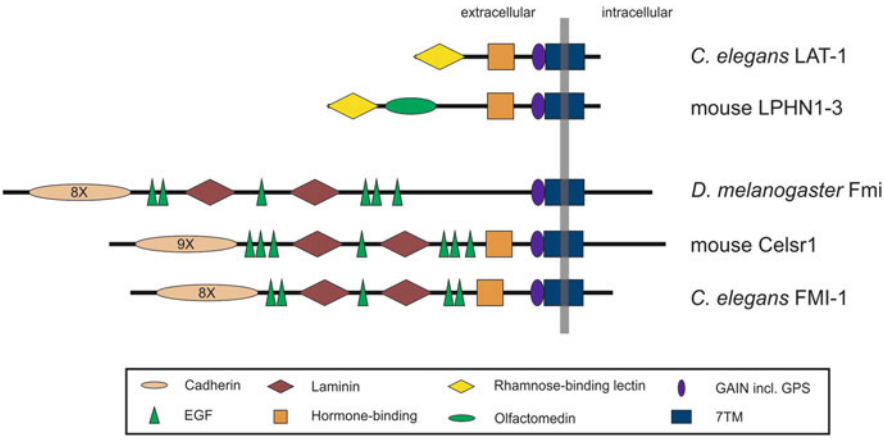


Fig. 1 Domain structure of invertebrate and mammalian Latrophilin and Flamingo/Celsr homologues. Both receptor families are conserved in invertebrates and vertebrates and have the GAIN (GPCR autoproteolysis inducing) domain containing the GPS (GPCR proteolysis site), which is a hallmark feature for aGPCRs. The Latrophilin family is determined by a Rhamnose-binding lectin domain, whereas Cadherins are a hallmark of the Flamingo group

book chapter will discuss the current state of knowledge on the physiological functions of aGPCRs in polarity of epithelia and cell migration as well as the molecular mechanisms they mediate.

2 Latrophilin Signals in Tissue Polarity of Embryogenesis

2.1 Embryonic Development of *C. elegans*

All life today is based on the division of cells, which in metazoans originates from the female gamete. Development is then just a modification of this cell. It is therefore an interesting question what the basal state of the original progenitor cell looks like. Does the embryo have to arrange the polarity of each cell division de novo? If so there should be specific cues or even forces to arrange the directions of mitotic spindles in space. Or is there an inherent polarity rule governing the directions of consecutive cell divisions? If so this may organise a basal arrangement of cells, which may only be modified in specific situations and one cue or force could suffice to do so. Two scenarios for the developmental programme of cells are conceivable. A cell can either get information through interaction with neighbouring cells or follows a preprogrammed series of divisions without any or much interaction with other cells (reviewed in [10, 11]).

The dogma that cells are solely specified autonomously in the roundworm *Caenorhabditis elegans* [12] was broken in the late 1980s [13, 14] suggesting that inductions via contacts with neighbouring cells also play a key role. The hermaphroditic *C. elegans* has always been an ideal organism to study polarity and cell division, especially after it was revealed that its cell lineage is invariant [12, 15]. The division planes and axes of blastomeric cleavages are highly reproducible and tightly controlled by various cell induction events. After fertilisation, the first division occurs in an anterior-posterior direction leading to the anterior AB and the posterior P1 blastomere (Fig. 2). In the second cleavage, P1 generates the two blastomeres EMS and P2, whereas AB divides into an anterior daughter ABa and a posterior daughter ABp. In the subsequent divisions up to the 12-cell stage embryo, the dorsal-ventral and left-right body axes of the embryo are formed through a set of controlled asymmetric and symmetric cell divisions. The

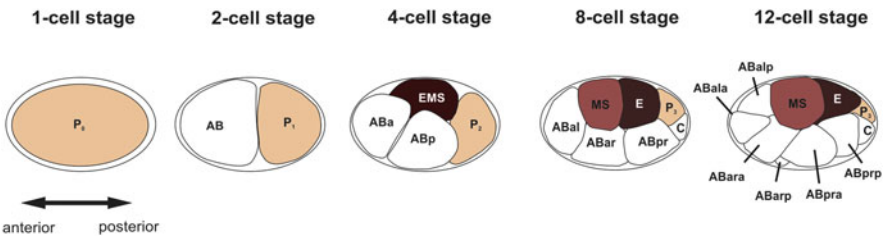


Fig. 2 Cell division in the early *C. elegans* embryo. Cell division in a *C. elegans* embryo occurs in a coordinated and unchangeable manner

mechanisms and pathways controlling these highly concerted and tightly regulated processes remained elusive. However, only 10 years after the first reports of the invariant cell lineage and cell inductions a sudden burst of studies established that the Wnt/Frizzled (Fz) pathway—or more accurately, pathway modules stitched together for different purposes—supplies the polarity required for anterior-posterior fate decisions and for controlling the spindle directions of the EMS and ABar blastomeres. In nematodes mutant for components of the Wnt pathway, spindles are tilted compared to spindles of wild-type embryos leading to severe defects including embryonic lethality [16–20]. This finally also disproved the notion that cell cleavage directions are specified merely autonomously [12]. The fact that Wnt/Fz also regulate spindle directions, which depend on an arrangement of cytoskeletal components, was a surprise as it had been shown before that the directions of early spindles depend on the *par* genes establishing the initial polarity of the zygote and the early blastomeres [21].

It is noteworthy that there has never been a *C. elegans* mutant identified, in which any of the other three AB-derived blastomeres of the eight-cell stage embryo (apart from ABar) cleaved specifically in an aberrant direction deranging the left-right inductions of anterior or even posterior blastomeres [22]. The exception is ABar where mutation of *ced-10*/*Rac* results in spindles turning in the same direction as in *wnt* mutants. It turned out to be the missing link between polarity conferred by the Wnt pathway and the modulation of the cell cytoskeleton during engulfment of cell corpses, spindle turning and cell migration [23].

In 2009, a novel player in the regulation of these highly complex processes of orienting spindles in the early embryo was discovered—the aGPCR Latrophilin [24]. Interestingly, the three Latrophilin homologues in mammals (ADGRL1–3/LPHN1–3) had so far only been associated with neuronal functions [25, 26]. They were first identified as targets of latrotoxin, a component of the black widow spider's venom [27, 28]. One of the two homologues in *C. elegans*, LAT-1 was now found to be essential for spindle orientation in certain AB descendants of the eight-cell stage of the early embryo (Fig. 3a).

2.2 LAT-1 Is Required for Spindle Orientation in the ABal Blastomere

Nematodes null for *lat-1* displayed several defects including embryonic and larval lethality yielding only about 25 % of the total brood to reach adulthood [24] which suggested an involvement of the receptor in cell division or similar processes. Subsequently, more detailed analyses revealed that indeed, *lat-1* mutant nematodes have a very distinct defect in spindle orientation in the early embryo (Fig. 3a). In embryos from homozygous *lat-1* mutant mothers, the ABal division direction is tilted from the normal $128° \pm 8°$ towards a direction of $90° \pm 18°$ (mean $\pm$ SD), which is almost perpendicular to the anterior-posterior axis [29]. Thus, the anterior daughter ABala is located closer to the centre of the embryo than it is in the wild type. This new position has fatal consequences for the embryo as now not only

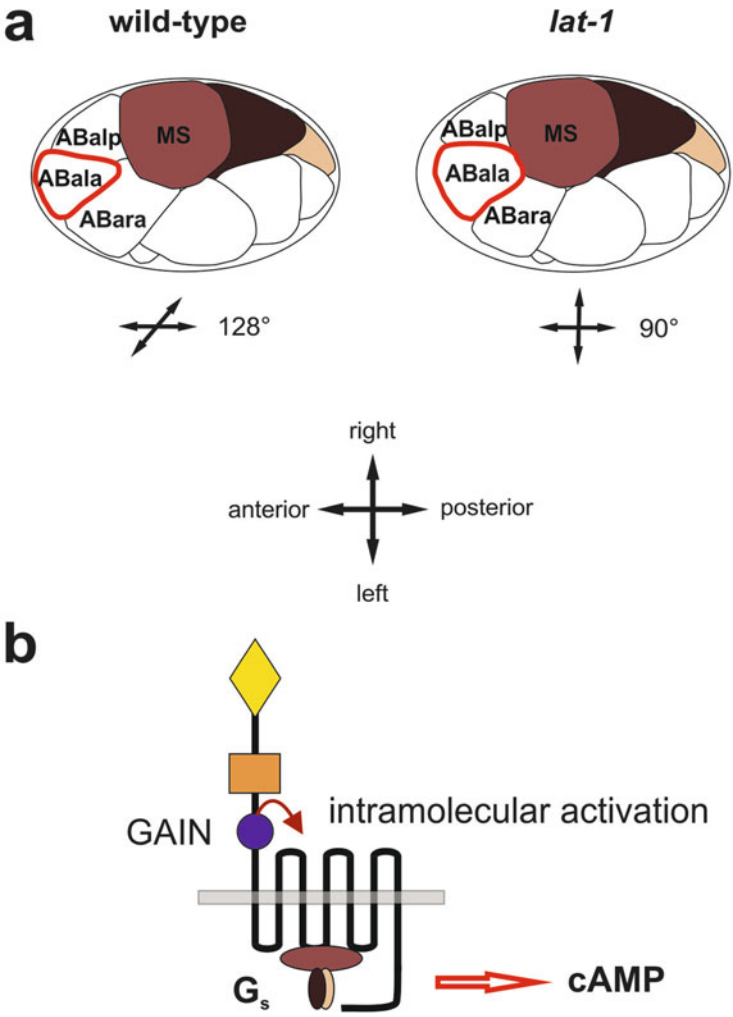


Fig. 3 Cleavage plane orientation defects in *lat-1* mutant embryos and LAT-1 signalling. (**a**) In wild-type *C. elegans* embryos, the mother blastomere of ABala and ABalp divides in an 128° angle to the anterior-posterior axis. This angle is shifted to 90° in *lat-1* mutant individuals. As a consequence, ABala is not displaced towards the anterior pole of the embryo as in the wild type but is located more towards the centre of the embryo. This leads to ABala contacting MS in *lat-1* mutants. (**b**) Schematic depiction of LAT-1 signalling. LAT-1 is activated by an intramolecular sequence within the GAIN domain which potentially interacts with the seven transmembrane region. Subsequently, the receptor triggers an elevation of cAMP levels via a G_s protein cascade leading to correct cell division plane orientation

ABalp, as in normal embryogenesis, but also ABala touches the MS blastomere (Fig. 3a). Through this contact, the MS blastomere generally induces left-right identity in the two cells ABara and ABalp. As in *lat-1* embryos, ABala is also in contact with MS; consequently, not only ABara is induced to execute the ABara

blastomere identity but by mistake also ABala, resulting in two ABara fates in the embryo. This effect is lethal for the embryo as subsequent errors in the following cell divisions, and thus, wrong induction events occur. In the original description of the *lat-1* phenotype, only the deletion of the ABala fate was documented [24]. Interestingly, cleavage orientation in the other blastomeres at this stage does not seem to be controlled by LAT-1 function.

It is a key question in the contemplation of LAT-1's role whether or not there is a basal system guiding spindle orientations in consecutive cell divisions in the absence of any external polarity. Earlier observations including the analysis of the development of isolated AB blastomeres were consistent with the notion that such a basal pattern (0° along the anterior-posterior axis followed by 90° along the left-right and dorsal-ventral axis, respectively) may exist [30, 31]. New investigations using much more sophisticated methods show that this basal system coordinates spindle directions during the first cleavages, and it is expected that parallel cleavages of the four AB-derived blastomeres in the eight-cell stage embryo reflects a basal state of the cells (R. Schnabel, unpublished results). Modulation of such a system in a specific new direction may only require one cue or force. In this scenario LAT-1 may be sufficient to direct the spindle of ABal away from MS to avoid a left-right induction. The same is true for the turning of the ABar spindle by the Wnt pathway.

2.3 Genetics of *lat-1*

The function of the Latrophilin gene in *C. elegans* was not identified in a mutant screen as for many other regulators of embryonic polarity [15] but genetically defined in a reverse genetic approach by isolating an induced deletion in the gene [24]. This approach is not unproblematic. If not all exons are deleted, there may be still some gene activity left. The mutation *lat-1(ok1465)* passed all classical and modern genetic tests of excluding residual activity; thus, it most likely is a null allele. The mutation has a maternal effect, which may be expected concerning its very early function at the eight-cell stage embryo. Correspondingly the mRNA of *lat-1* is present in the very early embryo. The fact that the homozygous mutation causes lethality of L1 larvae after a maternal rescue indicates that the gene also has zygotic function(s). A hallmark of all observed phenotypes is that they are not fully penetrant. Classically, this is seen as an indication that the mutation is not amorphic. However, if the *lat-1* mutation is indeed a null mutation, another attractive interpretation is that the gene serves in a "failsafe" process limiting variability in critical situations.

2.4 Oriented Cell Division Is Controlled by G Protein Signalling

The molecular mechanisms underlying the establishment of polarity in the early *C. elegans* embryo are intensely studied, but due to the complexity of the processes

involved, many are still poorly understood. Naturally, one central question that occurred after it became clear that LAT-1 is a novel component essential for the control of cell division and spindle orientation in the early *C. elegans* embryo was how the receptor mediated its function. While for the mammalian homologues of Latrophilins several ligands have been identified [32–34], LAT-1 was long considered to be an orphan receptor meaning that neither agonists nor signalling mechanisms were known. As this is the case for many aGPCRs, for very few a direct link between physiological function and signalling mechanisms has been demonstrated in vivo thus far. It was shown that for the regulation of spindle polarity, LAT-1 transduces a signal mostly via a G_s protein [29]. Biochemical as well as genetic analyses showed that via activation of this G_s protein, LAT-1 modulates intracellular cAMP levels (Fig. 3b). Consistently, cAMP concentrations in *lat-1* mutant embryos are lower than in wild-type individuals. Artificially raising cAMP levels in these *lat-1* embryos, for instance, by application of an adenylyl cyclase activator, leads to an amelioration of ABal blastomere cleavage defects showing that via LAT-1 a GPCR-dependent G protein signalling cascade is involved in oriented cell division. Further, it was shown that LAT-1 is activated by an intramolecular sequence within the GPS [29], possibly through an interaction with the seven transmembrane region (Fig. 3b) [35]. This concept of activation has already been shown for other aGPCRs [36–38] and might be a common principle for many members of this class (see also [39, 40] on this topic).

Interestingly, neither LAT-1 nor the signal immediately transduced by the receptor need to be distributed in a polarised manner to mediate this effect. Consistently, expression of a *lat-1::gfp* fusion shows no polarised distribution of the receptor in cells as seen for the components of the Wnt pathway [24]. However, it is possible that the signal is polarised further downstream of the cascade.

2.5 LAT-1 Functions in Later Embryonic Stages

There is evidence that LAT-1 function is not restricted to the ABal blastomere but also plays a role in later embryonic stages. *Lat-1* is expressed in various cells of the developing embryo [24]. Among others, this later expression is detected in pharyngeal cells. Initially it may be expressed at a lower level in most hypodermal (epidermal) cells but later shows a strong expression in the left ABarppa- and Caaa-derived hypodermal cells indicating LAT-1 function in these lineages. Several additional observations suggest additional LAT-1 functions. Phenotypes in *lat-1* mutants are not penetrant as evident by the observation that not all embryos display identical ABal cleavages and subsequent lineage fate deviations. Weak alterations of the ABal cleavage directions towards MS, as opposed to strong ones, may not cause ABala to touch MS significantly enough to cause a left-right induction [29]. Embryos also exhibit severe defects in the posterior far away from the anterior fate transformations caused by the turning of the ABal spindle. Interestingly, two embryos executed a wild-type ABal cleavage but nevertheless showed severe morphogenetic defects and developmental arrest, also supporting

the hypothesis that LAT-1 has essential functions independent of its very early activity. These functions can be manifold such as directing cell fate decisions or cell migration.

The descendants of the ABarp blastomere undergo the most extensive morphogenetic movements of all AB-derived cells to form a y-shaped structure on the dorsal side of the embryo, and they are therefore ideally suited to search for the causes of the observed morphogenetic problems in *lat-1* mutant embryos (Fig. 4). Langenhan et al. already showed that cells from the ABarp lineage, which form eight large hypodermal cells on the left and right side, each fail to move to their proper place and that two cells H2L and H2R, which are normally located on the left or right side of the embryo, stay together on the right side [24]. As the specification of left or right is based on anterior-posterior cleavages (anterior specifies left, posterior right) and LAT-1 fails to direct the ABal spindle in its proper anterior-posterior direction, one explanation for the observed phenomenon is that LAT-1, like the Wnt pathway, directs anterior-posterior decisions [24]. In another scenario LAT-1 could be involved in cell migration and cell sorting. This hypothesis is supported by occasional *lat-1* mutant embryos in which only certain anterior-posterior decisions occur, making it rather improbable that anterior-posterior fate alterations were a secondary consequence of transverse divisions.

As reported, above a certain number of *lat-1* mutant embryos survive, most probably because the ABal spindle is still (possibly by chance) in a direction preventing a significant contact of ABala to MS. Despite these occasional correct cell division orientations in the absence of LAT-1, the function of this receptor is essential for cell division plane orientation and subsequently, for establishing correct cell-cell contacts. Therefore, LAT-1 may serve in a "failsafe" system limiting variability in critical situations. How it could be involved in coordinating cell migration is a challenging question to be addressed in the future.

3 Flamingo/Celsr Are Key Components in Planar Cell Polarity Signalling

3.1 Flamingo/Celsr Homologues Are Developmental Patterning Genes

Members of the family of Celsr homologues (ADGRC1–3/CELSR1–3) were first functionally characterised following their discovery in the fruit fly *Drosophila melanogaster* by two groups in the late 1990s [41, 42]. The gene product was found to have an unusual molecular structure consisting of an extraordinarily long extracellular N terminus followed by a seven-pass transmembrane domain with similarity to members of the secretin family of GPCRs and an intracellular C terminus [41, 42]. This architecture led to the coining of the name Flamingo for the *Drosophila* homologue [42]. The receptor structure was found to be highly conserved throughout almost the entire animal kingdom [43, 44]. As the extracellular domain contains 9 Cadherin repeats, 3–5 EGF-like domains and 2 laminin G

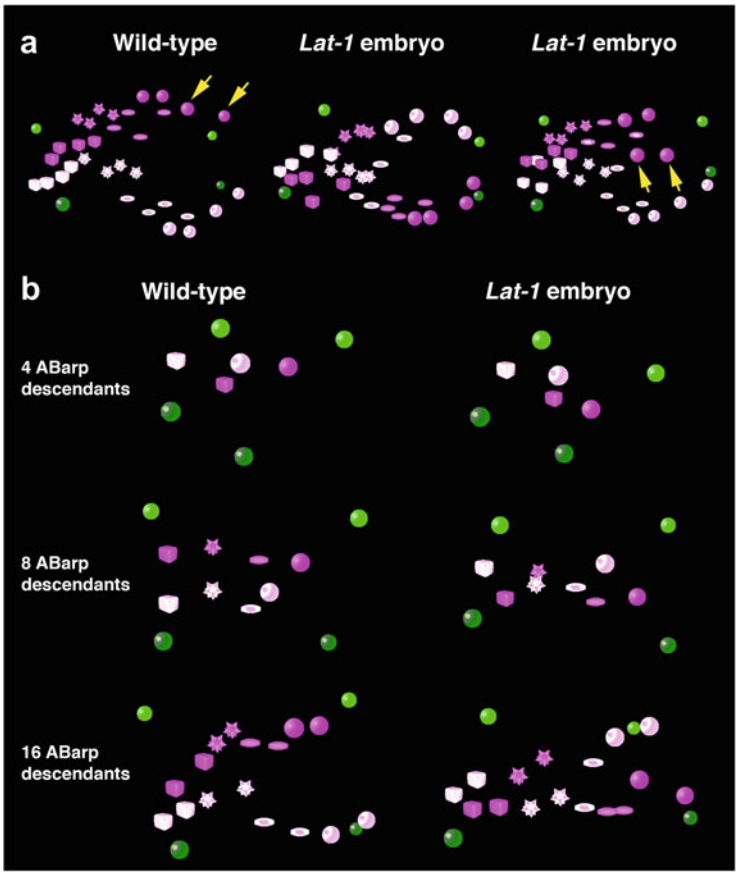


Fig. 4 LAT-1 functions in later embryonic stages. (**a**) In wild-type embryos, the descendants of ABarp form a y-shaped arrangement of 32 cells. The anterior (*left* in figure) contains some neurons, while the posterior part mostly consists of large hypodermal cells. The later left cells are derived from ABarpaa (a descends marked as *cubes*, p descendants marked as *spikes*) and ABarpap (a descends marked as *ufos*, p descendants marked as *spheres* all *white-purple* cells). The right cells are derived from ABarpap and ABarppp (*purple* cells), and the shapes follow the same system as the left cells. The *light green* cells are the most anterior and posterior descendants of ABpra, the *dark green* cells those of ABpla. These cells define the left-right axes of the embryos. In the *lat-1* embryo 19 cells of left or right identity exchanged their positions in a mosaic fashion. These may be explained by an exchange of the anterior-posterior polarities during the ABarpa and ABarpp cleavages. The *lat-1* embryo 41 shows a displacement of the two most posterior hypodermal cells on the *right side* (they moved up in a parallel position to their cousins *arrows*). These positions are consistent with the assumption that both mothers have an anterior fate. Since all cell divisions occurred properly on the anterior-posterior axis, one had to postulate that LAT-1 is like the Wnt pathway not only involved in specifying the cell cleavage directions but also in the specification of cell fates. (**b**) In wild-type embryos, descendants of ABarp with left identities are born to the right of their homologues with right identities but exchange immediately their positions to move into their final positions. In the *lat-1* embryo 19, only two cells exchanged their positions, while the others move to the wrong side. R. Schnabel, unpublished data

motifs, the name Cadherin EGF LAG seven-pass G-type Receptor (Celsr) was adopted in parallel to Flamingo for the human and mouse homologues [45–47] (Fig. 1; see also [48]).

The first clue as to the function of Fmi-Celsr homologues came through the analysis of *Drosophila* mutant phenotypes. Loss of *fmi* gene function leads to a disruption of the polarity of cuticular structures of the adult fly [41, 42, 49]. This phenotype is strikingly consistent with a loss of function of the Frizzled-dependent "core" planar polarity pathway and led to the idea of Flamingo/Celsr homologues being developmental patterning genes. Planar polarity (planar cell polarity, PCP) refers to the coordinated polarisation of cells in the plane of a tissue (commonly an epithelial sheet) orthogonal to the apicobasal axis of the tissue [50] (Fig. 5a, b). The core planar polarity pathway (or simply "core pathway") is one of the major developmental regulators of planar polarity throughout the animal kingdom

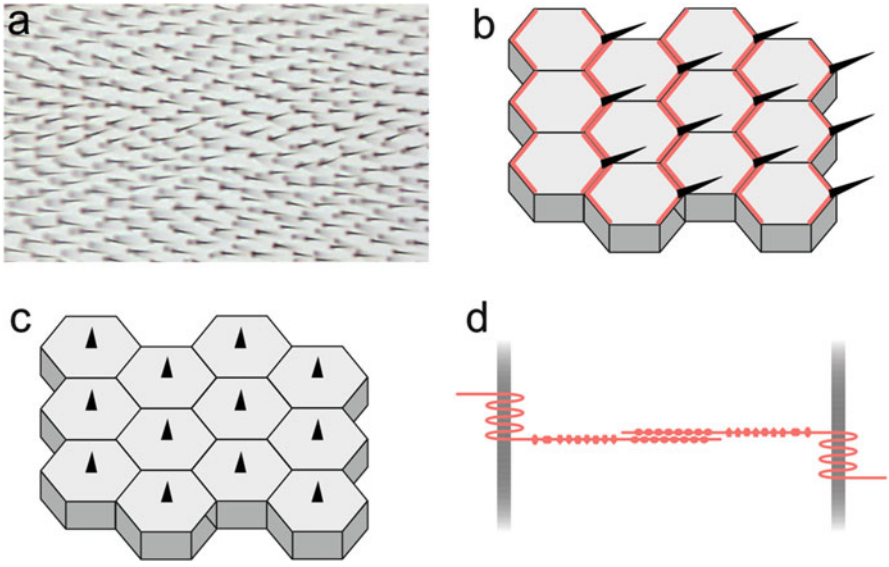


Fig. 5 *Drosophila* Flamingo and planar polarity specification in the wing. (**a**) Image of hairs (trichomes) on the surface of the adult wing of *Drosophila* (distal to the right). In wings of wild-type animals, each cell produces a single hair that projects distally, showing alignment of cellular polarity both with the overall proximodistal axis of the wing and with each neighbouring cell (D. Strutt, unpublished data). (**b**) Cartoon of cells in a wild-type wing at the time of trichome production, showing distally pointing trichomes (*black*) emerging from the distal vertex of each cell. Localisation of Flamingo protein is indicated in *red*, at the level of apicolateral junctions and enriched to both proximal and distal cell edges. (**c**) Cartoon of cells in a wing lacking Flamingo protein at the time of trichome production, showing production of a trichome in the centre of the apical surface of each cell, indicating loss of cell polarity cues. Note that subsequently in development, these mispolarised trichomes will adopt a swirling pattern. (**d**) Cartoon showing homophilic binding of two Flamingo molecules (*red*) between junctions of adjacent cells. Homophilic binding is dependent on the presence of the Cadherin repeats; however, the exact binding interfaces have not been mapped. See main text for respective references

[43, 50]. In *Drosophila* the loss of its activity leads to defects in the polarity of a variety of epidermal derivatives such as the hairs (also known as trichomes) on the surface of the wing (Fig. 5b, c), the orientation of bristles on the back and abdomen and the orientation of photoreceptor clusters in the compound eye.

It had long being recognised that the specification of planar polarity during tissue development requires the transmission of polarised signals between cells and that planar polarity genes were likely to encode proteins involved in such cell-cell signalling [51, 52]. However, little was known regarding the underlying mechanism. In this respect, the molecular characterisation of Fmi protein localisation and behaviour in *Drosophila* was a major step forward.

Generation of antibodies against Fmi revealed that during the process of cell polarisation in the fly wing, the Fmi protein became asymmetrically distributed within cells, being found at proximodistal cell junctions [42] (Fig. 5b). Further biochemical and genetic evidence indicated that Fmi bound homophilically to itself, with distally localised molecules binding to proximally localised molecules in the neighbouring cell (Fig. 5d).

This pattern of localisation generates a puzzle: Fmi is required for cell polarisation, such that each cell in the wing can distinguish its distal side from its proximal side, yet the localisation of the protein is equal at both distal and proximal cell edges. To address this, and based on elegant genetic and molecular analyses, Usui et al. proposed that through interactions with other planar polarity proteins, Fmi would exist in two forms with different activities on each side of the proximodistal cell boundaries and would be involved in feedback amplification of initially small differences in cell polarity in order to generate a robust polarity cue [42]. Furthermore, the ability of Fmi to link adjacent cells through homophilic binding provides an elegant mechanism for cell-cell coupling of planar polarity.

3.2 The Role of Flamingo/Celsr Activity in the Core Planar Polarity Pathway

Following the discovery of the asymmetric cellular localisation of Fmi in the developing *Drosophila* wing, a series of studies quickly followed showing that such asymmetric localisation was a hallmark of all the protein products of the genetically defined components of the core planar polarity pathway (reviewed in [53]). Furthermore, unlike Fmi which is found concentrated at both proximal and distal cell edges, each of the other components was found to preferentially localise either proximally or distally (Fig. 6a). Similar patterns of subcellular asymmetry for the core proteins were also found to occur in other *Drosophila* tissues such as the developing eye, suggesting that these polarised localisations are a general property of the core pathway components.

Consistent with the genetic requirement for activity of the core pathway components for correct planar polarisation of adult cuticular structures, the activity of each protein is also required for the asymmetric subcellular localisation of all the other core proteins (Fig. 6a–d). This supports the view that the core proteins all act

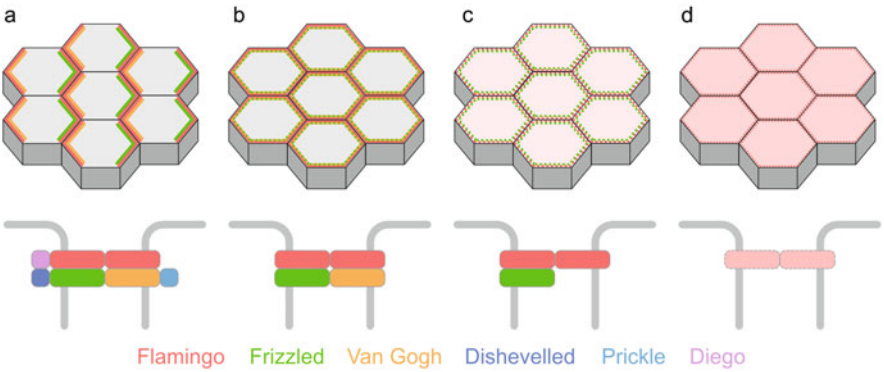


Fig. 6 Localisation of Flamingo and the core planar polarity proteins in the developing wing. (**a**) In a wild-type wing prior to trichome formation, the core planar polarity proteins are asymmetrically localised along the proximodistal axis in the apicolateral region of wing cells. Fz, Dsh and Dgo are localised at distal cell edges (all three represented in *green* in cartoon at *top*), where they associate with Fmi (*red*). Distally localised Fmi forms homophilic adhesions with Fmi molecules at the proximal edge of neighbouring cells, which are associated with proximally localised Vang and Pk (represented in *orange* in cartoon at *top*). (**b**) In the absence of the cytoplasmic core proteins, Fz, Vang and Fmi still tightly localise to the apicolateral junctional region but are no longer asymmetrically distributed on the proximodistal cell axis. (**c**) In the absence of Vang (with or without the cytoplasmic complex components), Fz and Fmi still localise largely to apicolateral cells junctions. Fz-Fmi preferentially bind homophilically to Fmi in neighbouring cells, although a small fraction of Fmi is also seen in apical cell membranes. The formation of Fz-Fmi:Fmi intercellular complexes appears to be the first symmetry breaking step in planar polarisation. (**d**) In the absence of Fz and Vang (with or without the cytoplasmic complex components), Fmi shows only weak homophilic junctional binding and is largely seen localised to apical cell membranes. See main text for respective references

together as part of a common molecular machinery for the planar polarisation of cells and suggests that it is the asymmetric localisation of the proteins themselves that signals the production of correctly polarised cuticular structures such as the trichomes of the wing. This latter hypothesis was validated by the subsequent discovery that a group of downstream "effector" proteins, which act on the cytoskeleton to produce polarised wing trichomes, are themselves asymmetrically localised within wing cells under the control of the core pathway components (reviewed in [53]).

Taking together the studies to date examining core protein behaviour, it is possible to construct a hierarchy of core protein function, with Fmi intercellular homodimers lying at the top, providing the central function of linking the localisation of core proteins in adjacent cells. As already noted, in common with most other cadherins, Fmi acts as a junctionally localised homophilic binding molecule, with Fmi in one cell binding to Fmi in adjacent cells, in a manner dependent on the presence of the extracellular cadherin repeats [42, 54]. Notably, this preferential homophilic junctional binding of Fmi is not an intrinsic property but depends on the presence of two of the other core proteins, the seven-pass transmembrane protein Frizzled (Fz) and the four-pass transmembrane protein

Van Gogh (Vang, also known as Strabismus [Stbm]) [55]. In the absence of both Fz and Vang, the majority of Fmi appears to be localised to apical cell membranes where it cannot bind homophilically to molecules in neighbouring cells (Fig. 6d) and from where it is subject to high rates of endocytic turnover [55, 56].

Mutual dependency between Fmi, Fz and Vang for junctional localisation is further evident in that in the absence of Fmi, Fz fails to show junctional localisation [57] and similarly Vang junctional localisation is noticeably compromised [58]. The promotion of these junctional localisations is believed to depend on direct protein-protein interactions between Fmi and Fz and Vang. Specific binding interfaces between Fmi and its putative partners have not been mapped, in part due to the inherent difficulties of carry out biochemical studies with large multipass transmembrane proteins. Nevertheless, the coincidence of their junctional colocalisation, their mutual dependence for such localisation and a number of cell culture and co-immunoprecipitation studies with both *Drosophila* and vertebrate homologues [55, 59, 60] lend support to the view that their interactions might be direct.

Interestingly, Fmi appears to preferentially form homophilic junctional complexes when Fz is only present in one of the two adjacent cells (Fig. 5c), consistent with the formation of a complex consisting of Fz-Fmi in one cell with Fmi in the neighbouring cell being the first symmetry breaking step in planar polarity complex formation [55]. Taken together with other evidence, this has led to the view that by association with different binding partners (in particular Fz and Vang), Fmi exists in two states, a "distal" form and a "proximal" form, which have different binding affinities both for each other in adjacent cells and for other partners in the same cell [55, 59].

Following this initial Fz-dependent symmetry breaking step that leads to formation of asymmetric complexes of the form Fz-Fmi:Fmi-Vang between the adjacent cells, the next key activity of the core proteins is the asymmetric distribution of these complexes at the cellular level (Fig. 6a). This requires the activity of a group of cytosolic core pathway components, notably Dishevelled (Dsh) which is recruited into the complex most likely by direct interactions with Fz [61, 62], Prickle (Pk) which interacts with Vang [58, 63] and Diego (Dgo) which is recruited in a Fz- and Dsh-dependent manner [64, 65]. The exact mechanism by which the core proteins mediate their own asymmetric distribution within cells is a subject of active investigation, but a large body of evidence supports the view that feedback interactions between core protein complexes lead to the amplification of initially small asymmetries to produce the final robust patterns of polarised subcellular localisation (reviewed in [4, 53, 66]) (Fig. 7a). The exact source of the initial small asymmetries has been (and continues to be) a subject of some controversy, although the likely situation is that both within and between different tissues, multiple cues play a role (reviewed in [4]).

This role of Fmi-Celsr proteins as key components of asymmetrically distributed intercellular protein complexes that mediate planar polarisation of cell sheets has been confirmed by numerous studies in vertebrates (reviewed in [4, 50]), and

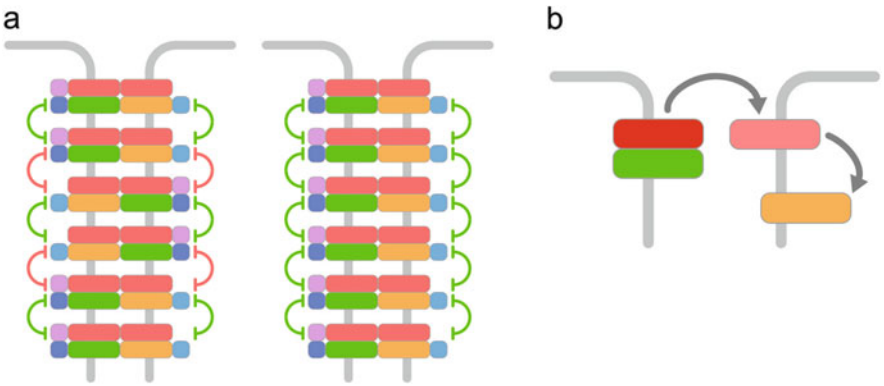


Fig. 7 Feedback models for amplification of asymmetry and possible mechanisms of Flamingo action. (**a**) Amplification of cellular asymmetry and the distribution of Fz and Vang containing complexes to opposite cell ends are believed to depend on feedback mechanisms that locally sort core protein complexes into polarised domains. On the *left* shows a situation where complexes are poorly sorted and asymmetry is low. It is believed that local interactions between complexes might lead to mutual stabilisation of complexes of the same orientation (*green* interactions) and destabilisation of complexes of opposite orientation (*red* interactions), leading to the situation shown on the *right* where complexes are locally sorted into domains of common orientation. Feedback interactions that promote stability might function by increasing cis multimerisation or transhomophilic binding between neighbouring Fmi molecules, whereas destabilising interactions might block cis- or trans-Fmi interactions or promote endocytosis of Fmi. (**b**) Model indicating that Fz-Fmi in one cell might act as a ligand for Fmi in a neighbouring cell, "activating" Fmi and causing it to interact with Vang, for instance, via a conformational change. See main text for respective references

functions in planar polarisation are also likely in other invertebrate groups (see [43]). In particular, studies in epithelia such as those of the inner ear, skin, airways and oviduct have revealed similar patterns of subcellular asymmetric localisation of Celsr homologues and other core planar polarity proteins and also evidence of mutual dependency of core protein function for this localisation and for planar polarisation of the tissue [60, 67–69].

To date, overlapping activities of the three Celsr homologues found in vertebrates have been implicated through genetic studies in knockout animals in a plethora of developmental processes (reviewed in [70, 71]). Based on either evidence for planar polarisation of the tissue, or the requirement for other components of the core planar polarity pathway, many of these seem likely to be genuine planar polarity functions, dependent on polarised cell-cell communication mediated by asymmetrically distributed core protein complexes: examples include polarised growth of hair follicles or polarised positioning of cilia in tissues such as the airways or oviduct (as noted above) but also polarised cell movements such as seen in gastrulation (including neural tube closure) [72], wound healing [73] and commissural axon guidance (reviewed in [74]).

However, it should be noted that in both *Drosophila* and vertebrates, there are examples of Fmi-Celsr functions that are either not evidently related to planar polarisation of tissues, which do not require the activity of the other core planar polarity proteins and/or which do not appear to require homophilic interactions between Fmi/Celsr molecules in neighbouring cells. In *Drosophila*, these include roles in dendritic tiling [54, 75] and photoreceptor axon spacing and targeting [76, 77]; and in vertebrates some roles in gastrulation movements [78, 79] or forebrain patterning [80] appear to be core pathway independent. While these alternative functions of Fmi/Celsr are of great interest, the existing lack of knowledge regarding the molecular mechanisms means that they currently add little to our overall understanding of Fmi/Celsr biology.

3.3 Molecular Mechanisms of Flamingo/Celsr Function

The large size and multidomain structure of Fmi-Celsr proteins hint at perhaps multiple functions for these molecules in various developmental processes, a view supported by the observation that mutations affect both planar polarity and non-planar polarity processes. Even regarding the better characterised functions in planar polarity, studies of the molecular mechanisms of Fmi-Celsr function are still in their infancy and in large part have been limited to simple structure-function analyses. Although belonging to the family of aGPCRs, no G protein-dependent function has been shown. Generally, very little is known about whether Fmi-Celsr proteins can signal via similar mechanisms to G protein-coupled receptors or whether alternatively (at the opposite extreme) they simply act as passive protein scaffolds onto which other signalling active proteins can assemble.

So far no specific functions in planar polarity have been assigned to the EGF-like domains, laminin G motifs or GPS region in the extracellular domain. However, in *Drosophila*, immunoblotting using antibodies against the Fmi protein shows that species of different molecular weights are present, consistent with the GPS providing a functional cleavage site [42].

Consistent with being aGPCRs, the extracellular cadherin repeats are required for homophilic adhesion interactions [42, 54], and this activity appears to be strongly modulated by the partner proteins Fz and Vang [55]. Intriguingly, similar to a construct lacking the cadherin repeats, a deletion construct lacking the hormone binding domain is non-functional when overexpressed [54], perhaps indicating a key role for the hormone binding domain in mediating cadherin-dependent homophilic adhesion. Overexpression of full-length Fmi in the *Drosophila* wing gives a phenotype similar to loss of Fz activity [42] in which cells repolarise towards the region of overexpression, and under these conditions, excess Fz is seen recruited to cell junctions [55] and Vang to the junctions of neighbouring cells [59]. Additionally, the intracellular domain of Fmi is not required for the overexpression phenotype to be seen [54, 55, 59], and if a form of Fmi lacking the intracellular domain is expressed at close to physiological levels, it

preferentially localises at distal cell edges with Fz and appears to be compromised in its ability to interact with Vang [55]. In this context, it is notable that normally Vang only appears to have strong interactions with Fmi homodimers if they are already bound to Fz in the neighbouring cell [55]. Conversely, overexpression of a form of Fmi lacking almost all the extracellular regions gives a phenotype similar to loss of Vang activity whereby cells repolarise away from the region [54].

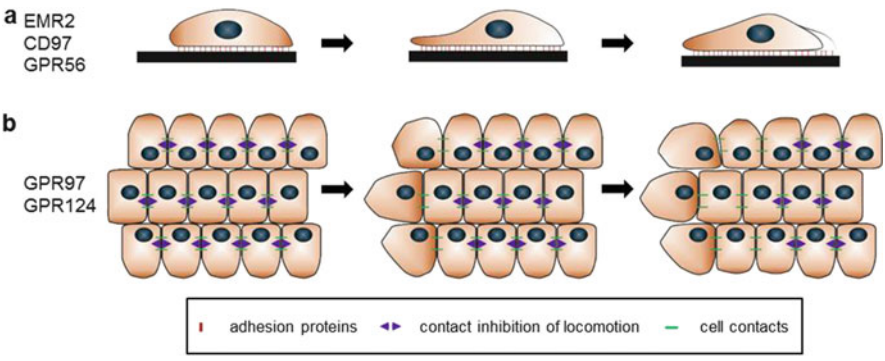
Taken together, these results suggest that Fmi has critical interactions with Fz that require either the extracellular or transmembrane regions, but not the intracellular domain. Furthermore, Vang interactions are promoted by (but not are not absolutely dependent on) an activity conferred by the intracellular tail of Fmi, and these interactions are stimulated by interactions with Fz-Fmi complexes in the neighbouring cell [55] (Fig. 7b). As it is generally assumed that the intracellular tail of GPCR-like molecules such as Fmi would be required for intracellular signalling activity, it is tempting to speculate that a Fz-Fmi complex in one cell acts as a ligand for a Fmi molecule in the neighbouring cell and induces a signalling event that leads to recruitment of Vang. The nature of such a signalling event is unclear, but by analogy to other GPCRs, it could involve a conformational change that exposes a binding site. Furthermore, in zebrafish, the intracellular domain of a Celsr homologue is able to affect planar polarised convergent extension movements in a manner dependent on a conserved cluster of potential serine-threonine phosphorylation sites, suggesting that an unknown kinase may be involved in Fmi/Celsr activation [78].

There is limited evidence that any signalling activity of Fmi-Celsr is dependent on these molecules acting as guanine nucleotide exchange factors (GEFs) for heterotrimeric G proteins in the normal manner of GPCRs. However, in rat brain slice culture, Celsr2 and Celsr3 can cause calcium release in phospholipase C-dependent manner in response to exogenously applied Celsr cadherin repeats bound to beads [81]. This supports the view that Fmi-Celsr molecules can act as homophilic ligands to activate their own signalling activity. Identification of a requirement for heterotrimeric G proteins would be strong evidence that such a mechanism was involved in planar polarity activity, but given the broad roles of such molecules in developmental and physiological signalling events, a specific function in planar polarity might be masked.

In addition to forming Fz-Fmi:Fmi-Vang intracellular complexes, planar polarisation requires the sorting of these complexes into locally polarised domains [56]. Plausible mechanisms underlying the feedback interactions that mediate such sorting include the modulation of dimerisation activity between complexes or regulation of endocytosis and trafficking of complex components (Fig. 7a). Interestingly, zebrafish Celsr2 has been shown to be able to dimerise in cis in a manner that does not require the complete seven-pass transmembrane region or intracellular domain [78], but how such dimerisation could be modulated to regulate planar polarisation is unclear. Similarly, mouse Celsr1 can be specifically internalised at the cell surface in response to cell cycle-dependent phosphorylation by Polo-like kinase 1 [82, 83], but again no role for this mechanism in sorting of planar polarity complexes is known.

3.4 Emerging Roles of Adhesion GPCRs in Cell Migration

The concept of polarity is not only essential for the development of epithelia and organs but is also a central element in cell migration. Migratory processes are essential for development, wound healing or immune responses and can lead to tumour formation when misregulated [84]. Naturally, cell migration belongs to the most important and tightly controlled biological mechanisms in multicellular organisms. In general, two types of migration can be distinguished: single cell migration and collective cell migration (Fig. 8a, b). However, the basic mechanisms underlying both processes are similar. Upon receiving a migration-promoting signal, a cell polarises by forming protrusions called lamellipodia or filopodia caused by actin polymerisation. By developing adhesion contacts to components of the cell's environment such as the extracellular matrix or surrounding cells, the lamellipodia or filopodia of the migrating cells get stabilised. Subsequently,



c

Adhesion-GPCR	tissue / cells involved in migration	Single cell/ Collective cell Migration	Reference
ADGRE2/EMR2	activated leukocytes	single	[86]
ADGRE5/CD97	activated leukocytes	single	[85]
ADGRG1/GPR56	radial glia cells and other neural progenitor cells	single	[90]
ADGRG3/GPR97	mouse lymphatic endothelium	collective	[95]
ADGRA2/GPR124	central nervous system	collective	[94]

Fig. 8 Schematic illustration of single cell and collective cell migration and the involvement of aGPCRs in these processes. (**a**) Single cell migration. After a migration-promoting signal, a cell forms protrusions generally driven by actin polymerisation. By forming new adhesion contacts to the environment of the cell, these protrusions are stabilised. A traction force translocates the cell over the substrate. In a cell layer migration is inhibited by contact inhibition of locomotion signals. After a migration-promoting signal, cells polarise at the edge of the free space. By exerting attractive force between neighbouring cells, the contact inhibition of locomotion is resolved and thus coordinates movement of the cell layer. (**b**) Collective cell migration displays the same underlying mechanisms as single cell migration but in a cellular environment. (**c**) Several aGPCRs are involved in cell migration

actin- and myosin-dependent contractions lead to a traction force which translocates the cell and retracts it from its contact (Fig. 8a, b) [85, 86].

Given that cell migration is highly dependent on the ability of cells to form adhesive contacts, it was not surprising to find that several aGPCRs are implicated in migratory processes. Group II was the first one to be linked with migration (Fig. 8c). Members of this group are very prominent in immune cells [87] and have been shown to be involved in single cell migration of various types of these cells. Single cell migration is an important mechanism in immunity, which allows immune cells to invade into inflamed tissue in a rapid manner and evoke an immune response [85, 88]. Expression of ADGRE2/EMR2 and ADGRE5/CD97 is tightly linked to activation of leukocytes [89, 90] and increased migration of these immune cells [90, 91]. While EMR2 shows a redistribution to the leading edge of activated leukocytes similarly to Rac, a redistribution of CD97 within the activated leukocytes was not observed [90, 92]. However, the details of the molecular mechanisms underlying the involvement of group II aGPCRs in immune cell migration remains to be determined. First steps towards an understanding of their signalling mechanisms revealed that C-terminally truncated versions of CD97 as well as EMR2 showed no significant migration to a chemotactic agent, whereas the full-length receptors showed migration towards a chemoattractant. This sheds light on possible signal transduction via the Rho family of small GTPases, which are a central player in migratory processes [85, 90, 93].

Besides immune cell migration, aGPCRs are also implicated in the migration of neurons. An example for inhibiting migratory processes of this cell type is ADGRG1/GPR56 which is expressed in migrating neurons [94, 95] (Fig. 8c). By interacting with collagen III, the receptor suppresses migration of neurons in the developing cerebral cortex [96]. In mice lacking *Gpr56*, neurons overmigrate beyond the pial basement membrane which leads to cerebral cortex malformation. This phenomenon is linked to bilateral frontoparietal polymicrogyria (BFPP) in humans, which is a recessive inherited development disorder of the cerebral cortex [94, 96, 97].

There is also evidence for aGPCRs playing roles not only in single cell but also in collective cell migration. The collective migration process, in which cells connected by cell-cell contacts move together as a tissue, often takes place in embryogenesis and wound healing [86]. Interestingly, Flamingo/Celsr are implicated in collective cell migratory processes such as gastrulation [72] and wound healing [73] as part of their PCP function (described above). Other prominent examples for the involvement of an aGPCR in collective cell migration are ADGRG3/GPR97 and ADGRA2/GPR124 (Fig. 8c). *Gpr124* is highly expressed in the endothelium of the central nervous system (CNS) [98]. Mice lacking *Gpr124* show severe CNS haemorrhage restricted to the forebrain but not in the mid- or hindbrain [98]. Further, it was shown that GPR124 regulates CNS angiogenesis in a cell-autonomous manner in vivo and in vitro. A brain endothelial cell line expressing *Gpr124* exhibits a directional migration towards gradients of conditioned medium of forebrain cortical cells in a Cdc42-dependent manner [98]. Again, the signals mediated by the receptor remain elusive. GPR97 is

expressed in lymphatic endothelial cells (LEC) (Fig. 8c). Interestingly, an enhanced single cell migration but a decreased collective cell migration was observed in these cells in vitro upon silencing of *Gpr97* [99]. As single cell migration can progress into collective cell migration, it is very possible that GPR97 acts as a linchpin between both migratory systems. Further analyses indicate that GPR97 is directly involved in migration without an intermediary soluble factor. Changes in the actin cytoskeleton and focal adhesions are possibly caused by GPR97 through influencing the balance between the small G proteins RhoA and Cdc42 [99].

Taken together, several aGPCRs have a role in migratory processes. Although details of the signalling mechanisms underlying their functions are yet to be elucidated, preliminary data indicate that they probably are directly involved in mediating cell adhesion. Especially for group II aGPCRs, the EGF domains in their N termini are potentially distinctive for the formation of adhesion contacts [87, 100]. Although signalling via heterotrimeric G proteins has not been shown for any of these receptors and cannot be excluded, there is evidence that the aGPCRs involved in migratory processes signal via members of the Rho family of small GTPases. These proteins cause cytoskeletal changes and focal adhesion rearrangements [88, 99].

4 Conclusion

Polarity in multicellular organisms is a fundamental concept for the development of tissues and organs but also for maintaining biological processes such as immune responses or wound healing. Due to the highly complex and tightly regulated processes ensuring the correct establishment and maintenance of polarity, the details on how they are governed especially on a molecular level are not fully understood.

Over the past years, intense research has revealed that aGPCRs play essential roles in various types of polarity. Interestingly, the members of the two evolutionarily oldest groups, Latrophilins and Flamingo/Celsr, are involved in tissue polarity. While for Flamingo/Celsr it has been shown that vertebrate homologues of this aGPCR group have similar functions to *Drosophila* Fmi, for homologues of the Latrophilin group, this has not yet been the case. Vertebrate Latrophilins are implicated in synaptic functions [25, 26, 34], but no role in establishing or maintaining polarity has been assigned to any of the three mammalian Latrophilin homologues (ADGRL1–3). It can be speculated that the reason for this is that due to the complexity of the mammalian system together with possible redundancy regarding the function of the different homologues, analyses of these receptors are still incomplete and some of their functions have not yet been discovered. It is, however, also conceivable that due to the existence of so many more aGPCRs in mammals (33 in humans compared to 3 in *C. elegans* [101, 102]), the invertebrate receptors have different roles than their vertebrate homologues.

Similarly there are major gaps in our knowledge regarding the actual mechanisms of signalling by aGPCRs in polarity processes. It has been shown for

several receptors of this family that they do have a function in these processes, but the details and the impact of their individual roles need further investigation. Especially the molecular details underlying their physiological functions remain elusive. It is quite possible that several if not all receptors follow similar mechanisms of signalling such as activation by an intramolecular agonist or transmission of signals through heterotrimeric or small G proteins. In particular for members of the group of Flamingo/Celsr, which have been characterised in great detail regarding their function in PCP, this information would be invaluable in understanding how they mediate polarity and also if and how their function in PCP is related to the one in migratory processes. Thus, future analyses need to focus on further clarifying the mechanisms of activation as well as signalling cascades of aGPCRs involved in the different types of polarity.

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Adhesion GPCRs as Novel Actors in Neural and Glial Cell Functions: From Synaptogenesis to Myelination

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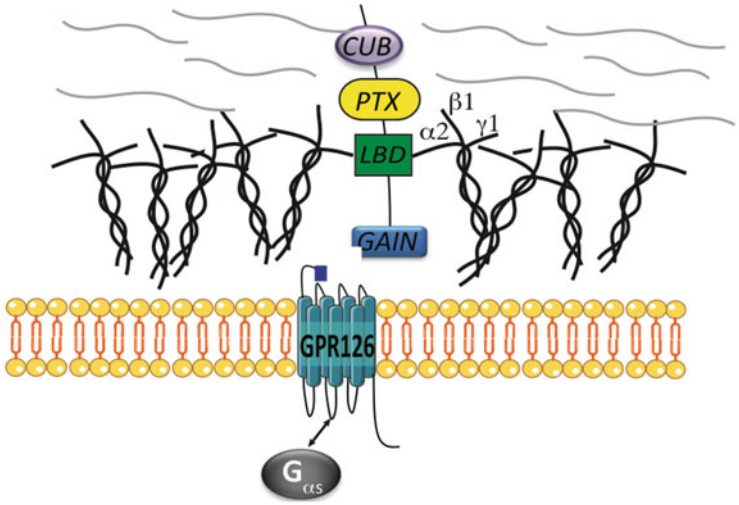
Graphical Abstract



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Contents

Abstract

Adhesion G-protein-coupled receptors (aGPCRs) are emerging as key regulators of nervous system development and health. aGPCRs can regulate many aspects of neural development, including cell signaling, cell-cell and cell-matrix interactions, and, potentially, mechanosensation. Here, we specifically focus on the roles of several aGPCRs in synapse biology, dendritogenesis, and myelinating glial cell development. The lessons learned from these examples may be extrapolated to other contexts in the nervous system and beyond.

Keywords

Adhesion GPCR • Neurodevelopment • Synaptogenesis • Dendritogenesis • Myelination

1 Introduction

The nervous system contains a multitude of complex cell-cell and cell-environment interactions. Neural cells—neurons and glia—utilize a battery of electrical, chemical, and mechanical signal transduction methods to rapidly transmit information across the nervous system and to other cells throughout the organism, often over very long distances. To do this, neurons and glia maintain close associations with one another to allow constant communication within individual cells, between cells and their extracellular matrix (ECM).

The adhesion G-protein-coupled receptor (aGPCR) family regulates many cellular processes in a wide variety of cell types and contexts [1]. Despite being the second largest family of GPCRs, aGPCRs are relatively understudied, in part owing to their complex structure. aGPCRs are defined by a large extracellular N-terminus and a GPCR autoproteolysis-inducing (GAIN) domain that can cleave the receptor at the GPCR proteolysis site (GPS) into an N-terminal fragment (NTF) and a 7-transmembrane (7TM)-containing C-terminal fragment (CTF) during the maturation process [2, 3]. The NTF and CTF are thought to remain non-covalently

attached at the cell surface [2, 4]. In addition to their characteristic autoproteolytic cleavage, aGPCRs are distinct from other GPCR families in that their N-termini often contain protein domains that are classically associated with cell-cell or cell-ECM adhesion, hence the name "adhesion GPCR" (see also [5, 6]). There are a total of 33 aGPCRs in humans, 31 in mice and rats [7], and over 50 in zebrafish [8]. In vertebrates, there are nine distinct subgroups of aGPCRs that can be separated based on the phylogeny of their 7TM regions [7] (see also [9, 10]).

This chapter will focus in detail on the roles of aGPCRs in two specific areas of neurobiology: synapto-/dendritogenesis and myelination. However, it is important to note that aGPCRs have proven to be critical for many different aspects of nervous system development including, but not limited to, cortical lamination and blood-brain barrier formation [11–13]. For an exhaustive overview of mammalian aGPCRs and their known functional roles, please refer to other chapters of this book and [1].

2 Adhesion GPCRs in Synaptogenesis and Dendritogenesis

Neurons communicate with each other at synapses, specialized junctions that ensure the transfer of neuronal information. Numerous types of neurons and synapses exist, characterized by different morphologies and properties, depending on their position in the network. Among the nine subfamilies of aGPCRs, three have come to light for their roles in synaptogenesis and/or dendrite morphogenesis: latrophilins (ADGRLs/LPHNs), Flamingo/cadherin, EGF-like, LAG-like, and seven-pass G-type receptors (ADGRCs/Fmi/CELSRs) and brain-specific angiogenesis inhibitors (ADGRBs/BAIs).

2.1 LPHN1 and LPHN3 Regulate Synapse Formation and Function

The LPHN subfamily is composed of three members, *LPHN1–3* [14]. Unlike LPHN2, LPHN1 and LPHN3 are highly enriched in the brains of zebrafish, rodents, and humans [8, 15, 16]. In rodents, expression of *Lphn1* and *Lphn3* is high until the third postnatal week and then decreases in older animals, suggesting a role during postnatal brain development [17]. Indeed, various studies have demonstrated that LPHN1 and LPHN3 regulate synaptic development and function, through their interactions with multiple partners.

2.1.1 LPHN1's Interaction with NRXs May Regulate Synapse Function

Originally identified as the endogenous receptor of α-latrotoxin, a component of black widow spider venom, LPHNs act as co-receptors for the neurotoxin together with the presynaptically localized single-pass transmembrane neurexins (NRX-1α, NRX-1β, NRX-2β, and NRX-3β) [15]. LPHNs bind to α-latrotoxin in a calcium-independent manner, whereas NRXs bind it in a calcium-dependent manner.

Disruption of LPHN1 and/or NRX-1α in mice confirmed that the two receptors bind α-latrotoxin independently but act cooperatively to regulate the effect of α-latrotoxin on neurotransmitter release [18]. Furthermore, LPHN1 interacts at high affinity with NRX-1α and NRX-1β, through its olfactomedin (Olf) domain, to form a transcellular adhesion complex in a calcium-dependent manner (Fig. 1). Together with the fact that LPHN1 co-enrich with postsynaptic markers in biochemical preparations [18], this suggests that LPHN1/NRX1 could produce a trans-synaptic contact between neurons [16].

2.1.2 LPHN1 Interacts with TEN2 to Stabilize Synapses and Induce Neurotransmitter Release

Other endogenous binding partners for LPHNs are the teneurins (TENs), single-pass transmembrane glycoproteins encoded by four different genes *(Ten1–4)*. In the rat brain, TEN2 binds with high affinity to LPHN1 and low affinity to LPHN2, but has no affinity for LPHN3. The interaction between LPHN1 and TEN2 was shown to mediate intercellular neuronal contact in a calcium-independent manner [16, 20]. In addition, in an artificial synapse formation assay, presynaptically enriched LPHN1 induced postsynaptic specialization in neurons, whereas the postsynaptically enriched TEN2 influenced axon-target interaction (Fig. 1) [20]. LPHN1 thus seems to regulate synapse stability and function through its interaction with TEN2. Similarly, TEN1 and TEN3 both interact with LPHN3 [21], although a specific role for these interactions in synapses has not yet been demonstrated.

2.1.3 LPHN/FLRT Trans-synaptic Complexes Regulate the Strength and Density of Synapses

Another group of LPHN interacting partners is the fibronectin leucine-rich transmembrane (FLRT) proteins, as shown by affinity purifications from rat synaptosome extracts using the LPHN3 or FLRT3 extracellular domain [21]. FLRT3 is a postsynaptic protein partially co-localizing with the postsynaptic scaffolding protein PSD95 at glutamatergic but not GABAergic synapses. While LPHN3, like LPHN1, is sufficient to induce postsynaptic differentiation, FLRT3 is not sufficient to induce presynaptic differentiation. However, *Lphn3* or *Flrt3* knockdown experiments in hippocampus, dentate gyrus, or cerebral cortex reveal a decrease in the strength and number of synapses, as well as a decrease in glutamatergic transmission, sometimes accompanied by a reduction in the number of dendritic spines [21, 23]. These results suggest that the LPHN3-FLRT3 complex positively regulates synaptic development and function.

In vertebrates, while NRXs are expressed ubiquitously, FLRTs and TENs are expressed in a cell-specific manner that is complementary and nonoverlapping [21]. Therefore, only certain combinations of LPHNs and FLRTs or TENs seem to be present at any given synapse, suggesting their role in the specific formation of different neuronal circuits. Indeed, *lphn3*-knockdown zebrafish larvae are reported to have defects in dopaminergic neurons, which control locomotor activity and impulsivity. This phenotype is reminiscent of *Lphn3* mutant mice, which are

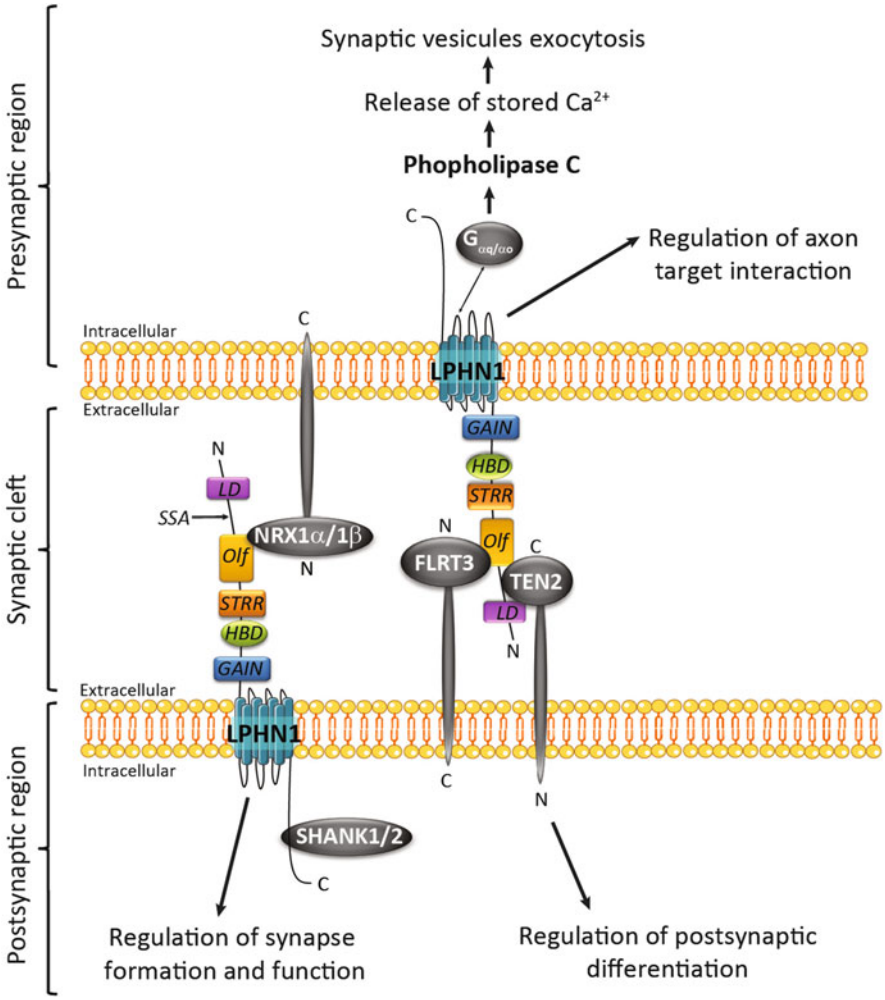

Fig. 1 Structure and functions of LPHN1 at the synapse. The extracellular N-terminal fragment (NTF) of latrophilins contains a rhamnose-binding lectin domain (RBL) [19], a central olfactomedin-like domain (Olf), a serine/threonine-rich region (STRR), and a hormone-binding domain (HBD), in addition to the characteristic GAIN domain containing the autoproteolytic GPS motif. The 7TM domain is followed by a long cytoplasmic tail where most of the alternative splicing occurs. One splicing site, referred as SSA, is also present between the lectin and olfactomedin domains in the N-terminal region. LPHN1 interacts extracellularly with neurexin1α and 1β (NRX1α/1β) [16], Teneurin 2 (TEN2) [20], and FLRT3 [21] and intracellularly with $G\alpha_q/\alpha_o$ [22] and the postsynaptic scaffolding proteins SHANK1/2 [17, 18]

hyperactive and display increased sensitivity to a locomotor stimulant. In both species, the defects can be reversed by ADHD medication [24].

Although LPHNs are conserved in invertebrates, their roles in synaptogenesis have not yet been demonstrated in these models. However, the *C. elegans* homolog

of LPHN1, LAT1, was shown to be involved in the modulation of neurotransmitter release in the pharyngeal nervous system [25], so some functions may be conserved. Interestingly, invertebrate LPHN homologs lack the Olf domain, which is required for forming the LPHN-NRX complexes as well as the functions of LPHN/FLRT and LPHN/TEN complexes in synapses [16, 23]. The Olf domain is absent in LPHNs in invertebrates and thus may confer a specific role for LPHNs in the vertebrate brain. Additionally, in *C. elegans*, TEN1 and LAT1 are possibly engaged in cis interactions and thus might have different modes of action than their vertebrate counterparts [3] (see also [26] on this topic).

2.2 FMI/CELSRs Regulate Synaptogenesis and Dendritogenesis

The aGPCR Flamingo (Fmi) is found in invertebrates and is evolutionary conserved, as mammals have three *Fmi* homologs named *Celsr1–3*. In *C. elegans*, *fmi-1* is expressed in neurons and localized in axons during development and adulthood [27]. In *Drosophila, fmi* is expressed at the neuromuscular junction and in dorsal sensory neurons of the peripheral nervous system (PNS) as well as in the central nervous system (CNS), where it is mostly present on dendritic and axonal membranes [28, 29]. Similarly, in rodents, *Celsr1–3* are predominantly expressed in the brain. While *Celsr1* is mostly restricted to regions of neural stem cell proliferation, *Celsr3* is preferentially expressed in postmitotic neurons, and *Celsr2 is* expressed in both proliferating and postmitotic neurons of the postnatal brain [30, 31].

2.2.1 Fmi Regulates Axon Guidance and Synaptogenesis in Invertebrates

In the ventral nerve cord of *C. elegans*, pioneer axon growth allows the formation of a path that will be followed by later growing follower axons. In *fmi-1* mutants, the navigation of both pioneer and follower axons is perturbed. The navigation of pioneer axons requires the CTF of FMI-1, whereas the navigation of follower axons requires the NTF [27], suggesting that FMI-1 controls axon navigation through signal transduction for pioneer axons and through axon-axon adhesion and/or *trans* signaling for follower axons.

At the larval neuromuscular junction of *Drosophila fmi* mutants, Fmi prevents the formation of inappropriate ectopic synapses. *fmi* mutants also present defective synaptic responses in a subset of muscles, as well as an age-dependent loss of muscle innervation due to drastic degeneration of axons in the third larval stage. Importantly, Fmi restoration in neurons rescues these synaptic and axonal defects. Fmi is thus required in neurons for the selection of synaptic targets, for synaptogenesis, and for the maintenance of axons and synapses in adulthood (Fig. 2) [28].

In the sensory system of *Drosophila*, processes of dendritic avoidance or repulsion occur during early development and are required to obtain a specific dendritic branching pattern for each of the dorsal sensory neurons. *fmi* mutant embryos show

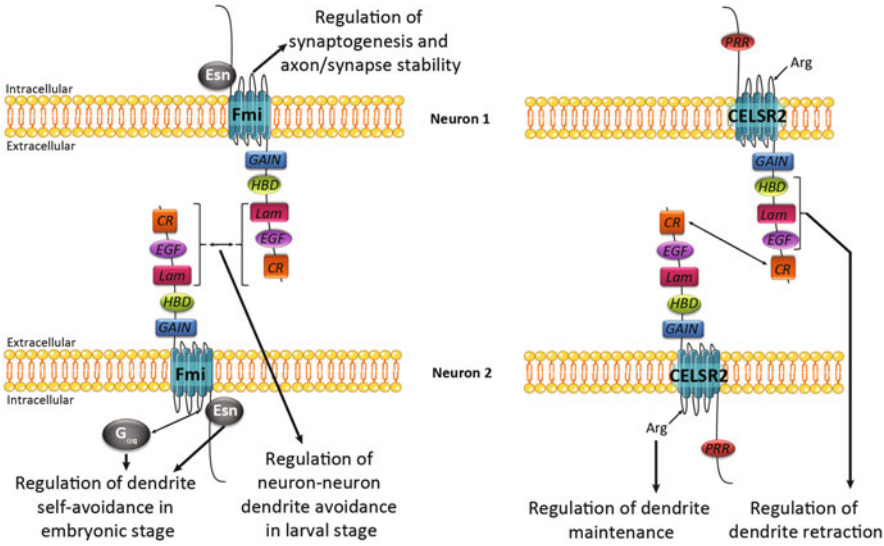

Fig. 2 Structure, partners, and functions of FMI and CELSR2 in neurons. In the NTF, Fmi/CELSR proteins contain nine cadherin repeats (CR), six EGF-like domains (EGF), two laminin globular-like domains (Lam), a hormone-binding domain (HBD), and the GPS-containing GAIN domain. The three CELSR proteins share 50 % sequence identity in their N-terminal and 7TM regions but have different C-terminal regions: 300 residues long for CELSR1–2 and 590 residues long for CELSR3. The C-terminal intracellular domain does not contain any conserved motif between invertebrates and vertebrates, except for a proline-rich region (PRR). In addition, CELSR2 has two splice variants, one long and one short, which differ based on splicing of exon 31, affecting 20 residues in the cytoplasmic tail. Fmi interacts with Espinas (Esn) [32] and $G\alpha_q$ [33]. CELSR2 and CELSR3 are localized both pre- and postsynaptically and are engaged in homophilic interactions [34]

dendritic overgrowth and overlap of dorsal sensory neurons [29, 34]. These defects can be partially rescued by the specific expression of an *fmi* construct containing only the extracellular hormone-binding domain (HBD) that impairs Fmi homophilic binding (Fig. 2). Intracellularly, genetic experiments suggest a signaling pathway (Fig. 2) involving the proteins Fmi, Esn, and Van Gogh [32] that could be localized in dendrites and locally activate RhoA upon contact of two branches to redirect dendrites away from each other. In addition, neuronal G protein $G\alpha_q$ may also function downstream of Fmi to inhibit dendritic growth [33]. At the larval stage, *fmi* mutants show dendritic outgrowth from the contralateral sides with overlapping of dendrites that can only be rescued by Fmi-containing cadherin repeats (Fig. 2). Thus, Fmi first restricts dendritic growth in embryos and then controls neuron-neuron dendritic avoidance through homophilic binding at the larval stage.

2.2.2 CELSRs Modulate Dendritic Growth in the Mammalian Brain

In rodents, CELSR2 is distributed in dendrites and axons of various embryonic and postnatal neuronal cells [35]. *Celsr2* loss-of-function manipulations, performed on organotypic brain slices from rat, caused simplification of the dendritic arborization of hippocampal and cortical pyramidal neurons and cerebellar Purkinje cells (PCs). This decrease in dendritic complexity is likely due to dendrite retraction, indicating that CELSR2 is required for dendritic maintenance [36]. Rescue experiments suggest a dual role of CELSR2: promotion of dendritic maintenance through homophilic interactions mediated by the extracellular cadherin repeats and decreasing dendritic complexity through the EGF-HBD region (Fig. 2). This suggests that different ligands of CELSR2, binding to different domains, could exert opposite effects on dendrites. This property of CELSR2 could be important for the control of dendritic complexity depending on environmental molecular cues.

In contrast to CELSR2, CELSR3 suppresses dendritic growth [37]. This functional difference may be determined by a single amino acid in the 7TM domain: *Celsr1*, *Celsr3*, and *Fmi* possess a histidine in the first intracellular loop of the 7TM domain, whereas *Celsr2* has an arginine. This residue switch is correlated with an inversion of the roles of the corresponding proteins in dendritogenesis, perhaps suggesting the presence of different second messengers initiating different signaling pathways. The role of Celsr3 in dendritogenesis in vivo remains to be confirmed since *Celsr3* knockout mice lack a dendritic phenotype, in contrast with the reported knockdown phenotype [37, 38].

2.3 BAIs Regulate Dendrite, Spine, and Synapse Formation

Three different BAI receptors are encoded by *Bai1–3* and are only present in vertebrates. Their expression increases in the brain after birth, with a peak in rodents at postnatal day 1 for *Bai3* and at postnatal day 10 for *Bai1* and *Bai2*. *Bai1* and *Bai3* are specifically expressed in the brain, whereas *Bai2* is also found in many other tissues [8, 39–42]. Although suggested by the antidepressant phenotype of the *Bai2* knockout mice [43], the involvement of BAI2 in neuronal circuit formation remains to be demonstrated. In contrast, roles for BAI1 and BAI3 in dendritogenesis and synaptogenesis have been described by several studies.

2.3.1 BAI1 Regulates Synaptogenesis and Synaptic Plasticity

BAI1 is enriched in the postsynaptic density (PSD) of excitatory synapses. Here, it co-localizes with the AMPA receptor GluR1 [44, 45] and potentially interacts with a variety of PDZ proteins such as PSD95, PSD93, the MAGUKs MAGI1–3, and the synapse-associated protein 97 (SAP97). BAI1 also binds to MDM2, an E3 ubiquitin ligase, and prevents PSD95 ubiquitination and degradation (Fig. 3). In *Bai1*-null animals, the synaptic ultrastructure is characterized by a decreased PSD thickness associated with increased PSD95-associated MDM2 and PSD95 ubiquitination. This modification is associated with defects in hippocampal-dependent learning and memory and in synaptic plasticity. The restoration of PSD95 to physiological

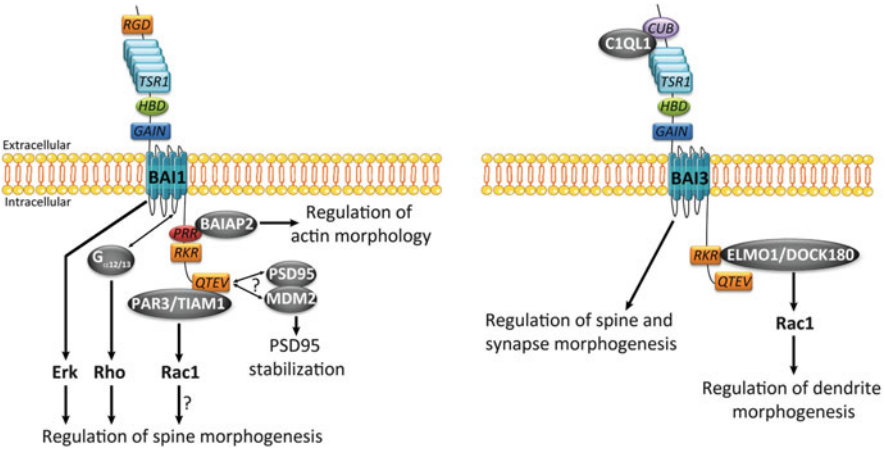

Fig. 3 Structure, partners, and functions of BAI1 and BAI3 in neurons. In the NTF, BAI proteins contain thrombospondin type 1 repeats (TSR1; five TSRs in BAI1 and four TSRs in BAI2 and BAI3), a HBD, and a GAIN domain containing the GPS site for autoproteolysis. At the N-terminal extremity, BAI3 also possesses a CUB (complement C1r/C1s, Uegf, Bmp1) domain, and BAI1 has an integrin-binding RGD motif. The N-terminal region of the BAIs is followed by the 7TM domain and a cytoplasmic domain containing an α-helical RKR motif and a terminal PDZ-binding motif (QTEV residues). BAI1 also presents a C-terminal proline-rich region (PRR). BAI1 interacts intracellularly with PAR3/TIAM1 [44], BAIAP2 [46], and $G\alpha_{12/13}$, as well as PSD95 [45] and MDM2 [47], possibly through the QTEV domain. BAI3 interacts with the complement C1Q-related molecule C1QL1 [48] extracellularly and with ELMO1/DOCK180 [41] intracellularly

levels in the hippocampus of *Bai1*-null mice restores normal synaptic plasticity, showing that plasticity defects are directly linked to the destabilization of PSD95 [47]. Knockdown experiments in cultured neurons further revealed the role of BAI1 in spinogenesis and synaptogenesis and, to a lesser extent, in synapse maintenance [45]. Rho GTPases and ERK have been previously shown to regulate the morphogenesis of dendritic spines as well as synapse formation and plasticity [38, 49]. Interestingly, BAI1 activates Rho via $G\alpha_{12/13}$ [45] and activates Rac1 by recruiting the Par3/Tiam1 complex at the PSD [50, 51]. Finally, BAI1 promotes the phosphorylation of ERK in a PDZ-motif-dependent manner. BAI1 seems thus required for the proper assembly of actin in developing spines *via* Rho GTPases and ERK signaling.

2.3.2 BAI3 Regulates Dendritogenesis and Synaptogenesis in Early Brain Development

Dendrite morphogenesis relies on the stabilization of neurites and the regulation of the actin cytoskeleton via the modulation of Rho GTPases [52, 53]. *Bai3* knockdown during early neuronal development leads to dendrite overgrowth in various types of neurons in vitro, as well as in PCs in vivo [41]. No major dendritic defects are observed in adult mice with a specific disruption of *Bai3* in PCs [54]. This

discrepancy could be due to developmental timing differences in the inactivation of BAI3 in these experiments. This would suggest a requirement of BAI3 preferentially at an early stage of dendritogenesis and/or a subsequent recovery of the major defects in the adult. Dendrite morphogenesis relies on the stabilization of neurites and the regulation of the actin cytoskeleton via the modulation of Rho GTPases. BAI3 controls dendritic growth by regulating the activity of the Rho GTPase Rac1, partially through binding to the ELMO1/DOCK180 complex, a guanylate exchange factor of Rac1 (Fig. 3) [41]. Thus, BAI3 might control the rearrangement of dendritic actin cytoskeleton via Rac1, a potential role reinforced by the fact that BAI3 co-localizes with actin in growing dendrites.

Proteomic analysis of synaptic fractions revealed the localization of BAI3 at excitatory synapses in the forebrain and in the cerebellum [55, 56]. Knockdown or cell-specific knockout experiments in PCs show that BAI3 promotes the connectivity of its two types of excitatory afferents, the parallel and the climbing fibers. Indeed, *Bai3* loss of function leads to a decrease in the number and size of parallel fiber presynaptic boutons [42], as well as a decrease in the number, size, and extension territory of climbing fiber presynaptic boutons [42, 54]. During synaptogenesis, each PC is first contacted by multiple climbing fibers around embryonic day 19 in mouse. Then, only one climbing fiber "wins" and supernumerary climbing fibers are eliminated. In the PC-*Bai3*-null mice, many PCs remain multi-innervated in the adult [54]. This defect in climbing fiber elimination could be a direct effect of BAI3 in determining the "winner" climbing fiber or an indirect effect consecutive to the defective establishment of parallel fiber connections [42], since normal transmission at parallel fiber synapses is necessary for proper climbing fiber elimination.

The only extracellular partners of BAI3 reported so far are the secreted C1QL proteins from the complement C1Q-related family (Fig. 3) [48]. The four C1QLs, generated from four genes, are expressed almost exclusively in the mammalian brain, each one with a specific and partially overlapping expression pattern [42, 57]. C1QLs-BAI3 interaction was first reported to inhibit synapse formation or maintenance in cultured hippocampal neurons [48]. Two subsequent studies show that in the cerebellar cortex, C1QL1 needs to be secreted specifically at the climbing fiber to allow proper formation of climbing fiber/PC synapses [42, 54]. In addition, C1QL1 is transiently expressed in the cerebellum and promotes PC spinogenesis in a BAI3-dependent manner. Thus, BAI3 could regulate the number of parallel fiber/PC synapses early during development [42]. Both *C1ql1* and *Bai3* knockout mice exhibit defects in climbing fiber elimination and motor learning defects, highlighting the importance of the signaling pathway formed by C1QL1 and BAI3 for proper CF/PC synaptogenesis and cerebellar function [54].

3 Adhesion GPCRs in Myelinating Glia

While synapses permit direct communication between neurons, the glial-derived myelin sheath enables the rapid propagation of action potentials along axons; thus, myelinating glia are also key players in information transfer in the nervous system. Myelin is made by Schwann cells (SCs) in the PNS and oligodendrocytes (OLs) in the CNS. Myelination increases the conduction velocity of action potentials; additionally, OLs and SCs provide trophic support to aid in the proper functioning and survival of neurons [58, 59]. Relatively less is known about myelinating glia compared to neurons. Similarly, little is known about the roles of aGPCRs in myelinating glia compared to roles in synaptogenesis and dendritogenesis. Nevertheless, recent studies have established two aGPCRs, Gpr126 and Gpr56, as key regulators of both SC and OL development.

3.1 GPR126 Is Essential for Schwann Cell Myelination

Previous work has defined several steps in SC development, leading from neural crest progenitors to mature myelinating SCs [58]. During embryogenesis, SC precursors migrate with axons in the developing PNS and differentiate into immature SCs that remain associated with many axons. In a process called radial sorting, immature SCs insert cytoplasmic projections into the axon bundle and begin to envelop and separate individual axons away from the rest of the bundle according to axon diameter. Some immature SCs will remain associated with many small axons and become nonmyelinating SCs. However, immature SCs that will eventually make myelin will first become promyelinating SCs that associate in a 1:1 ratio with a single axonal segment. Upon additional cues, myelinating SCs then iteratively wrap their plasma membrane around the associated axonal segment, ultimately generating a mature myelin segment with a thickness proportional to the diameter of the axon they wrap.

The first evidence of a role for aGPCRs in myelination was the discovery that Gpr126 is required for the normal progression from a promyelinating SC to a fully mature myelinating SC in both zebrafish and mice [60, 61]. In *Gpr126* mutants, SCs express the transcription factor *Sox10*, which is required for SC specification, maintaining SC identity and proper progression of the lineage [62–64]. However, *Gpr126* mutant SCs fail to express later-stage transcription factors Oct6 (Pou3f1) and Krox20 (Egr2). Oct6 and Krox20 are required to activate the expression of many myelin-associated genes, including *myelin basic protein* (*Mbp*), which encodes a structural component of the myelin sheath. Ultrastructural analyses demonstrated that *Gpr126* mutant SCs can associate with axons in the proper 1:1 ratio, but are arrested at the promyelinating stage of development, and fail to ever elaborate a myelin sheath [60, 61].

In vitro, SCs cocultured with neurons follow a similar developmental progression as described above—they migrate and proliferate along nascent axons, perform radial sorting, and myelinate axons [65]. However, in the absence of axons, SCs fail

to differentiate. This requirement for neurons can be overcome by the addition of axonal membrane fragments [66, 67] or by increasing levels of cAMP. Elevation of cAMP induces SC maturation, as illustrated by their upregulation of myelin-related gene expression and downregulation of immature SC markers [68–70]. Thus, an in vivo role for cAMP elevation in SC myelination had long been hypothesized, although the mechanism by which cAMP might be elevated has been elusive. Interestingly, myelination defects in *gpr126* mutant zebrafish can be suppressed by treating the animals with forskolin, an adenylate cyclase activator that potently elevates cAMP. Specifically, SCs of forskolin-treated *gpr126* mutant zebrafish express *oct6*, *krox20*, and *mbp* and produce ultrastructurally normal myelin [60]. Additionally, elevating cAMP levels and activating protein kinase A (PKA) restored myelination in myelinating cultures from *Gpr126*$^{-/-}$ knockout mice [71], and PKA activation also rescues myelination in zebrafish [72]. Moreover, cAMP is downregulated in the sciatic nerves of mutant mice in which *Gpr126* has been deleted specifically in SCs [71]. Finally, GPR126 was shown to directly couple to the G_s-protein and G_i-protein families [71], which are associated with modulating cAMP levels. All together, these data suggest that Gpr126 is a major, if not the sole receptor, that elevates cAMP in SCs to initiate myelin-gene expression and terminal differentiation.

3.1.1 Gpr126 Has cAMP-Independent Roles in Schwann Cell Development

Until quite recently, it was unclear if aGPCRs functioned as adhesion molecules by virtue of the NTF, as traditional GPCRs signaling through heterotrimeric G proteins by virtue of the 7TM, or if the same molecule could perform both functions. Notably, studies on Gpr126 have contributed to answering this question, as Gpr126 SC myelination studies strongly suggested that aGPCRs could behave as traditional GPCRs [71, 73]; later, the Gpr126-NTF was reported to have a CTF-independent role in heart development [74]. Together, this suggested that a given aGPCR can have domain-specific roles, at least in different cellular contexts. Interestingly, recent structure-function studies of Gpr126 have shown that an aGPCR can also have multiple independent functions in modulating the development of a single cell [73].

Preliminary analyses of *gpr126* zebrafish mutants suggested that Gpr126 functions specifically in the final maturation of SCs. This function of Gpr126 is conserved in mammals; however, both global and SC-specific *Gpr126* mutant mice display additional defects that were not observed in *gpr126* zebrafish mutants, including severe delays in radial sorting, axon degeneration, and limb contracture abnormalities [61, 71]. While species differences may certainly be at play, there are key differences between the alleles in *Gpr126* mutant mice and zebrafish. The targeted deletion in *Gpr126* null mice produces an early premature stop codon that results in the near absence of *Gpr126*-NTF mRNA, and these mice have severely impaired radial sorting and completely lack peripheral myelination as mentioned above [61, 71]. In contrast, two previously studied mutant alleles in zebrafish were likely not null alleles; the *gpr126*^{st49} allele is a point mutation resulting in a

premature codon near the GPS [60], such that the NTF might be intact, while the CTF signaling domain is absent. Similarly, the *gpr126*^{st63} allele is a missense mutation that disrupts a highly conserved cysteine in the 7TM, thus the NTF and CTF both remain intact [60]. Importantly, neither *gpr126*^{st49} nor *gpr126*^{st63} displays defective radial sorting. In contrast, recently generated *gpr126*^{stl47} mutants represent a very early premature stop codon (amino acid position 96), which is more similar to the targeted deletion in *Gpr126* mutant mice [73]. Interestingly, SCs in *gpr126*^{stl47} mutants phenocopy the radial sorting defects observed in mouse mutants. These defects could be rescued by genetically "adding back" the Gpr126-NTF but could not be rescued by cAMP elevation, suggesting that Gpr126 has CTF-independent roles in SC development. These findings are consistent with earlier studies highlighting NTF- and CTF-specific functions for aGPCRs in *C. elegans* axon migration, fertility, and viability [3, 27], though future work is required to determine how the Gpr126-NTF might direct radial sorting in a cAMP-independent fashion.

3.1.2 Control of Gpr126 Signaling in Schwann Cells

Given that aGPCRs have both CTF-dependent and independent roles, an important question centers around binding partners that might modulate these functions. Notably, most aGPCRs are orphaned [1, 75], meaning their ligands and activation methods are unknown. aGPCRs are unique relative to other GPCRs in that they undergo an autoproteolysis event at the GPS motif that separates the protein into an NTF and CTF, which then remain non-covalently associated with one another at the cell membrane [2, 4]. Interestingly, a spate of recent studies have shown that several aGPCRs, including GPR126, generate a tethered agonist ligand, termed the *Stachel* sequence (German word for "stinger"), which potently activates the CTF of the receptor in vitro [76–79]. Moreover, analysis of zebrafish mutants suggested that activation of Gpr126 by the *Stachel* sequence is required for SC myelination [76], and it was recently suggested that Gpr126-interacting proteins in the extracellular matrix (ECM) might modulate availability of the *Stachel* sequence (see also [80, 81] on this topic).

The NTFs of several aGPCRs can bind to ECM molecules; Gpr126 is no exception, as the Gpr126-NTF can bind to type IV collagen and Laminin-211 [73, 82], which are both components of the SC basal lamina. In rodent SCs and GPR126-expressing heterologous cells, exogenous application of type IV collagen induced cAMP elevation, which is required for SC differentiation. Additionally, GPR126 derivatives lacking the NTF were constitutively active, suggesting that the NTF is important for inhibiting Gpr126-CTF signaling [82]. Interestingly, Laminin-211 also binds Gpr126 in a different region of the NTF than the collagen-IV binding site. Overexpression of *lama2* rescued myelination defects in hypomorphic *gpr126*^{st63} zebrafish mutants in a cAMP-dependent manner [73]. Intriguingly, and in contrast to type IV collagen, addition of Laminin-211 to GPR126-expressing heterologous cells suppressed cAMP accumulation under static conditions. However, under dynamic culture conditions, Laminin-211 activated GPR126. The in vivo relevance of this result was underscored by the observation that a

polymerization-defective mutant form of *lama2* could no longer restore myelination in *gpr126* zebrafish mutants. Taken together, these studies suggest that ECM molecules in the basal lamina may modulate the NTF and impact *Stachel* sequence signaling. Moreover, the Laminin-211/Gpr126 studies suggested that aGPCRs may be mechanically sensitive, consistent with contemporaneous work in *Drosophila melanogaster* [83]. For an in-depth discussion of the emerging concept of aGPCRs as mechanosensors, the reader is referred to [84].

Importantly, these data tie in with the model that Gpr126 has CTF-independent functions in SC development. Low levels of cAMP promote SC proliferation and facilitate an immature state, while elevated cAMP drives myelination [85]. Perhaps in early SC development, the Gpr126-NTF remains tightly associated with the CTF such that cAMP elevation is suppressed. Changes in ECM proteins in the SC basal lamina, including Laminin-211 polymerization, may modulate the NTF such that the *Stachel* sequence can bind the 7TM and induce Gpr126 signaling cascades through G_s, which elevate cAMP, activate myelin-related gene expression, and allow terminal maturation of myelinating SCs (Fig. 4).

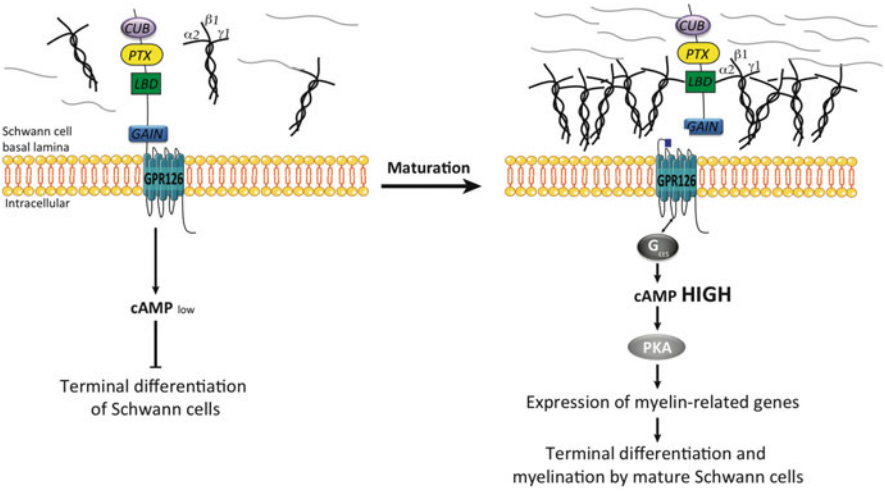

Fig. 4 Interactions with the ECM modulate Gpr126 throughout Schwann cell development. In addition to the canonical aGPCR domains (GAIN, GPS, and 7TM), GPR126 also possesses CUB and Pentraxin (PTX) domains in the NTF. Recent data showing that Gpr126-NTF interacts with Laminin-211 (α2, β1, γ1) [73] supports the addition of a novel Laminin-binding domain (LBD) in the NTF. Altogether, current data support a model in which molecules in the SC basal lamina (Laminin-211 in *black*, collagens in *gray*) interact with GPR126-NTF to stabilize the receptor in an inactive state during early development. This prevents cAMP accumulation by suppressing GPR126 G_s signaling to prevent SC differentiation. Following basal lamina maturation and Laminin-211 polymerization, an active conformation of GPR126-CTF is facilitated to induce G_s signaling and cAMP elevation, possibly through mechanical modulation of the receptor. This results in PKA activation, which promotes myelin-related gene expression and terminal SC differentiation

3.2 GPR56 Regulates Oligodendrocyte Development

OLs are the myelinating glia of the CNS that, as mentioned above, share many characteristic features with their PNS counterparts, the SCs. Like SCs, OLs generate myelin by iteratively wrapping their plasma membranes around axon segments [86], although OLs can myelinate many axonal segments, in contrast to the single axon segment myelinated by an SC. Accordingly, OLs and SCs perform similar functions using an overlapping, but not identical, set of genes [87]. Moreover, while SCs are derived from neural crest, the OL lineage is derived from neural stem cells, which ultimately give rise to all resident cells in the CNS except for microglia [88]. OL precursor cells (OPCs) migrate in waves from their germinal zones and become immature OLs as they begin extending cytoplasmic processes and associating with axons [86]. Finally, upon incompletely understood cues, immature OLs terminally differentiate into mature myelinating OLs that spiral and compact their plasma membrane around as many as 40 different axon segments [89].

Although Gpr126 is essential for SC development and myelination, it has no known function in OLs, suggesting that there might be another aGPCR that is similarly important for OL maturation and myelination. Previously published data sets indicate that *Gpr56*, an aGPCR in the same subfamily as GPR126, is highly expressed in OPCs in the developing mouse CNS [90, 91]. In humans, mutations in *GPR56* cause bilateral frontoparietal polymicrogyria (BFPP) [11], a cortical brain malformation associated with a variety of devastating neurological impairments such as epilepsy and mental retardation. Interestingly, brains of BFPP patients also exhibit reduced white matter volume by MRI and signal changes in T2-weighted images [12, 85, 92], which are indicative of myelin defects. Two complementary studies have recently defined key functions for GPR56 in OL development [93, 94].

In these studies, the authors analyzed both zebrafish and mouse *Gpr56* mutants. $gpr56^{stl13}$ mutant zebrafish harbor a 6 bp deletion that removes a completely conserved tryptophan residue in the GPS motif that is essential for autoproteolysis [94]. Since previous reports show that disruption of autoproteolytic cleavage of GPR56 prevents the protein from being trafficked to the membrane and thus severely impairs GPR56 signaling [95], *stl13* is likely a strong loss-of-function allele. Although loss of Gpr56 function in *stl13* mutants did not result in abnormal OPC specification or initial OPC numbers, both *Gpr56* knockout mice and $gpr56^{stl13}$ zebrafish displayed significantly fewer OL lineage cells over time [93, 94]. GPR56 is expressed in many CNS cell types including oligodendrocyte precursor cells (OPCs) and immature oligodendrocytes [93], which could impact some of the OL phenotypes observed in *Gpr56* mutants. Giera et al. also used OL-specific deletion of *Gpr56* to demonstrate that this aGPCR functions cell autonomously in OLs to regulate their development. Ultrastructural analyses show that zebrafish and mouse *Gpr56* mutants possess fewer myelinated axons as compared to controls in the spinal cord and corpus callosum, respectively. However, axons that are myelinated appear to be myelinated normally, suggesting that GPR56 regulates the number of OL lineage cells but is not required for myelination per se. Marker analyses in both species demonstrated that the reduction in OL

number is not due to increased OPC cell death but instead a lack of OPC proliferation [93, 94]. Additionally, overexpression of *gpr56* in wild-type zebrafish larvae induced increased OPC proliferation, while the ventral spinal cord was precociously myelinated in *gpr56*^{stl13} zebrafish mutants at early developmental stages [94]. Together, these studies support the notion that loss of GPR56 results in OPCs prematurely exiting the cell cycle in favor of differentiation.

3.2.1 Gpr56 Regulates Oligodendrocyte Development via $G_{\alpha 12/13}$ and RhoA

Previous studies established that GPR56 couples to $G_{\alpha 12/13}$ and activates RhoA to control migration of neural precursor cells in vitro [96, 97]. RhoA is highly expressed in mammalian OPCs but is downregulated at later stages to facilitate terminal differentiation of OLs [98], paralleling the early function of GPR56 in these cells. In zebrafish, knockdown of the genes encoding $G_{\alpha 12/13}$ proteins or overexpression of a dominant-negative RhoA phenocopied OL lineage defects in *gpr56*^{stl13} mutants, while overexpression of constitutively active RhoA rescued the defects [94]. In mouse, levels of active RhoA were significantly reduced in *Gpr56* knockout optic nerves while there were no observable changes in PKCα, the only other effector reported to be downstream of GPR56 [93]. Taken together, these experiments strongly support a model in which GPR56 couples to $G_{\alpha 12/13}$ and signals through RhoA to promote OPC proliferation and inhibit terminal differentiation to ultimately regulate appropriate levels of CNS myelination (Fig. 5). In the future, it will be interesting to determine if this activation is dependent on the *Stachel* sequence as well as what ligand(s) might influence GPR56-dependent OL development.

3.3 Other aGPCRs in Myelinating Glia

Beyond GPR126 and GPR56, VIGR/GPR98 (ADGRV1) is expressed in OLs, and modulation of this aGPCR can affect levels of a key myelin protein, MAG (myelin-associated glycoprotein). In vitro, VIGR knockdown reduces, while overexpression increases, MAG expression [99]. Additionally, expression studies suggest that there may be yet undiscovered aGPCR players in glial cell development and myelination as RNAseq analyses in the mouse CNS indicates that *Gpr125* (*Adgra3*), *BAI1–3*, *Lphn1, Lphn3*, and *Celsr2* are all expressed (FPKM > 5) in OL lineage cells [100]. Future studies can elucidate the roles of additional aGPCRs in myelinating glia.

4 Discussion

Thus far, three groups of aGPCRs, latrophilins, Flamingo/CELSRs, and BAIs, have been implicated in morphogenesis of various neurons and/or their connectivity. Similarly, two aGPCRs from the group VIII (ADGRG) subfamily, GPR126 and

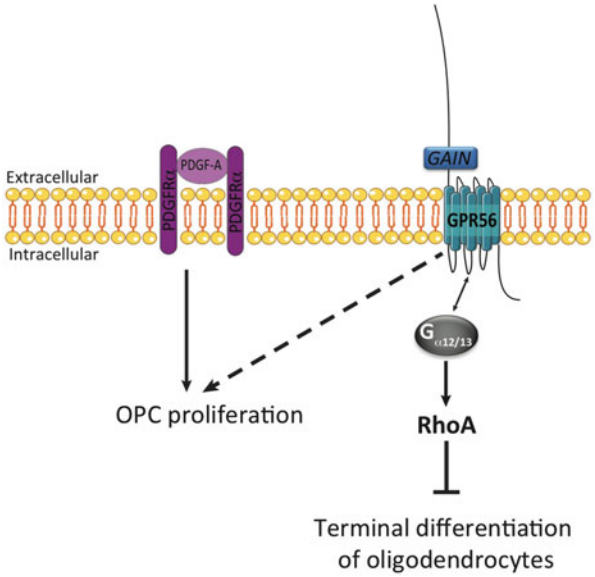

Fig. 5 Gpr56 regulates oligodendrocyte development by promoting precursor proliferation and inhibiting premature differentiation. Although GPR56 contains all of the classic aGPCR domains (GAIN, GPS, and 7TM), other protein domains in either the NTF or the intracellular tail region have not been described. Recent data from zebrafish and mouse models suggest that GPR56 couples to $G\alpha_{12/13}$ [93, 94] and activates RhoA to prevent terminal differentiation of OLs. In doing so, GPR56 indirectly promotes OPC proliferation (*dashed arrow*) by maintaining the OPC in an immature, proliferative state

GPR56, have proven to be critical regulators of myelination. These molecules are thus crucial for the establishment of a properly functioning nervous system.

The importance of understanding the molecular basis underlying dendrite and synapse formation is highlighted by the fact that any modification in neuronal microcircuits can lead to neurodevelopmental defects and brain pathologies. This is underscored by the fact that some aGPCRs regulating dendritogenesis and synaptogenesis are also associated with neurodevelopmental pathologies. In humans, deletion of *LPHN1* is associated with various symptoms including mental retardation, psychomotor and language delay, and hearing impairment [101]. SNPs in *LPHN3* increase the risk for attention deficit/hyperactivity disorder (ADHD) and substance addiction [102, 103]. In addition, human *BAI1* is present in a "hot spot" for de novo germline mutations in autism patients [104], and the BAI1-associated protein BAIAP2 is also linked to autism [105]. Finally, genetic association studies reveal that BAI proteins could contribute to behavioral defects in psychiatric disorders. Indeed, SNPs and CNVs in the *BAI3* gene have been associated with schizophrenia [106, 107], bipolar disorder [108], and addiction [109].

Similarly, the importance of understanding the molecular mechanisms that govern proper myelination is emphasized by the fact that damage to or loss of

this insulation leads to debilitating symptoms in many neurological disorders. For example, in multiple sclerosis, OL myelin is attacked and destroyed by the body's immune system [110]. As noted above, BFPP patients present with white matter abnormalities; perhaps GPR56 represents a new candidate target to modulate OL differentiation in the CNS. Similarly, GPR126 may represent a viable target for patients with peripheral nerve disease as this aGPCR is required for proper myelin-gene expression in humans [111]. Furthermore, mutations in *LAMA2*, which encodes the α2 chain of Laminin-211, cause merosin-deficient congenital muscular dystrophy (MDC1A) in humans [112]. Given that GPR126 binds to Laminin-211, activation of GPR126 could be pursued in MDC1A patients. GPCRs are the most widely studied targets for the development of pharmacological drugs. Therefore, studying aGPCRs is highly pertinent to increase our understanding of these receptors in the context of human neurodevelopmental and myelin diseases and to find efficient treatments for human disorders.

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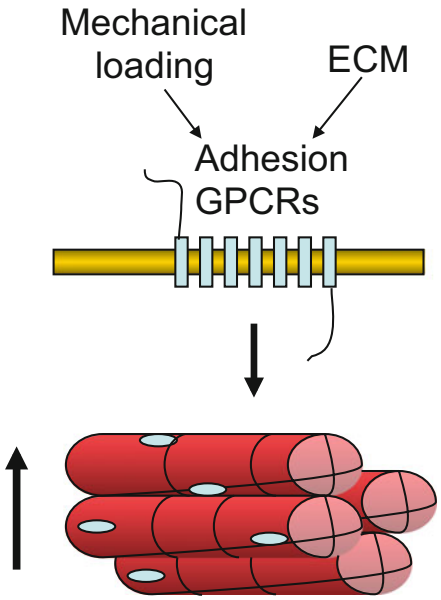
Control of Skeletal Muscle Cell Growth and Size Through Adhesion GPCRs

James P. White

Graphical Abstract



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Contents

Abstract

Skeletal muscle homeostasis is regulated by a constant influx of chemicals and exposure to mechanical stimuli. A number of key signaling pathways that translate these stimuli into changes in muscle physiology have been established. The GPCR family known as adhesion GPCRs (aGPCRs) has largely elusive roles in skeletal muscle biology; however, their unique capacity to activate adhesion and G protein signaling pathways makes them an attractive point of investigation. The skeletal muscle myofiber contains a highly organized cyto-architecture to ensure contractile function. This requires intricate interactions with components of the extracellular matrix (ECM) surrounding each fiber. aGPCRs possess extended N-termini known to interact with ECM proteins and complexes suggesting a compatible role in skeletal muscle biology. Furthermore, recent work demonstrated the involvement of certain aGPCRs in whole muscle hypertrophy and differentiation of muscle progenitor cells. Signaling pathways downstream of aGPCRs are still incompletely understood; however, initial findings show involvement of the Gα12/13 subunit signaling to the pro-anabolic Akt/mTOR pathway. Together, this chapter will review the emerging role of aGPCRs in skeletal muscle biology and putative mechanism (s) employed to regulate skeletal muscle growth.

Keywords

Skeletal muscle hypertrophy • Mechanical overload • mTOR • Adhesion GPCRs • Myogenesis

1 Introduction

Skeletal muscle is a highly plastic tissue capable of responding to both chemical and mechanical stimuli. The balance of chemical stimuli derives from factors that promote muscle growth [e.g., growth hormones, anabolic steroids, insulin-like growth factor 1 (IGF-1)] or atrophy [e.g., myostatin, interleukin 6 (IL-6), tumor necrosis factor α (TNF-α)] [1]. These factors along with their downstream signaling pathways have been heavily researched over the last several decades. In addition to these chemical stimuli, mechanical loading/unloading constitutes a potent regulator of muscle growth and atrophy [2]. However, unlike the chemical affecters of

muscle mass, the load-sensitive mechanism(s) that regulate muscle size remain ill-defined. Despite the growing body of research investigating the mechanisms of muscle growth, the role of G protein-coupled receptors (GPCRs) and specifically aGPCRs in this process is currently unclear. Intriguingly, aGPCRs are structurally similar to integrin receptors yet functionally signal through G protein pathways allowing for potentially novel signaling mode, which could integrate with known anabolic pathways in muscle. Further investigation of the biology of aGPCRs in skeletal muscle is certainly warranted.

1.1 Molecular Regulators of Muscle Mass

Under physiological conditions, skeletal muscle regulates mass by increasing/decreasing myofibrillar protein accumulation. This can be achieved through expansion or depletion of myofibrillar proteins with or without adding or subtracting myonuclei. The addition of myonuclei is a process referred to as myogenesis. Muscle stem cells, called satellite cells, are located around the periphery of the myofiber and remain quiescent until the local environment cues for satellite cell activation and fusion into the adjacent myofiber [3]. The additional myonuclei can increase the capacity to transcribe mRNA and facilitate accretion of more myofibrillar protein. The process of myogenesis is particularly crucial in conditions of muscle injury when protein synthesis is not sufficient for myofiber repair (REF). The IGF-1/Akt/mTOR pathway (Fig. 1) is a well-established signaling cascade of muscle protein balance (reviewed in [4, 5]). The IGF-1 signaling pathway is well established and known to regulate skeletal muscle mass by controlling protein synthesis and degradation as well as apoptotic pathways [4, 5]. Binding of IGF-1/insulin activates the receptor tyrosine kinase IGF-1 receptor and recruits insulin receptor substrate (IRS). Subsequently, this leads to the activation of phosphatidylinositol 3′-kinase (PI3K) and potentially serine-threonine kinase Akt (PKB) through a phosphorylation of a serine residue at position 473 [6–8]. Interestingly, during skeletal muscle hypertrophy, Akt activity is increased when examined in vivo [9] and in cultured myotubes [10]. In addition, a genetically altered, constitutively active Akt was able to induce muscle hypertrophy without growth factor stimulation [9, 11]. A known downstream target of Akt is the mammalian target of rapamycin (mTOR). mTOR signals downstream to enhance translational capacity through p70S6K and 4E-BP1 to promote muscle protein accretion. In addition to activating anabolic signaling, the IGF-1 pathway can also suppress atrophic signaling through inhibition of FOXO, a key transcriptional regulator of atrophy-related gene expression [12]. Together, the Akt/mTOR pathway drives muscle hypertrophy by increasing protein synthesis and suppressing protein breakdown.

In addition to growth factor stimulation, skeletal muscle is very sensitive to mechanical load. Overload-induced muscle hypertrophy is evident with continuous stretch [13], mechanical overload [14, 15], and intermittent lengthening contractions [16, 17]. In terms of load-sensitive signaling, mTOR plays a central

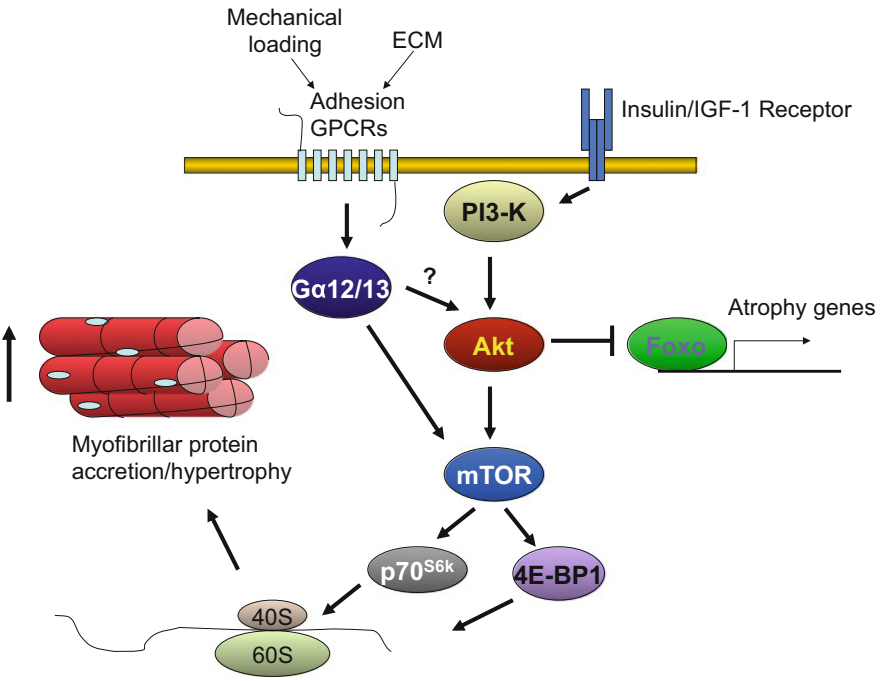

Fig. 1 Anabolic signaling pathways in skeletal muscle. The canonical insulin/IGF-1 pathway signals through the PI-3k/Akt/mTOR pathway to induce ribosomal translation and increased myofiber protein accretion. Activation of the adhesion GPCR is initiated through binding to ECM components and/or mechanical stress. Interaction of adhesion GPCR signaling and Akt/mTOR signaling pathways is mediated by the Gα12/13 subunit and downstream activation of mTOR

role in regulating mechanical overload-induced muscle adaptations [18]. Overload-induced activation of mTOR and its target p70S6K is independent of PI3K or Akt [19, 20], which suggests dispensability of growth factors for overload-induced hypertrophy. Mechanical overload in both mice [21] and humans [22] increases mTOR phosphorylation and its downstream target p70S6K. Integrin signaling has been implied as a mechanism to sense mechanical load and integrate downstream signaling events [23]. Currently, no evidence directly links canonical integrin signaling to skeletal muscle mTOR activation. However, in nonmuscle cells, integrin signaling activates Ras and its major effectors phosphoinositide-3 kinase (PI3K) and raf [24] suggesting a potential integrin-mTOR link in muscle.

In terms of physiological hypertrophy, overexpression of α7β1 integrin in skeletal muscle enhances the hypertrophic effect of downhill running exercise (an eccentric contraction-induced hypertrophy model) [25]. However, no signaling mechanism was implied to explain these observations. aGPCRs have integrin-like signaling capacity and could conceivably act as a load sensor in the muscle. The extended N-terminal tail can signal to ECM proteins such as collagen I/IV [14, 26]

similar to integrins. Furthermore some aGPCRs signal through Rho [26, 27], another well-established downstream effector of integrin activation. GPCRs can signal through the Akt pathway in 3T3 fibroblast [28]; however, this connection has not been described in skeletal muscle. The role of aGPCRs in both canonical Akt/mTOR signaling and mechanical loading-induced hypertrophy is unclear and could act as a focal point to connect several known anabolic pathways.

2 Adhesion GPCRs and Skeletal Muscle Biology

2.1 Muscle Mass Regulation by Adhesion GPCRs

aGPCRs form one of five GPCR families and have been shown to facilitate cell–cell and cell–matrix interaction [29]. One such aGPCR, GPR56, has been heavily investigated in the brain as mutations in *GPR56* result in a condition known as bilateral frontoparietal polymicrogyria (BFPP), an autosomal recessively inherited developmental disorder of the brain [30] (see also [31]). In terms of skeletal muscle, *GPR56* was recently discovered to be a transcriptional target of the PGC-1α isoform 4 (PGC-1α4) known to promote muscle hypertrophy and to accumulate during resistance-type exercise [32]. Furthermore, GPR56 is necessary for PGC-1α4-induced muscle hypertrophy and regulates mechanical loading-induced hypertrophy [14]. GPR56 can independently cause muscle hypertrophy as overexpression of GPR56 resulted in myotube hypertrophy in vitro. In addition, mRNA expression of GPR56 and its ligand collagen III were increased in muscle during various mouse models of loading-based muscle hypertrophy and human resistance exercise. Interestingly, *GPR56* loss of function mice did not display an overt muscle phenotype until challenged with an overload stimulus. Under overload conditions, the *GPR56* KO mice did not hypertrophy to the same extent as wild types [14]. This suggests that GPR56 may function as a "load-sensitive" driver of muscle growth, which is not active during sedentary conditions. In terms of myogenesis, GPR56 is highly expressed during the initial days of in vitro myoblast differentiation [33]. Knockdown of *GPR56* attenuates myotube formation, expression of myogenic genes, and nuclear factor of activated T cell (NFAT) (Fig. 2). Furthermore, *GPR56* null mice were able to regenerate from muscle injury similar to wild-type mice [33].

The aGPCR brain-specific angiogenesis inhibitor (BAI3) has also been shown to have a role in myogenesis [34]. Knockdown of BAI3 in C2C12 myotubes results in a severe suppression of myotube formation. The function of BAI3 in myotube formation depends on binding to the ELMO1/DOCK1 complex, a downstream component of integrin signaling. The third and most recently identified adhesion GPCR associated with skeletal muscle biology is CD97. CD97 localizes to the muscle fiber sarcolemma and the sarcoplasmic reticulum. In mouse, deletion of CD97 was shown to alter the morphology of the sarcoplasmic reticulum, while the skeletal muscle function was unaltered in vivo [35]. The signaling mechanism for CD97 is currently unknown in skeletal muscle. Together, the role of aGPCRs in

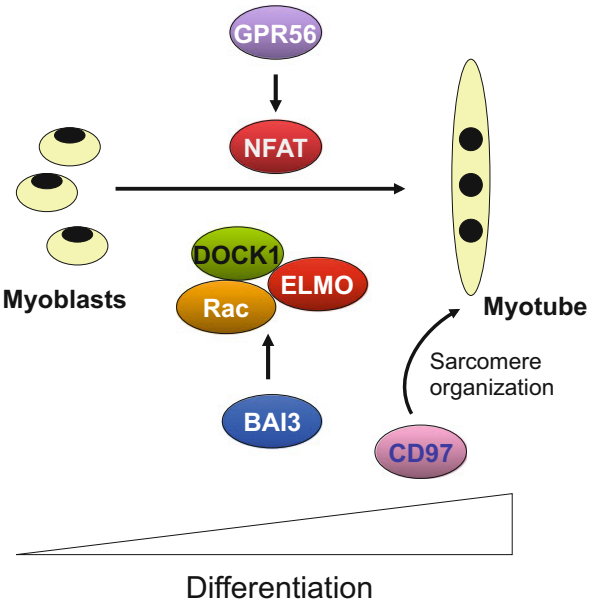

Fig. 2 Regulation of myogenesis by adhesion GPCRs. The differentiation process of myoblasts into mature myotubes is a highly complex process in which certain mechanisms remain unclear. aGPCRs are known to regulate myogenesis through different pathways. GPR56 regulates myotube formation through expression of NFAT and coordination of myogenic regulatory factors. BAI3 binds to the Rac/DOCK1/ELMO complex to promote differentiation, while CD97 has a role in sarcomere organization during myotube formation

skeletal muscle is slowly expanding and interestingly can be identified throughout various locations within the myofiber. In addition, aGPCRs effect various functions in muscle ranging from anabolic signaling (Fig. 1) to myoblast differentiation (Fig. 2).

2.2 Adhesion GPCR Signaling in Skeletal Muscle

The convergence between aGPCR signaling and established regulatory pathways in muscle is currently unknown and could elucidate novel points of regulation. Outside the aGPCR family, there is evidence for Gα signaling, specifically through $G\alpha_{i2,}$ to promote skeletal muscle hypertrophy and muscle stem cell (satellite cell) myogenic capacity [36, 37]. The anabolic effect of $G\alpha_{i2}$ is dependent on PKC to inhibit GSK-3β, a negative regulator of protein synthesis, and to activate p70S6k, a positive regulator of translation initiation [37]. The functional role of GPR56 in skeletal muscle is dependent on Gα12/13 activity [14]. Interestingly, the Gα13/Rho pathway has been associated with muscle hypertrophy in pressure overloaded cardiac muscle [38], supporting the concept of the Gα12/13 pathway being an integrin-like "mechano"-sensitive anabolic pathway (see also [39]). The Rho/SRF pathway is sensitive to mechanical loading in muscle and associated with muscle hypertrophy [40, 41]. In addition, the load-sensitive Rho pathway has been shown to regulate satellite cell proliferation [42] and differentiation [43]. GPR56 overexpression in myotubes was associated with an increase in mTOR activation, while inhibition of the Gα12/13 subunit was sufficient to block GPR56-induced

mTOR activation (summarized in Fig. 1) [14]. Together, GPR56 activation may promote muscle anabolism through multiple pathways. The direct mechanism for Gα12/13 to activate mTOR is unclear and may require additional signaling arms specific to muscle, as Gα12/13 activation in 293 cells inhibited Akt signaling [44] suggesting this pathway may not be so straightforward. Elucidating downstream signaling through other aGPCRs in skeletal muscle deserves further investigation.

2.3 Endocrine Signaling from ECM to Adhesion GPRCs

An intriguing concept of aGPCRs especially in the case of GPR56 is the idea of trans-activation through ECM fragments originated from other muscle groups. As previously mentioned, collagen III is a known ligand of GPR56, which activates Rho signaling through the Gα12/13 pathway [26]. The N-terminal pro-peptide of collagen type III (P-III-NP) has been suggested as a biomarker of muscle anabolism with testosterone and growth hormone treatment [45]. There are two possible hypotheses for this observation: (1) the correlation of muscle growth could simply be a by-product of collagen synthesis used for muscle hypertrophy, or (2) it could be a novel "feed-forward" endocrine mechanism to promote muscle anabolism. This paracrine/endocrine mechanism could work in parallel with secretion of typical growth factors such as IGF-1 [46] and other myokines including IL-6 [47], IL-10 [48], irisin [49], and meteorin-like [50].

3 Conclusions

The integrin/adhesion-like properties of aGPCRs make for an easy transition into the biology of skeletal muscle. Integrin signaling is an established "load-sensitive" pathway associated with muscle anabolism; however, direct mechanisms are currently unclear. aGPCRs may represent a novel functional unit to ensure adequate and reliable translation of mechanical stimuli into anabolic signaling events. The second role for aGPCRs is in muscle precursor cell function including regeneration after injury. The investigation of GPR56, BAI3, and CD97 function in muscle is the start of an understanding for the role of these novel receptors. Interestingly, despite a small literature base, aGPCRs play a role in both whole muscle in vivo and myoblasts in vitro suggesting their role in regulating myofiber growth and myogenic progenitor function.

In addition to promising functional capacity in skeletal muscle, in terms of pharmacology, GPCRs are often druggable targets due to their expression on the cell membrane. Currently, no pharmacological agents are available to treat muscle disease. Therefore, investigating the role of adhesion, and other GPCR family members, in skeletal muscle is warranted and could lead to new pharmacol targets to improve muscle anabolism.

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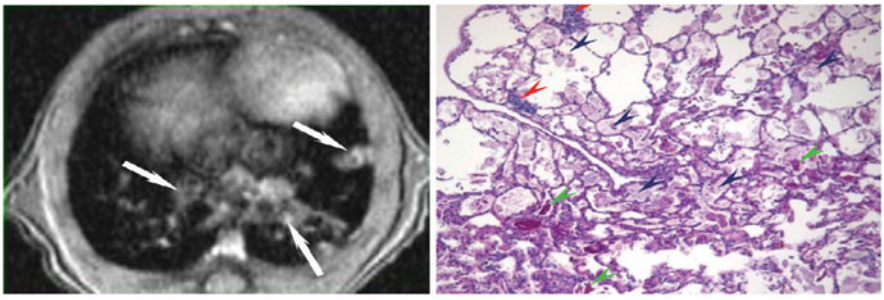
Adhesion GPCR Function in Pulmonary Development and Disease

Marie-Gabrielle Ludwig, Klaus Seuwen, and James P. Bridges

Graphical Abstract



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Abstract

Classic G-protein-coupled receptors (GPCRs) control multiple aspects of pulmonary physiology as demonstrated by loss-of-function experiments in mice and pharmacologic targeting of GPCRs for treatment of several pulmonary diseases. Emerging data demonstrate critical roles for members of the adhesion GPCR (aGPCR) family in pulmonary development, homeostasis, and disease. Although this field is still in its infancy, this chapter will review all available data regarding aGPCRs in pulmonary biology, with a particular focus on the aGPCR for which the most substantial data to date exist: Adgrf5.

Keywords

Lung development • Lung homeostasis • Lung disease • COPD • Pulmonary surfactant • Alveolar epithelium

1 Introduction: Overview of Lung Development

The lung is comprised of approximately 40 distinct cell types that are derived from the endoderm (epithelial cells) or the mesoderm (fibroblasts, vascular and airway smooth muscle cells, pericytes, endothelial cells, and mesothelial cells). A complex interplay of multiple signaling pathways between epithelial and mesenchymal cells is essential for branching morphogenesis and maturation of the lung (reviewed in [1]). Anatomically speaking, the lung can be broadly parsed into two compartments: the proximal and distal lung. Epithelial cell types within the proximal lung include basal cells, club cells, ciliated cells, mucous cells, and neuroendocrine cells, while the distal lung epithelium consists of two epithelial cell types, type I and type II epithelial cells. Structural and functional maturation of the developing distal lung is required for the transition of a fluid-filled fetal lung to one that is equipped for air breathing at birth. Key processes during this period of development include: (1) thinning of the mesenchyme within the presumptive alveolar septae, culminating in a tight juxtaposition of the capillary vascular bed with the distal epithelium to promote efficient gas exchange at birth, (2) differentiation of alveolar type 1 (AT1) and type 2 epithelial cells (AT2), and (3) maturation of the pulmonary surfactant system within AT2 epithelial cells. Defects in any one of these processes contribute to pulmonary dysfunction at birth or in early perinatal life.

At birth, the lung must rapidly transition from a fluid-filled organ to one that is compatible with air breathing. This multifaceted process is achieved through increased respiratory rates, closing of the ductus arteriosus resulting in increased blood flow through the pulmonary vasculature, adsorption of fluid from the airways and airspaces via active trans-epithelial fluid transport and lymphatic drainage, and increased secretion of pulmonary surfactant from AT2 cells. Surfactant is a mixture of 90 % lipids/10 % protein by weight and is unequivocally required for air breathing as evidenced by the fact that premature infants are susceptible to respiratory distress at birth due to insufficient surfactant levels in the distal airspaces. Following birth, the lung continues to grow and mature as nascent alveoli are formed in the distal lung, a process that continues throughout childhood and adolescence in humans [2]. Recent studies have provided insight into the multiple stem/progenitor cell populations that exist in the fetal lung to promote branching morphogenesis and differentiation and in the adult lung to mediate homeostasis and repair following injury (reviewed in [3]). The potential role of aGPCR family members in these developmental, homeostatic, and reparative processes in the lung is just beginning to be explored.

2 Non-adhesion GPCRs in Lung Homeostasis and Disease

Several classic GPCRs, including members of the adrenergic, muscarinic, protease-activated receptor (PAR), endothelin, and purinergic families, regulate various aspects of pulmonary physiology including airway and vascular tone, mucous production, and modulation of the immune system (reviewed in [4–6]). The clinical importance of the adrenergic and muscarinic GPCR families in pulmonary disease is exemplified by the fact that agonists and antagonists for this family of receptors comprise the mainstay therapy for chronic lung diseases including chronic obstructive pulmonary disease (COPD) and asthma [4, 7].

Recent data implicate bitter taste GPCRs, specifically those in the TAS2R family, as regulators of airway smooth muscle tone in mice and humans [8, 9]. Bitter taste GPCRs are expressed on solitary chemosensory epithelial cells lining the digestive and respiratory tract, ciliated cells in the upper airways, airway smooth muscle cells, and on subsets of immune cells [10]. Stimulation of TAS2Rs with agonists including saccharin, chloroquine, or denatonium elicited increased intracellular calcium in a Gβγ-, PLCβ-, and IP3-receptor-dependent manner that resulted in relaxation of isolated smooth muscle cells and decreased airway contraction in a mouse model of allergen-induced asthma [8]. This area of research represents an interesting and unexpected role for bitter taste GPCRs in airway physiology, opening up potential new avenues for pharmacologic intervention in pulmonary disease.

3 Adhesion GPCR Expression and Function in the Developing Lung and in Adult AT2 Cells

Review of the published literature for all identified aGPCRs revealed that three receptors in this GPCR subfamily are expressed in the developing lung including Adgrf5, Adgra2, and Adgrc1 [11–13]. To determine the repertoire of aGPCR expression in distal pulmonary epithelial cells, we performed a GPCR screen on isolated murine AT2 cells using a microfluidic card-based approach. As shown in Fig. 1a, several aGPCRs were detected in wild type (WT) AT2 cells, in addition to Adgfr5, including Adgrg1, Adgre5, Adgrc1, Adgrl2, Adgrl4, Adgra3, Adgre1, Adgrg3, Adgrd1, Adgra2, Adgrl3, and Adgrc3. Of the aGPCRs that were detected at a level consistent with potential functionality (Adgrf5, Adgrg1, Adgre5, and Adgrc1), two have reported lung phenotypes following gene deletion: Adgrf5 and Adgrc1 [11, 14]. We performed qPCR analysis on separate samples of murine WT AT2 cells (1) to confirm the top five expressing aGPCRs identified in the microfluidic screen data, (2) to analyze an aGPCR that was absent from the chip (Adgrg6), and (3) to determine the relative expression levels of the G alpha protein family members Gnaq, Gna11, Gna12, Gna13, and Gna15. As shown in Fig. 1b, the top five highly expressed aGPCRs identified in the microfluidic screen were confirmed by conventional qPCR analysis. Of the Gna proteins, Gna11 is the most abundant isoform, followed by Gna13, Gna12, and Gnaq. Gna15 mRNA levels are barely detectable in AT2 cells (Fig. 1c), suggesting little if any functionality of this Gna family member in AT2 cell function. Below is a summary of the current published data regarding the role of aGPCRs in pulmonary fetal and perinatal development.

3.1 Adgrf5 (Gpr116)

Adgrf5 mRNA is developmentally regulated in the murine lung, reaching peak expression in newborn animals with maintenance of high expression levels throughout adulthood [11]. The sharp increase in Adgrf5 expression in the late gestation lung is temporally consistent with maturation of the surfactant system in AT2 epithelial cells. Regarding cell-specific expression, Adgrf5 mRNA is readily detected in pulmonary endothelial cells and alveolar epithelial cells. Expression of Adgrf5 protein in AT2 cells has been demonstrated by multiple groups [11, 15, 16], while Adgrf5 protein expression in pulmonary endothelial cells remains controversial; Adgrf5 protein has been detected in areas consistent with endothelial cells in mouse and rat lung sections using immunohistochemical staining approaches [15, 16], while Adgrf5 protein was undetectable in isolated CD31+ primary mouse lung endothelial cells [11]. Interestingly, a recent report demonstrated a role for Adgrf5 in regulating blood-brain barrier permeability and retinal regrowth following oxygen-induced injury [17]. A definitive role for Adgrf5 in pulmonary endothelial cells has yet to be determined.

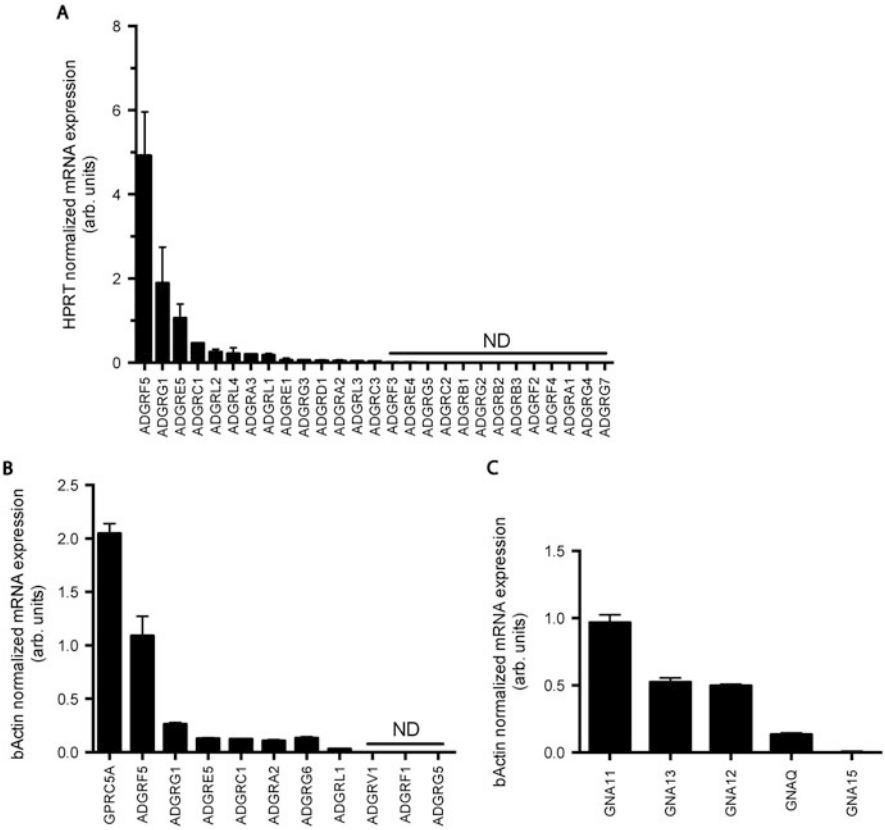

Fig. 1 aGPCR and G-protein alpha mRNA expression in alveolar type 2 (AT2) epithelial cells. (**a**) aGPCR expression in primary wild type, murine AT2 cells isolated from 4-week-old animals as determined by qPCR-based microfluidic card platform (TaqMan® Array Mouse GPCR Panel, Thermo Fisher Scientific). Samples were analyzed in triplicate and data were normalized to HPRT expression. *ND* not detected. (**b**) Taqman-based qPCR validation of aGPCR expression in murine AT2 cells. Samples were analyzed in triplicate and data were normalized to β-actin expression. (**c**) Expression of selected G-protein alpha subunit mRNAs in murine AT2 cells by TaqMan®-based qPCR method. Samples were analyzed in triplicate and data were normalized to β-actin expression

Despite the developmental increase in Adgrf5 mRNA expression in whole lung tissue and enrichment of Adgrf5 in AT2 cells, fetal lung development is normal in the absence of Adgrf5 and Adgrf5$^{-/-}$; mice are born at expected Mendelian ratios. While Adgrf5$^{-/-}$ mice do not display any overt respiratory phenotype at birth or in the early postnatal period, the pulmonary phenotype in juvenile and adult mice is profound and is discussed in detail in the following section.

3.2 Adgrg6 (Gpr126)

In addition to expression in the heart and peripheral nervous system, Adgrg6 mRNA is detected at high levels in rat and mouse lungs [12, 13]. In the developing lung, Adgrg6 mRNA expression appears to be enriched pericytes and smooth muscle cells (mural cells) at embryonic day 16.5 (E16.5) (unpublished data, https://research.cchmc.org/pbge/lunggens/). Germline deletion of Adgrg6 in mice results in uniform embryonic lethality associated with cardiac defects in one line [18] and partial embryonic lethality in a separate line [19]. Recent reports have identified laminin-211 as an Adgrg6 ligand in the developing peripheral nervous system of mice [20]. While Adgrg6 is known to be important for peripheral nervous system and heart development, the cell-specific expression pattern and potential function of this receptor in pulmonary tissues has yet to be explored.

3.3 Adgrc1 (Celsr1)

Adgrc1 has been implicated in planar cell polarity and is abundantly expressed in the embryonic murine lung. While Adgrc1$^{-/-}$ mice are viable and devoid of gross respiratory deficits, embryonic Adgrc1$^{-/-}$ mice have hypoplastic lungs characterized by condensed mesenchyme and decreased distal airspaces [14]. Alveolar type II cell maturation, as demonstrated by expression of the differentiation marker surfactant protein C, appeared to be normal although a thorough assessment of AT2 epithelial cell maturation was not performed in this particular study.

4 Adhesion GPCR Function in Lung Cancer and Disease

According to the American Cancer Society, lung cancer is the second most common type of cancer in both men and women and is the leading cause of cancer death in both sexes. To date, six aGPCRs have been associated with human lung cancer, either in primary tissues or in transformed cell lines. Adgrg2 (Gpr64) expression was found to be upregulated in human lung carcinomas and in transformed lung cancer cell lines; inhibition of Adgrg2 led to decreased cell growth and metastasis, associated with reduced cellular matrix protein degradation and invasiveness in vitro [21]. Similarly, Adgrf1 (Gpr110) has been identified as an oncogene in a mouse mutagenesis study and was found to be overexpressed in pulmonary adenocarcinoma specimens in two separate studies [22, 23]. A high frequency of somatic mutations in Adgrg5 (Gpr114) have been associated with air pollution-related lung cancer in a cohort of patients in China [24]. Expression of Adgrb1 (Bai1) mRNA was detected in resected pulmonary adenocarcinoma specimens and expression levels correlated with high vascular density in comparison to Adgrb1-negative carcinomas [25]. Adgrb3 (Bai3), in combination with CDX2 and VIL1 expression, serves as a diagnostic lung cancer biomarker, differentiating highly aggressive small cell lung carcinomas (SCLC) from large-cell neurocrine carcinomas (LCNEC)

[26]. In addition to its role in alveolar homeostasis, Adgrf5 has also been associated with lung cancer as it was found to be overexpressed in adenocarcinomas [27]. The precise role of the aforementioned aGPCRs in the initiation and/or progression of lung cancer remains unknown and requires further mechanistic studies.

In addition to the association of certain aGPCRs with lung tumorigenesis, three aGPCR family members, Adgrg6, Adgrg1, and Adgrf5, have been associated with pulmonary disease in humans and/or have been experimentally proven to be regulators of pulmonary homeostasis in animal models. Below is a summary of these aGPCRs in relation to perinatal and adult lung disease.

4.1 Adgrg6 (Gpr126)

As stated previously, Adgrg6 mRNA is highly expressed in embryonic and perinatal mouse lung tissue with enrichment in pericytes and smooth muscle cells at E16.5. Interestingly, two independent studies have reported single nucleotide polymorphisms (SNPs) in the Adgrg6 locus associated with severe chronic obstructive pulmonary disease (COPD) in humans [28, 29]. While a specific lung phenotype has not been reported in Adgrg6$^{-/-}$ mice, based on human genetic data, it is interesting to speculate that Adgrg6 may play an important role in adult pulmonary function and/or disease.

4.2 Adgrg1 (Gpr56)

Our aGPCR expression screen determined that Adgrg1 mRNA is highly expressed in murine AT2 cells (Fig. 1a, b). Loss-of-function studies demonstrated that Adgrg1-null mice are viable and display central nervous system defects that closely recapitulate those observed in human patients with mutations in the Adgrg1 locus [30]. While pulmonary defects in Adgrg1$^{-/-}$ mice have not been reported, decreased expression of Adgrg1 has been associated with extracellular matrix production and migration in primary fibroblasts isolated from patients with idiopathic pulmonary fibrosis (IPF), a devastating lung disease with a mean survival rate of approximately 5 years [31]. Furthermore, methylation of the Adgrg1 promoter was found in the lung adenocarcinoma epithelial cell line A549 treated with the chemotherapeutic cisplatin [32], suggesting that modulation of Adgrg1 levels may contribute to fibroblast migration/invasion in the context of lung fibrosis, and/or tumorigenesis when repressed in pulmonary epithelial cells.

4.3 Adgrf5 (Gpr116)

To ascertain the function of Adgrf5, transgenic mice were generated in which exon17 of Adgrf5, encoding the seven transmembrane domains of the receptor, was deleted from the germline (Adgrf5$^{\Delta exon17}$). Homozygous loss of Adgrf5 in this

model resulted in a fully penetrant, rapid, and progressive accumulation of pulmonary surfactant in the distal airspaces and lung tissue [11] (Fig. 2). This phenotype is accompanied by massive inflammatory cell infiltrates, including lipid-laden alveolar macrophages, neutrophils, and B-cells, as evidenced by histopathological analysis and the predominance of inflammatory gene induction in microarray analysis comparing WT and Adgrf5$^{-/-}$ AT2 cells from our previous study [11]. In Adgrf5$^{\Delta exon17}$ mice, increases in the predominant surfactant phospholipid species, dipalmitoylphosphatidylcholine (SatPC), were first detected as early as 2 days of age. Augmented alveolar and tissue surfactant phospholipid pools correlated with age in Adgrf5$^{\Delta exon17}$ mice, associated with lipid-laden ("foamy") alveolar macrophages, increased inflammatory infiltrates and alveolar simplification (emphysematous-like structural alterations), ultimately resulting in compromised gas exchange and respiratory distress in aged mice. The primary pulmonary pathological features in Adgrf5$^{\Delta exon17}$ mice were remarkably consistent with three other mouse models of Adgrf5 deficiency in which distinct exons were targeted [16, 17, 33].

Analysis of surfactant lipid and protein composition in Adgrf5$^{-/-}$ mice revealed commonalities as well as differences between the distinct models. For example, all of the major phospholipid species present in alveolar surfactant, namely, phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylserine (PS),

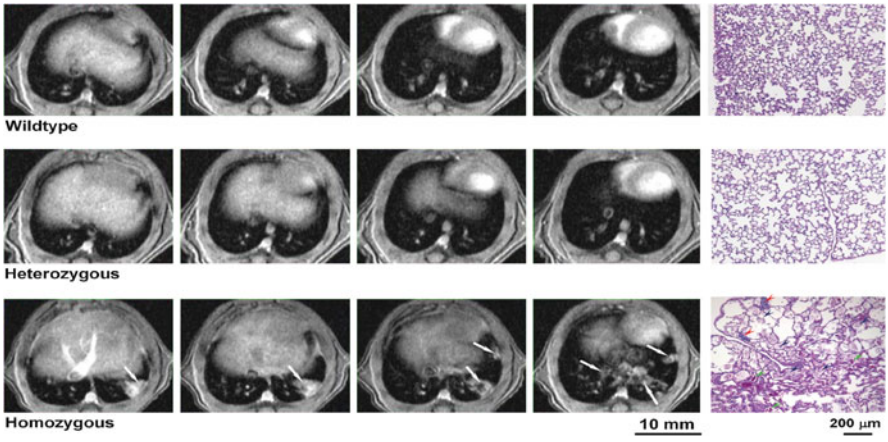

Fig. 2 Pulmonary imaging and pathology of adult Adgrf5$^{-/-}$ mice. Analysis of Adgrf5$^{-/-}$ mice at 3 months of age by magnetic resonance imaging (MRI) and histology. (*Left*) Axial MRI images from three representative mice. Acquisitions were performed at 4.7 T on spontaneously breathing, isoflurane-anesthetized animals without administration of contrast material. A single-slice image (0.75 mm thickness) was acquired in 74 seconds. Fluid signals (*white arrows*) were seen only in homozygous Adgrf5$^{-/-}$ mice. (*Right*) Corresponding PAS-stained histological slices. Lung anatomy was normal in wild-type and heterozygous animals. In contrast, eosinophilic amorphous material in the alveoli (*green arrowheads*), phagocytic macrophages (*blue arrowheads*), and infiltration foci (*red arrowheads*) were detected in the lungs of homozygous Adgrf5$^{-/-}$ knockout mice

phosphatidylinositol (PI), and phosphatidylethanolamine (PE), were increased in both Adgrf5$^{-/-}$ mouse lines for which they were reported, with modest increases noted in PS, PI, and PE ratios in one line [16] and lyso-PC in another [11] between knockout and control animals. Interestingly, a mass spectrometry-based lipidomic analysis showed alterations in the saturation status of individual PC and lyso-PC species in Adgrf5$^{-/-}$ surfactant, with a marked shift from the saturated and monosaturated species normally found in wild type surfactant to polyunsaturated species [16]. Large increases in cholesterol neutral lipid were also detected in Adgrf5$^{-/-}$ lavage fluid of adult mice [16]. It remains to be determined if alterations in the saturation status of surfactant phospholipids or increased cholesterol levels in Adgrf5$^{-/-}$ mice impact surfactant function.

In contrast to the lipidosis phenotype, surfactant protein levels differed across the Adgrf5$^{-/-}$ models. In two of the knockout lines, surfactant protein levels were increased proportionate to phospholipid levels, while in two other lines, surfactant proteins appeared discordant with phospholipid levels [11, 16]. The reason for the reported discrepancy is unknown but may be related to differences in sample isolation, preparation, and/or normalization procedures among individual labs. Irrespective of surfactant protein levels, mRNA levels for the four major surfactant proteins were unchanged in Adgrf5$^{-/-}$ mice [11, 16, 34]. In addition to the phospholipidosis phenotype, Ariestanti et al. have reported that the emphysematous-like alterations in Adgrf5$^{-/-}$ mice were associated with increased oxidative stress, phospholipid oxidation and increased expression of activated MMP2 and MMP9, two metalloproteinases associated with macrophage activation, and lung remodeling [33]. The temporal order of the pathological sequelae downstream of alveolar surfactant accumulation in the lungs of Adgrf5$^{-/-}$ mice greater than 1 month of age has yet to be determined.

Epithelial-specific deletion of Adgrf5 using Adgrf5$^{flox/flox}$ mice mated to mice carrying *Sftpc*-rtTA and *TetO7*-Cre alleles, which specifically targets AT2 cells [16], or to mice with an *Shh*Cre allele (our unpublished data), which uniformly targets all epithelial lineages in the lung, results in a phenotype nearly identical to that observed in Adgrf5$^{-/-}$ mice. Surprisingly, given the high level of Adgrf5 mRNA expression in endothelial cells, deletion of Adgrf5 in this lineage had no effect on pulmonary or alveolar homeostasis at the histological level [16]; likewise, alveolar surfactant phospholipid levels were normal in 4-week-old mice in which Adgrf5 was deleted in endothelial cells using the *Tie2*Cre driver (our unpublished data). Furthermore, bone marrow transplant of WT donor cells into Adgrf5$^{-/-}$ hosts failed to rescue the lung phenotype [16]. Taken together, these data demonstrate that cell-specific expression of Adgrf5 in alveolar epithelial cells is critical for alveolar homeostasis.

Pulmonary surfactant levels within the lumen of the alveolus are tightly controlled through a balance of three interconnected mechanisms: (1) surfactant synthesis and secretion by the AT2 cell; (2) surfactant uptake, recycling, and secretion by the AT2 cell; and (3) surfactant uptake and catabolism by alveolar macrophages. Perturbations in any one of these three mechanisms can result in either surfactant overload or surfactant deficiency in the distal lung. The surfactant phospholipidosis

phenotype in Adgrf5$^{-/-}$ mice is phenotypically similar to that observed in mice homozygous deficient for Sftpd [35, 36], Csf [37], Csf2rb [38], or Slc34a2 [39]; however, the severity and mechanistic basis of the phenotype varies significantly between the models with Adgrf5$^{-/-}$ and Slc34a2$^{-/-}$ being the most severe. Patients with mutations in Csf2rb in Csf2ra, or in those that autoantibodies against Csf2, present with a surfactant overload pathology that closely resembles the disease seen in correspondingly targeted mice, a rare disease known as pulmonary alveolar proteinosis (PAP) (for review, see [40]). Studies in genetically modified mice, and in primary cells from patients with PAP, have shown that surfactant accumulation related to defects in the Csf2 signaling pathway are due to alveolar macrophage dysfunction resulting in impaired surfactant uptake/catabolism [41, 42]. Conversely, in vivo surfactant catabolism studies in Sftpd$^{-/-}$ mice have demonstrated that the primary defect lies in decreased surfactant uptake by alveolar epithelial cells, owed to altered ultrastructure of surfactant in the airway [43, 44].

The endogenous ligand and intracellular pathways by which Adgrf5 signals in AT2 cells to control alveolar homeostasis have yet to be identified. In the transformed human breast cancer cell line MDA-MB-231, endogenous Adgfr5 regulates cell motility, F-actin dynamics, and mammary tumor metastases in a Gq-p63RhoGEF-RhoA-dependent manner [45]. Whether these intracellular pathways are operative in AT2 cells downstream of Adgrf5 to modulate surfactant secretion and/or uptake remains unknown. Hirose et al. have postulated that Sftpd may be a putative ligand for Adgrf5 based on co-immunoprecipitation of Sftpd with Adgrf5 in a heterologous overexpression system [34]. The fact that Sftpd$^{-/-}$ mice and Adgrf5$^{-/-}$ mice display a similar pulmonary phenotype, albeit at differing degrees of severity, and the abundance of Sftpd protein in the alveolar lumen supports this hypothesis. However, transgenic mice that overexpress Sftpd in the distal lung do not have decreased alveolar surfactant pools nor do they exhibit signs of respiratory distress [46], as would be predicted if Sftpd activated Adgfr5 signaling based on Adgrf5 loss-of-function data. In addition, it remains to be determined if purified Sftpd protein is sufficient to activate G-protein signaling downstream of Adgrf5 in transfected cells or in primary AT2 cells.

5 Transcriptional Responses in Adgrf5$^{-/-}$ Lung Tissue

We undertook an additional microarray analysis of Adgrf5$^{-/-}$ lung tissue from 3.5-month-old mice versus WT controls in order to determine the transcriptional responses in Adgrf5$^{-/-}$ lungs at the whole lung level (Table 1). In addition to induction of inflammatory pathways consistent with previously published data [33], several additional physiological responses were identified including increased expression of genes involved in lipid synthesis and vesicle secretion (LIPA, LRP2, LCN2, FABP5, PLIN2, LPL, FADS2, SCD1, LPCAT, members of the SLC27A and SCAMP families, and the V-ATPase ATP6V), as well as increased expression of CSF2, CSF2RA, two genes known to be essential for macrophage function, and surfactant uptake/catabolism in both mice and humans [37, 47, 48].

Table 1 Genes differentially expressed in adult Adgrf5$^{-/-}$ lung tissue

Gene symbol	Adgrf5 KO/WT log fold change	Description	No. probe sets[a]
Aqp11	−1.3	Aquaporin 11	1
Aqp3	2.2	Aquaporin 3	2
Aqp9	2.4	Aquaporin 9	2
Atp1a1	0.5	ATPase, Na+/K+ transporting, alpha 1 polypeptide	1
Atp1a2	−0.1	ATPase, Na+/K+ transporting, alpha 2 polypeptide	4
Atp1a3	3.5	ATPase, Na+/K+ transporting, alpha 3 polypeptide	1
Atp1a4	−0.7	ATPase, Na+/K+ transporting, alpha 4 polypeptide	1
Atp1b1	0.1	ATPase, Na+/K+ transporting, beta 1 polypeptide	1
Atp1b2	0.4	ATPase, Na+/K+ transporting, beta 2 polypeptide	1
Atp1b3	0.2	ATPase, Na+/K+ transporting, beta 3 polypeptide	1
Atp2a2	−0.8	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	2
Atp2b1	−0.2	ATPase, Ca++ transporting, plasma membrane 1	1
Atp2b4	−1.3	ATPase, Ca++ transporting, plasma membrane 4	1
Atp2c1	−0.6	ATPase, Ca++-sequestering	4
Atp6ap1	0.2	ATPase, H+ transporting, lysosomal accessory protein 1	1
Atp6ap2	0.2	ATPase, H+ transporting, lysosomal accessory protein 2	2
Atp6v0a1	0.6	ATPase, H+ transporting, lysosomal V0 subunit A1	3
Atp6v0b	0.4	ATPase, H+ transporting, lysosomal V0 subunit B	3
Atp6v0c	0.6	ATPase, H+ transporting, lysosomal V0 subunit C	3
Atp6v0d1	0.6	ATPase, H+ transporting, lysosomal V0 subunit D1	1
Atp6v0d2	1.6	ATPase, H+ transporting, lysosomal V0 subunit D2	2
Atp6v0e2	0.3	ATPase, H+ transporting, lysosomal V0 subunit E2	1
Atp6v1a	0.4	ATPase, H+ transporting, lysosomal V1 subunit A	2
Atp6v1b2	0.8	ATPase, H+ transporting, lysosomal V1 subunit B2	3
Atp6v1c1	0.7	ATPase, H+ transporting, lysosomal V1 subunit C1	3
Atp6v1c2	0.4	ATPase, H+ transporting, lysosomal V1 subunit C2	1
Atp6v1e1	0.4	ATPase, H+ transporting, lysosomal V1 subunit E1	3
Atp6v1f	0.5	ATPase, H+ transporting, lysosomal V1 subunit F	1
Atp6v1g1	0.3	ATPase, H+ transporting, lysosomal V1 subunit G1	1
Cacna1a	0.4	Calcium channel, voltage-dependent, P/Q type, alpha 1A subunit	1
Cacna1c	−0.3	Calcium channel, voltage-dependent, L type, alpha 1C subunit	1
Cacna1d	−0.5	Calcium channel, voltage-dependent, L type, alpha 1D subunit	1
Cacna2d1	−0.7	Calcium channel, voltage-dependent, alpha 2/delta subunit 1	5
Cacna2d4	−0.8	Calcium channel, voltage-dependent, alpha 2/delta subunit 4	1

(continued)

Table 1 (continued)

Gene symbol	Adgrf5 KO/WT log fold change	Description	No. probe sets[a]
Cacnb2	−0.3	Calcium channel, voltage-dependent, beta 2 subunit	1
Cacnb3	0.7	Calcium channel, voltage-dependent, beta 3 subunit	1
Cacnb4	−1.4	Calcium channel, voltage-dependent, beta 4 subunit	2
Car10	0.6	Carbonic anhydrase 10	1
Car13	1.2	Carbonic anhydrase 13	3
Car14	0.5	Carbonic anhydrase 14	1
Car2	0.2	Carbonic anhydrase 2	1
Car4	−0.6	Carbonic anhydrase 4	2
Car5b	0.4	Carbonic anhydrase 5b, mitochondrial	1
Car7	0.2	Carbonic anhydrase 7	1
Car8	0.3	Carbonic anhydrase 8	3
Cftr	−0.8	Cystic fibrosis transmembrane conductance regulator homologue	1
Clca1	1.2	Chloride channel calcium activated 1	3
Clca3	3.8	Chloride channel calcium activated 3	2
Clca5	1.6	Chloride channel calcium activated 5	1
Clcc1	−0.8	Chloride channel CLIC-like 1	1
Clcn5	0.6	Chloride channel 5	3
Clcn7	0.6	Chloride channel 7	1
Clic1	0.3	Chloride intracellular channel 1	1
Clic4	−0.1	Chloride intracellular channel 4 (mitochondrial)	2
Clic5	−0.3	Chloride intracellular channel 5	4
Clic6	0.3	Chloride intracellular channel 6	2
Ece1	−1.2	Endothelin converting enzyme 1	1
Ece2	0.9	Endothelin converting enzyme 2	1
Edn1	0.5	Endothelin 1	1
Ednra	−1.0	Endothelin receptor type A	3
Ednrb	−1.6	Endothelin receptor type B	3
Fxyd2	1.0	FXYD domain-containing ion transport regulator 2	3
Fxyd3	0.3	FXYD domain-containing ion transport regulator 3	1
Fxyd4	2.5	FXYD domain-containing ion transport regulator 4	1
Fxyd5	0.6	FXYD domain-containing ion transport regulator 5	1
Fxyd6	1.0	FXYD domain-containing ion transport regulator 6	1
Hvcn1	2.1	Hydrogen voltage-gated channel 1	1
Kcnab1	0.4	Potassium voltage-gated channel, shaker-related subfamily, beta member 1	1
Kcnab2	1.7	Potassium voltage-gated channel, shaker-related subfamily, beta member 2	1
Kcnd3	−0.5	Potassium voltage-gated channel, Shal-related family, member 3	1
Kcne2	−0.5	Potassium voltage-gated channel, Isk-related subfamily, gene 2	1

(continued)

Table 1 (continued)

Gene symbol	Adgrf5 KO/WT log fold change	Description	No. probe sets[a]
Kcnf1	−1.4	Potassium voltage-gated channel, subfamily F, member 1	1
Kcnh1	0.4	Potassium voltage-gated channel, subfamily H (eag-related), member 1	1
Kcnip4	−0.9	Kv channel interacting protein 4	3
Kcnj13	−0.6	Potassium inwardly-rectifying channel, subfamily J, member 13	1
Kcnj15	−1.3	Potassium inwardly-rectifying channel, subfamily J, member 15	1
Kcnj5	-0.6	Potassium inwardly-rectifying channel, subfamily J, member 5	1
Kcnj8	0.4	Potassium inwardly-rectifying channel, subfamily J, member 8	1
Kcnk1	0.2	Potassium channel, subfamily K, member 1	1
Kcnk13	0.3	Potassium channel, subfamily K, member 13	1
Kcnk2	−1.0	Potassium channel, subfamily K, member 2	1
Kcnmb2	−0.5	Potassium large conductance calcium-activated channel, subfamily M, beta member 2	2
Kcnn4	3.1	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4	2
Kcnq1	0.4	Potassium voltage-gated channel, subfamily Q, member 1	1
Kcnq4	−1.3	Potassium voltage-gated channel, subfamily Q, member 4	1
Kcnq5	−1.6	Potassium voltage-gated channel, subfamily Q, member 5	2
Kcnt1	−2.8	Potassium channel, subfamily T, member 1	1
Kctd1	0.4	Potassium channel tetramerization domain containing 1	1
Kctd11	0.3	Potassium channel tetramerization domain containing 11	1
Kctd17	0.6	Potassium channel tetramerization domain containing 17	1
Kctd18	−0.7	Potassium channel tetramerization domain containing 18	2
Kctd20	0.3	Potassium channel tetramerization domain containing 20	1
Kctd3	−0.7	Potassium channel tetramerization domain containing 3	1
Kctd5	0.2	Potassium channel tetramerization domain containing 5	2
Kctd6	−0.1	Potassium channel tetramerization domain containing 6	1
Kctd8	−1.4	Potassium channel tetramerization domain containing 8	1

(continued)

Table 1 (continued)

Gene symbol	Adgrf5 KO/WT log fold change	Description	No. probe sets[a]
Kctd9	−0.2	Potassium channel tetramerization domain containing 9	1
Nppb	2.4	Natriuretic peptide precursor type B	1
Nppc	1.3	Natriuretic peptide precursor type C	1
Npr2	−0.4	Natriuretic peptide receptor 2	1
Npr3	−1.1	Natriuretic peptide receptor 3	3
Ptgds	0.8	Prostaglandin D2 synthase (brain)	1
Ptger2	0.2	Prostaglandin E receptor 2 (subtype EP2)	1
Ptger3	1.0	Prostaglandin E receptor 3 (subtype EP3)	2
Ptger4	0.8	Prostaglandin E receptor 4 (subtype EP4)	2
Ptges	1.1	Prostaglandin E synthase	3
Ptges3	−0.2	Prostaglandin E synthase 3 (cytosolic)	2
Ptgfr	−0.7	Prostaglandin F receptor	3
Ptgir	1.9	Prostaglandin I receptor (IP)	1
Ptgr1	0.3	Prostaglandin reductase 1	1
Ptgs1	1.1	Prostaglandin-endoperoxide synthase 1	2
Ptgs2	0.3	Prostaglandin-endoperoxide synthase 2	1
Sclt1	−2.0	Sodium channel and clathrin linker 1	2
Scn1b	0.6	Sodium channel, voltage-gated, type I, beta	1
Scn2b	0.9	Sodium channel, voltage-gated, type II, beta	1
Scn3a	−1.0	Sodium channel, voltage-gated, type III, alpha	1
Scn3b	−1.6	Sodium channel, voltage-gated, type III, beta	2
Scn7a	−1.4	Sodium channel, voltage-gated, type VII, alpha	3
Scn8a	−3.2	Sodium channel, voltage-gated, type VIII, alpha	1
Scnm1	0.3	Sodium channel modifier 1	1
Slc12a2	−0.5	Solute carrier family 12, member 2	3
Slc12a4	0.4	Solute carrier family 12, member 4	1
Slc12a5	−0.8	Solute carrier family 12, member 5	1
Slc12a6	−0.5	Solute carrier family 12, member 6	3
Slc12a9	0.4	Solute carrier family 12 (potassium/chloride transporters), member 9	1
Slc19a1	0.3	Solute carrier family 19 (sodium/hydrogen exchanger), member 1	1
Slc19a3	−0.8	Solute carrier family 19 (sodium/hydrogen exchanger), member 3	1
Slc26a11	0.4	Solute carrier family 26, member 11	1
Slc26a3	0.5	Solute carrier family 26, member 3	2
Slc26a4	3.8	Solute carrier family 26, member 4	1
Slc27a1	0.8	Solute carrier family 27 (fatty acid transporter), member 1	2
Slc27a2	0.7	Solute carrier family 27 (fatty acid transporter), member 2	1

(continued)

Table 1 (continued)

Gene symbol	Adgrf5 KO/WT log fold change	Description	No. probe sets[a]
Slc4a1	1.9	Solute carrier family 4 (anion exchanger), member 1	2
Slc4a10	−2.1	Solute carrier family 4, sodium bicarbonate cotransporter-like, member 10	1
Slc4a11	0.4	Solute carrier family 4, sodium bicarbonate transporter-like, member 11	1
Slc4a4	−0.4	Solute carrier family 4 (anion exchanger), member 4	3
Slc4a5	−0.5	Solute carrier family 4, sodium bicarbonate cotransporter, member 5	1
Slc9a1	0.6	Solute carrier family 9 (sodium/hydrogen exchanger), member 1	1

[a]The number of probe sets on the genechip for each gene

Alveolar epithelial cells are critical regulators of fluid balance between the surface epithelium and the surfactant layer present at the air liquid interface. In addition to the lipid transcriptional response, several electrolyte channels and solute transporters were differentially expressed in Adgrf5$^{-/-}$ lung tissue, suggesting adaptation of the lung to maintain fluid homeostasis in the absence of Adgrf5. The major electrolytes involved in maintaining homeostasis of the alveolar fluid layer include Na^+, Cl^-, and K^+. Levels of these ions within the lining fluid are primarily regulated by the following channels and transporters: ENAC, CNG, and Na^+/K^+ ATPase for Na^+; CFTR, CLCs, NKKC and KCC transporters, and $HCO3^-/Cl^-$ exchangers for Cl^-; and voltage-dependent, Ca^{2+}-activated, 2-pore domain, and inward-rectifying channels for K^+.

Management of Na^+ flux is the principal regulator of fluid resorption in the distal lung, in conjunction with aquaporin-mediated water transport. Na^+ trans-epithelial transporters (mainly ENAC, CNG, and Na^+/K^+ ATPase), as well as the major aquaporins, were not modulated at the transcriptional level in Adgrf5$^{-/-}$ mice, which correlates with seemingly normal alveolar fluid clearance in these animals following birth [49, 50].

Although K^+ channels are known to play an important role in ion and liquid transport, only KCNQ5 and KCNK2 were decreased significantly, suggesting a minor role in the Adgrf5$^{-/-}$ phenotype. Similarly, classical Cl^- channels known to maintain electrolyte balance in the alveoli (CFTR, CLCs, NKKC and KCC transporters, $HCO3^-/Cl^-$ exchangers) were not strongly modulated in the lungs of Adgrf5$^{-/-}$ mice. In contrasts, the Ca^{2+}-activated Cl^- channel CLCA3, virtually undetectable in the WT lung, was significantly upregulated in Adgrf5$^{-/-}$ lung tissue, suggesting it may influence currents by modulating the activity, rather than the expression, of Ca^{2+}-activated Cl^- channels present in AT2 pneumocytes [51].

Upregulation the $HCO3^-/Cl^-$ exchangers SLC26A4 and SLC4A1 in Adgrf5$^{-/-}$ lungs may play an important role in fluid as well as acid-base homeostasis in the

distal lung [52, 53]. The observed higher expression of HVCN1 further supports the hypothesis of an alteration in the pH of the alveolar lining fluid, possibly toward a more alkaline state, and the need for adaptations [54]. The increase in regulatory subunits of V-ATPases may be part of these coordinated adjustments for maintenance of extra- and intracellular pH, leading to acidification of lamellar bodies and potentially participating in the increased synthesis, secretion, and/or recycling of surfactant in Adgrf5$^{-/-}$ lungs [55–57].

With regard to Ca^{2+} management, most voltage-dependent Ca^{2+} channels were slightly suppressed in Adgrf5$^{-/-}$ lungs (CACNA/D). However, the regulatory subunit of voltage-dependent calcium channels, CACNB3, was upregulated, while the Ca^{2+}-sequestering ATPase ATP2C1 was downregulated [58], which may result in increased Ca^{2+} entry and cytosolic concentration in AT2 cells, thereby augmenting Ca^{2+} signaling and Ca^{2+}-dependent intracellular processes including membrane and vesicle trafficking [56, 59]. It remains to be determined if membrane and vesicular trafficking are altered in Adgrf5$^{-/-}$ AT2 cells and if these processes contribute to the phospholipidosis phenotype observed in Adgrf5$^{-/-}$ mice.

In summary, these data highlight important transcriptional responses induced by Adgrf5 deficiency, including the modulation of several ion transporters and channels, possibly functioning to promote proper alveolar fluid levels and pH to maintain tissue homeostasis in the context of Adgrf5 loss. Dissecting the molecular mechanisms responsible for these transcriptional responses in Adgrf5 knockout mice requires further study to assess the functionality of channels through modulation with agonists or antagonists and/or through assessment of alveolar fluid clearance capacity in vitro and in vivo. Early responses following Adgrf5 loss may involve sensing changes in oxygenation levels and/or modulation of fluid acid-base balance, linked directly or indirectly to increased surfactant accumulation and associated sequelae observed in the lungs of Adgrf5$^{-/-}$ mice.

6 Conclusions

The field of aGPCR research is still in its infancy, particularly with respect to pulmonary physiology and disease. To date, a handful of aGPCRs have been associated with human lung disease and numerous questions remain as to the precise role of these particular receptors, and others within this family, in the field of pulmonary biology. The aGPCR for which the most lung data exists is Adgrf5, primarily owed to the surprising robust pulmonary phenotype in loss-of-function mouse models. As additional reagents for aGPCRs are generated, including genetic mouse models, and ligands are identified, the contribution of other aGPCRs to various aspects of pulmonary development, function, and disease are likely to be revealed.

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Adhesion GPCRs as Modulators of Immune Cell Function

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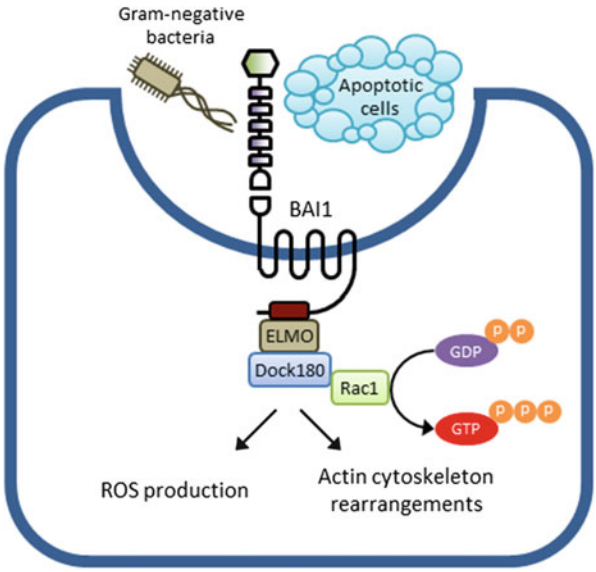
Graphical Abstract



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Contents

Abstract

Immune cells express several adhesion G protein-coupled receptors (aGPCRs), including the ADGRE subfamily members EMR1 (F4/80, ADGRE1), EMR2 (ADGRE2), EMR3 (ADGRE3), EMR4 (FIRE, ADGRE4), and CD97 (ADGRE5), the ADGRB subfamily member BAI1 (ADGRB1), and the ADGRG subfamily members GPR56 (ADGRG1), GPR97 (Pb99, ADGRG3), and GPR114 (ADGRG5). Expression of these molecules in hematopoietic stem and progenitor cells, monocytes/macrophages (Mφs), dendritic cells, granulocytes, and lymphocytes depends on lineage diversification and maturation, making them suitable markers for individual leukocyte subsets (e.g., F4/80 on mouse Mφs). Recent studies revealed intriguing activities of aGPCRs in tolerance induction (EMR1), granulopoiesis (CD97), engulfment of apoptotic cells and bacteria (BAI1), hematopoietic stem cell formation (GPR56), and control of cytotoxicity (GPR56). Here, we review these findings and discuss their biological and translational implications.

Keywords

Adhesion GPCRs • Immunity • Macrophages • Granulocytes • T cells • Phagocytosis

Abbreviations

7TM	Seven transmembrane
Ab	Antibody
ABCA	ATP-binding cassette transporter
ACAID	Anterior chamber-associated immune deviation
Ag	Antigen
AML	Acute myeloid leukemia

BAI	Brain-specific angiogenesis inhibitor
BDCA	Blood dendritic cell antigen
CTF	C-terminal fragment
DC	Dendritic cell
EGF	Epidermal growth factor
ELMO	Engulfment and cell motility
EMR	EGF-like module-containing mucin-like hormone receptor-like
GAIN	GPCR autoproteolysis inducing
GEF	Guanine nucleotide exchange factor
GPCR	G protein-coupled receptor
GPS	GPCR proteolysis site
HDL	High-density lipoprotein
HRM	Hormone receptor motif
HSPC	Hematopoietic stem and progenitor cell
IFN	Interferon
IL	Interleukin
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
Mφ	Macrophages
NK	Natural killer
NTF	N-terminal fragment
PtdSer	Phosphatidylserine
ROS	Reactive oxygen species
SIRS	Systemic inflammatory response syndrome
TGF	Tumor growth factor
TNF	Tumor necrosis factor
Treg	Regulatory T
TSR	Thrombospondin type 1 repeat

1 Adhesion GPCRs in the Immune System

Though regulating numerous cellular processes, G protein-coupled receptors (GPCRs) in the immune system are mostly known for their ability to steer cell migration and homing in response to chemokine gradients [1]. However, leukocytes express many more GPCRs, including members of the adhesion family. In fact, the first aGPCRs that have been described—F4/80 and CD97—are molecules expressed on mouse macrophages (Mφs) and human activated T cells, respectively [2, 3]. Since then, ADGRE, ADGRB, and ADGRG subfamily members on hematopoietic cells have been identified and extensively studied, both in vitro and in vivo. Here, we review current ideas about the role of these molecules in relation to immune cell development and function and discuss the biological and translational implications of these findings.

2 EGF-TM7/ADGRE Subfamily

The five members of the ADGRE subfamily, also known as EGF-TM7 receptors, are encoded by two gene clusters on chromosome 19p13.3 (EMR1 and EMR4) and chromosome 19p13.1 (EMR2, EMR3, and CD97) [4–6]. EGF-TM7 receptors possess N-terminal epidermal growth factor (EGF)-like domains encoded by a single exon. Alternative splicing of these exons in EMR1, EMR2, and CD97 gives rise to isoforms with a different EGF-domain composition. Expression of EMR1–4 is restricted to myeloid cells. In contrast, CD97 is found on virtually all hematopoietic cells as well as on many non-hematopoietic cell types (Fig. 1).

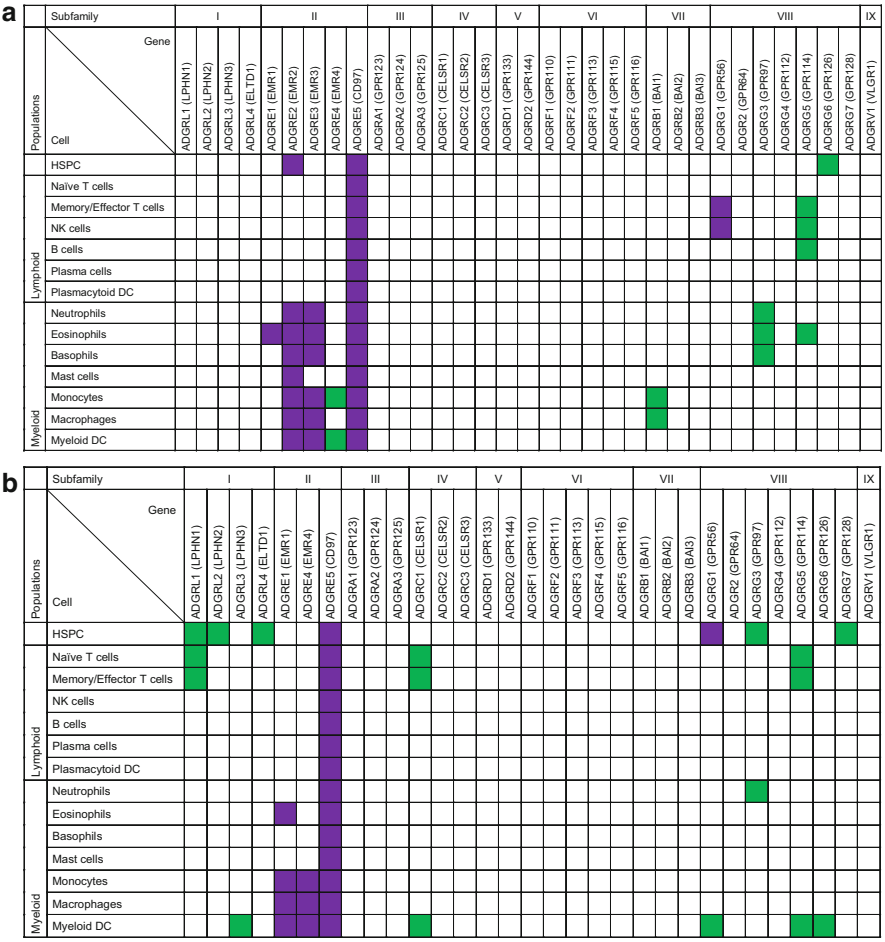

Fig. 1 Expression of adhesion GPCRs in immune cells. Summary of available published data on the expression of aGPCRs in hematopoietic cells in (**a**) human and (**b**) mice, obtained by transcriptional profiling (*green squares*) and protein analysis techniques (*purple squares*). For additional information and references, see the descriptions of individual aGPCRs at www.guidetopharmacology.org/

2.1 EMR1 (F4/80, ADGRE1)

The F4/80 antigen (Ag) was initially described as a novel cell surface marker of mouse Mφs by Jon Austyn and Siamon Gordon in 1981, following the development of the F4/80 monoclonal antibody (mAb) in rats against isolated thioglycollate-elicited mouse peritoneal Mφs [2]. Subsequent experiments established the limited expression pattern of F4/80 in myeloid cells, including monocytes, most tissue Mφs, and some dendritic cells (DCs) [7, 8]. Eosinophils also express the F4/80 Ag, whose expression is enhanced after parasitic infection [9]. No F4/80 reactivity was detected in other cells of the myeloid lineage, such as neutrophilic or basophilic granulocytes and osteoclasts, in cells of the lymphoid lineage, and in non-hematopoietic cells. Due to its robust reactivity and specificity in restricted Mφ subsets, the F4/80 Ag has been extensively used as a tissue Mφ marker in mice ever since [10–12]. The F4/80 Ag is detected as early as embryonic day 8/9 in Mφs in the aorta–gonad–mesonephros region and yolk sac, in fetal livers, and in splenic red pulp and bone marrow before birth [13]. In the adult mouse, F4/80 is weak on circulating monocytes, but is highly expressed on sinusoidal Mϕs in the liver (Kupffer cells), spleen, and adrenal glands and on almost all tissue extravascular Mϕs. F4/80 has also been used to identify tumor-associated Mφs [14].

F4/80 was identified as the murine ortholog of human EMR1 through genomic and phylogenetic analyses [4–6]. F4/80 and EMR1 possess seven and six N-terminal EGF-like domains, respectively [15–17]. They are the only members of the ADGRE subfamily that are not proteolytically processed due to the presence of an atypical GPCR proteolysis site (GPS) sequence. Indeed, both F4/80 and EMR1 are expressed as single-chain polypeptide receptor molecules. Notably, EMR1 is not expressed in Mφs, but instead is a specific marker of human eosinophilic granulocytes [18, 19]. Therefore, it is believed that F4/80 and EMR1 are structural orthologs that possess different cell-type- and species-specific expression patterns and functions.

Several approaches, including Ab treatment and gene targeting, have been used to investigate the physiological function(s) of F4/80 ex vivo and in vivo. Using the F4/80 mAb in an in vitro model of facultative intracellular pathogen infection, Warschkau et al. demonstrated a role for F4/80 in interferon (IFN)-γ production by severe combined immunodeficiency (SCID) mouse splenic cells in response to heat-killed *Listeria monocytogenes* [20]. IFN-γ is critical for the control of bacterial growth during *L. monocytogenes* infection in vivo. It was shown that heat-killed *L. monocytogenes*-stimulated Mϕs secreted tumor necrosis factor (TNF) and interleukin (IL)-12, which activated natural killer (NK) cells to produce IFN-γ. It was concluded that F4/80 is critical in the Mφ–NK cell interaction during *L. monocytogenes* infection, because the F4/80 mAb treatment downregulated TNF, IL-12, and IFN-γ production [20]. The F4/80 molecule was believed to interact with a potential cellular ligand on NK cell, which delivered a stimulatory signal for cytokine release.

Two strains of F4/80 knockout mice were generated using different gene targeting approaches; one involved the production of F4/80–Cre knock-in mice, where the first coding exon of F4/80 was replaced with the cDNA of the Cre recombinase, while the other used a conventional method by deleting the first coding exon [21, 22]. Interestingly, the F4/80-deficient animals were healthy and phenotypically normal. No obvious defect in the maturation and development of tissue Mϕs was noted in the mutant mice either. This indicated that F4/80 is not required for the differentiation and development of cells of the monocyte–Mφ lineage. In contrast to what was found in vitro, the F4/80-deficient animals show normal responses to infectious challenge with *L. monocytogenes*, suggesting a minimum role for F4/80 in the immune responses against *L. monocytogenes* infection in vivo [22].

In another study, the F4/80-deficient mice were employed to confirm the role of F4/80 in the induction of peripheral immune tolerance [21]. Indeed, the F4/80-deficient mice did not develop anterior chamber-associated immune deviation (ACAID) because the animals failed to generate functional Ag-specific efferent $CD8^+$ regulatory T (Treg) cells. Similarly, it was shown that efferent Treg cells were only produced when the APCs are $F4/80^+$ in an in vitro ACAID model using both the F4/80 mAb and F4/80-deficient APC. Furthermore, the F4/80-deficient animals were found unable to produce an efficient immune tolerance response in a low-dose oral tolerance model. Importantly, reconstitution of the F4/80-deficient animals with the $F4/80^+$ APCs restored their ability to induce ACAID and low-dose oral tolerance [21]. Therefore, the F4/80 molecule is required for the generation of Ag-specific efferent Treg cells [23]. The molecular mechanism mediated by the F4/80 receptor to induce peripheral immune tolerance remains elusive, but a putative cellular ligand of F4/80 on interacting immune cells is a likely candidate.

2.2 EMR2 (ADGRE2)

EMR2 seems to be a chimera of CD97 and another EGF-TM7 receptor, EMR3, as a result of a relatively recent evolution, comprising gene conversion and duplication [24]. Indeed, EMR2 shares ~97 % amino acid sequence identity in the EGF-like domains with CD97 and ~90 % identity in the seven-transmembrane (7TM) region with EMR3, respectively [6, 24–26]. This suggests that EMR2 might interact with similar cellular ligand(s) as CD97 while sharing with EMR3 comparable signaling activities. The EMR2 gene is lost in rodents, suggesting a specific need for EMR2 in the immune reactions of human [24].

Unlike F4/80 and human EMR1 in Mφs and eosinophils, EMR2 is more widely expressed in myeloid cells, including monocytes, Mφs, DCs, and granulocytes, suggesting a role for EMR2 in these innate immune cells [27]. Resting or activated lymphocytes do not express any detectable EMR2. $CD16^+$ blood monocytes, Mφs, and BDCA-3^+ myeloid DCs express the highest levels of EMR2. Interestingly, EMR2 expression is upregulated during the differentiation and maturation of Mφs

in vitro, but is downregulated during DC maturation [28]. Lipopolysaccharide (LPS) treatment upregulated EMR2 expression in monocytes and Mφs in an IL-10-dependent manner. In tissues, EMR2 expression was detected in certain Mφ subpopulations of the lung, skin, spleen, tonsil, and placenta, but not in those of the liver and kidney. In inflamed tissues, EMR2 staining was found in subpopulations of Mφs and neutrophils [28]. Recently, high levels of EMR2 were detected in foamy Mϕs in atherosclerotic vessels and in Gaucher cells in the spleen. On the contrary, multiple sclerosis brain foam cells express little if any EMR2 [29].

The full-length EMR2 protein contains a total of five EGF-like motifs. Alternative splicing results in four isoforms: EMR2(EGF1,2), EMR2(EGF1,2,5), EMR2 (EGF1,2,3,5), and EMR2(EGF1–5) [25]. EMR2(EGF1–5) shares with CD97 (EGF1–5) a glycosaminoglycan ligand, chondroitin sulfate B (dermatan sulfate), while EMR2(EGF1,2,5) binds to CD55 with a tenfold weaker affinity than does CD97(EGF1,2,5) [30, 31]. These findings suggest possible overlapping, but also specific functions for EMR2 and CD97.

Studies using the mAb 2A1, which binds downstream of the EGF domains, have demonstrated a role for EMR2 in potentiating the activation and recruitment of human neutrophils [32]. Specifically, ligation of EMR2 by 2A1 enhanced neutrophil adherence and migration under both static and shear stress conditions. In addition, ligation and activation of EMR2 was shown to work synergistically with a number of pro-inflammatory mediators in potentiating neutrophil respiratory burst and degranulation [32]. Patients suffering from noninfectious systemic inflammatory response syndrome (SIRS) and sepsis often die through multiple organ failure, caused by increased numbers and hyperactivation of neutrophils. A more recent study revealed higher EMR2 expression in neutrophils of SIRS patients and a positive correlation between the percentage of $EMR2^{+}$ neutrophils and the occurrence of organ failure. In fact, EMR2 in neutrophil was considered a potential biomarker for SIRS [33]. Huang et al. have investigated the potential role of EMR2 in Mφ activation. They demonstrated that cross-linking of EMR2 in Mφs by 2A1 induced the translocation and colocalization of N-terminal fragment (NTF) and the C-terminal fragment (CTF) within lipid rafts. This in turn led to receptor signaling and production of inflammatory cytokines, such as IL-8 and TNF [34]. Therefore, EMR2 seems to act as an activation receptor in myeloid cells, including neutrophils and Mφs.

Notably, a search for genetic variants causing the autoinflammatory disorder vibratory urticaria recently identified a missense substitution 26 amino acids upstream from the GPS in EMR2 [35] (see also [36, 37]). This C492Y variant destabilized the association between the NTF and the CTF, such that shear stress that is incurred during vibration disrupted their noncovalent attachment. The remaining CTF elicited greater degranulation from transfected LAD2 mast cells, which is consistent with numerous observations indicating enhanced signaling activity of the CTF of aGPCRs upon removal of the NTF (Fig. 2a) (see also [38, 39]).

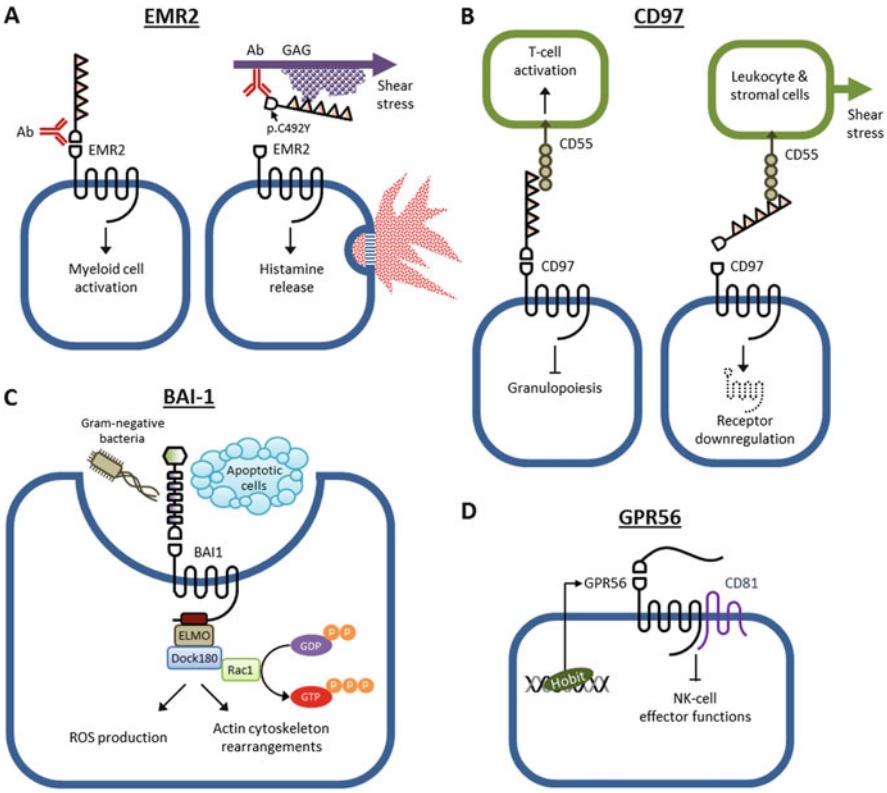

Fig. 2 Functions of adhesion GPCRs in immune cells. (**a**) *Left*: A mAb binding at the NTF of EMR2 underneath the EGF-domain region potentiates the activation and recruitment of human neutrophils and Mφ. *Right*: A mutation near the GPS of EMR2 facilitates sheer stress-induced dissociation of the NTF from the CTF upon ligation by antibody or glycosaminoglycan (GAG). The remaining CTF enhances vibration-induced degranulation of mast cells in patients suffering from vibratory urticaria. (**b**) *Left*: CD97 triggers different activities. Mice lacking either CD97 or CD55 display a comparable phenotype with about twofold more circulating granulocytes. Augmented numbers of Gr-1-positive cells in cell cycle in the bone marrow indicate a higher granulopoietic activity. In addition, the NTF of CD97 can activate T cells by engaging CD55. *Right*: In the circulation, CD97 expression is constantly downregulated by contact with CD55 on blood and stromal cells, possible to restrict CD97–CD55-mediated cell adhesion to tissue sites. (**c**) BAI1 on Mφ binds PtdSer on apoptotic cells and LPS on gram-negative bacteria via the TSRs in its extracellular domain. BAI1 interacts with the N-terminal domain of ELMO via an alpha-helical domain in its intracellular tail. ELMO recruits Dock180 and promotes guanine nucleotide exchange factor activity. The GEF activity of the ELMO–Dock180 complex promotes Rac1 association with GTP and thereby mediates actin cytoskeletal reorganization during engulfment and ROS production. (**d**) GPR56 on NK cells forms a complex with the tetraspanin CD81 to inhibit cytotoxicity. Expression of GPR56 is induced by the transcription factor Hobit and terminated upon physiological activation through shedding of the NTF and transcriptional downregulation

2.3 EMR3 (ADGRE3)

EMR3 was first identified in a DNA database, based on its high similarity with EMR2. cDNA cloning revealed that EMR3, due to concerted evolution of the encoding genes, shares ~90 % identity in the 7TM region with EMR2 [26]. EMR3 possesses two N-terminal EGF-like domains that may recognize a ligand on human Mφs and activated neutrophils [26]. Flow cytometric analysis revealed that all types of myeloid cells express EMR3 [40]. In peripheral blood, the highest expression of EMR3 was found on polymorphonuclear granulocytes. Moreover, mature $CD16^+$ monocytes express high levels of EMR3, while $CD16^-$ monocytes and myeloid DCs are $EMR3^{dim/low}$. Lymphocytes and plasmacytoid DCs lack EMR3. Interestingly, $CD34^+CD33^-/CD38^-$ committed hematopoietic stem cells and $CD34^+CD33^+/CD38^+$ progenitors in bone marrow do not express EMR3, which is only upregulated during late granulopoiesis. Accordingly, EMR3 can be used to monitor granulocyte maturation [41]. In rodents, the EMR3 gene has been lost together with gene for EMR2 [24].

2.4 EMR4 (FIRE, ADGRE4)

Like EMR1, EMR4 was first identified in mouse, where it was called F4/80-like receptor (FIRE) [42]. FIRE has two N-terminal EGF-like domains and is expressed exclusively in myeloid cells, including $CD8^-$ DCs, but not by $CD8^+$ DCs, monocytes, or Mφs [42, 43]. Immunohistochemical analysis revealed that $FIRE^+$ cells reside predominantly in the red pulp and in the marginal zone of the spleen; yet, very few $FIRE^+$ cells are seen in the white pulp. FIRE is downregulated during DC and Mφ activation, although the latter is not conclusive [43]; also the distribution of EMR4 on tissue Mφ warrants further investigation. A ligand for FIRE, present on the B lymphoma cell line A20, remains to be identified [43].

Human EMR4 is not expressed at the cell surface. Due to a one-nucleotide deletion in exon 8, translation of EMR4 would result in a truncated 232-amino acid protein, lacking the entire 7TM region [44]. Whether human cells express a soluble secreted fragment of EMR4 is not clear; transcripts are present in BDCA-1^+ DCs and monocytes, but protein expression remains to be demonstrated [45]. Of note, the deletion disabling EMR4 surface expression is not found in nonhuman primates, including chimpanzees, suggesting that the gene became nonfunctional only after human speciation, about 5 million years ago [44]. Thus, EMR4 accounts for a genetic difference between humans and primates related to immunity.

2.5 CD97 (ADGRE5)

CD97 is a prototypical member of the EGF-TM7 subfamily of aGPCRs [46]. Evolutionary conserved, the gene encoding CD97 is found in all vertebrate genomes analyzed so far. Moreover, the structure and cellular distribution of CD97 are

similar in human and mouse, indicating a conserved biological role. Initially, CD97 has been described as an activation-dependent Ag on lymphocytes [3]. Later, it became clear that hematopoietic stem and progenitor cells (HSPCs) and all leukocytes in human and mouse express CD97$^+$, with highest levels found in myeloid cells [47, 48]. Moreover, many normal and malignant non-hematopoietic cell types express CD97 [49, 50].

Like EMR2, CD97 possesses up to five N-terminal EGF-like domains. Alternative splicing gives rise to three isoforms in human, namely CD97(EGF1,2,5), CD97 (EGF1,2,3,5), and CD97(EGF1–5) [51]. CD97 isoforms expressed in the mouse are CD97(EGF1,2,4), CD97(EGF1,2,3,4), and CD97(EGF1,2,X,3,4), with X indicating a 45-amino acids sequence that does not correspond to any known protein module [52, 53]. EGF-like domains 1 and 2 interact with CD55 (decay-accelerating factor), a membrane-based regulator of complement activation [54, 55], also deposited on collagen fibers [56]. EGF-like domain 4, which is present only in the largest isoform, binds chondroitin sulfate B, a glycosaminoglycan that is found abundantly on cell surfaces and extracellular matrix [31, 57]. An RGD (Arg-Gly-Asp) motif 3′ to the EGF-domain region of human CD97 is bound by the integrin α5β1 and, possibly, αvβ3 [58]. Finally, through the GPCR autoproteolysis-inducing (GAIN) domain, CD97 interacts with the stromal and T-cell Ag Thy-1 (CD90) [59]. Thus, CD97 interacts with at least four matricellular ligands.

Based on its molecular structure and ligand interactions, a role for CD97 in leukocyte adhesion and/or trafficking has been proposed [54]. To investigate this assumption, mAbs directed against individual EGF domains were generated, interfering with either CD55 or chondroitin sulfate B binding. The functional consequences of targeting CD97 and blocking its ligand interactions were studied using several mouse inflammation models, each representing different aspects of innate and adaptive immunity. CD97 mAbs inhibited the accumulation of neutrophils at sites of inflammation, thereby affecting antibacterial host defense, inflammatory disorders, and stem cell mobilization from bone marrow [60–62]. Unexpectedly, they did so independent of the ligand interaction they interfered with. Moreover, the tested mAbs had no impact on Ag-specific (adaptive) responses, such as delayed-type hypersensitivity or experimental autoimmune encephalomyelitis [63]. Comparison of the consequences of Ab treatment and gene targeting implied that CD97 mAbs actively inhibit the innate response, presumably at the level of granulocyte and/or Mφ recruitment to sites of inflammation. Detailed investigation showed that CD97 mAbs deplete polymorphonuclear granulocytes in bone marrow and blood, which involved Fc receptors, but not complement activation, and was associated with an increase in serum levels of TNF and other pro-inflammatory cytokines [64]. Of note, depletion of granulocytes by CD97 mAbs requires acute inflammation, suggesting a mechanism of conditional, Ab-mediated granulocytopenia.

Two independently developed CD97-deficient mice showed no overt phenotype, except for a mild granulocytosis that increased under inflammatory conditions [50, 65]. A comparable phenotype with about twofold more circulating

granulocytes was found in mice deficient for the CD97 ligand CD55 [66]. Augmented numbers of Gr-1-positive cells in cell cycle in the bone marrow indicated a higher granulopoietic activity in mice lacking either CD97 or CD55 (Fig. 2b). Concomitant with the increase in blood granulocyte numbers, CD55 knockout mice, challenged with the respiratory pathogen *Streptococcus pneumoniae*, developed less bacteremia and died later after infection [66], while CD97 knockout mice displayed an improved immune response toward acute infection with *L. monocytogenes* [65]. Moreover, possibly related to the interaction of CD55 with CD97, amelioration of collagen-induced arthritis and a trend toward less severe K/BxN serum transfer arthritis was found in mice that lack CD97 or CD55 [67].

Cellular and molecular assays have proved the interaction between CD97 and its various ligands in vitro. For example, soluble CD97 can cross-link CD55 on T cells in vitro, thereby increasing $CD4^+$ T-cell proliferation, activation, and IL-10 and granulocyte–macrophage colony-stimulating factor (GM-CSF) secretion [68]. Yet, it has been difficult to prove ligand interactions of CD97 in vivo. Conclusive evidence was obtained when leukocytes from CD55-deficient mice were shown to express significantly increased levels of cell surface CD97 that normalized after transfer into wild-type mice because of contact with CD55 on both leukocytes and stromal cells [69]. Downregulation of both CD97 subunits occurred within minutes after first contact with CD55 in vivo and was strictly dependent on shear stress in the circulation. Of note, de novo ligation of CD97 did not activate signaling molecules constitutively engaged by CD97 in cancer cells, suggesting that CD55 downregulates CD97 surface expression on circulating leukocytes by a process that requires physical forces, but may not induce receptor signaling (Fig. 2b).

3 BAI/ADGRB Subfamily

The members of the ADGRB subfamily, commonly referred to as brain-specific angiogenesis inhibitors (BAIs), possess a large NTF that contains, next to the GAIN domain, a single hormone receptor motif (HRM) and 4–5 thrombospondin type 1 repeat (TSR) domains [70–72]. Moreover, BAIs contain a long C-terminal tail, downstream of the 7TM region, which comprises the PDZ-binding motif QTEV (Gln-Thr-Glu-Val) for intracellular signal transduction. BAIs were initially known for their ability to inhibit angiogenesis and tumor formation. Later studies unraveled key roles in the clearance of apoptotic cells and bacteria and in the regulation of immune responses. Finally, BAIs have been involved in synaptogenesis and dendritic spine formation. Below, we focus on the roles of BAI family proteins in cell clearance and immune regulation.

3.1 BAI1 (ADGRB1)

BAI1 (human chromosome location at 8q24) was first cloned in 1997 as a target of the tumor suppressor gene p53 [73]; yet, it is not clear whether the expression of BAI1 indeed is dependent on p53 [74]. Abundantly found in the brain on neurons, astrocytes, and microglia, BAI1 is also expressed in other tissues (bone marrow, spleen, testis, and colon) and in distinct cell types (Mφs, skeletal muscle myoblasts) [70, 72] (Fig. 1). The TSR region of BAI1 comprises five repeats, which can bind phosphatidylserine (PtdSer) and LPS [75, 76]. Moreover, an N-terminal RGD motif can interact with the integrin α5β1 that regulates extracellular matrix attachment and motility [77]. Autoproteolysis of BAI1 at the GPS or behind the first TSR generates soluble ectodomains with antiangiogenic properties, known as vasculostatin 120 (kDa) and vasculostatin 40 (kDa) [78–80].

Park et al. identified BAI1 as an engulfment receptor for apoptotic cells that can both, bind the corpses and signal intracellularly in the phagocyte to facilitate their internalization [75]. BAI1 was found to function as a phagocytic receptor both, in Mφs and nonprofessional phagocytes, such as fibroblasts and epithelial cells. In embryonic zebra fish brains, BAI1 mediates engulfment of dying neurons by microglia [81]. BAI1 directly binds PtdSer, which is exposed on the outer leaflet of the plasma membrane of cells undergoing apoptosis, via the TSR regions, as determined by a combination of biochemical approaches [75]. Upon PtdSer binding, BAI1 transmits signals via the downstream intracellular engulfment molecule ELMO1 (engulfment and cell motility 1). The specific interaction occurs via an α-helical motif within the cytoplasmic tail of BAI1 and the N-terminal region of ELMO1 [75]. In fact, all three ELMO family members (ELMO1, ELMO2, and ELMO3) can bind to the cytoplasmic tail of BAI1. ELMO1 acts as a guanine nucleotide exchange factor for Rac and activates Rac, which promotes actin cytoskeletal rearrangements, necessary to mediate the phagocytosis of apoptotic cells (Fig. 2c). When phagocytes engulf apoptotic cells, they release anti-inflammatory mediators, such as tumor growth factor (TGF)β, IL-10, and prostaglandin E2, and these, in turn, reduce the levels of pro-inflammatory TNF in the local tissue. BAI1-mediated apoptotic cell clearance has been implicated in anti-inflammatory response.

Upon stimulation with apoptotic cells, BAI1 signaling in Mφs leads to upregulation of ABCA1 (ATP-binding cassette transporter 1) [82]. ABCA1 is a large transmembrane transporter that mediates cholesterol efflux from Mφs and is a key player for high-density lipoprotein (HDL) biogenesis. Genetic loss of BAI1 prevented ABCA1 upregulation in response to apoptotic cells and reduced serum levels of total cholesterol, HDL, low-density lipoprotein (LDL), and triglycerides [82]. Furthermore, transgenic mice that overexpressed BAI1 had an improved HDL/LDL ratio [82]. HDL has potent anti-inflammatory functions. Therefore, BAI1 in Mφs can regulate lipid homeostasis and anti-inflammatory responses via mediating apoptotic cell clearance.

A more recent study revealed a novel role for BAI1 in colonic epithelial cells [83]. Lee et al. showed that healthy epithelial cells mediate the engulfment of their

apoptotic neighbors that arise due to acute injury, and this BAI1-mediated apoptotic cell removal beneficially influenced the level of tissue inflammation. Genetic deficiency of BAI1 caused increased numbers of uncleared apoptotic cells, greater inflammatory colon regions, and higher inflammatory cytokine levels (IL-1α and TNF) in the dextran sulfate sodium-induced colitis model. Conversely, transgenic overexpression of BAI1 in colonic epithelial cells resulted in a reduction of uncleared apoptotic cells, smaller inflammatory colon regions, and lower inflammatory cytokine levels. Furthermore, transgenic mice expressing a mutant version of BAI1 that cannot signal failed to protect mice suggesting that BAI1 signaling (BAI1–ELMO) is required for the beneficial effects in experimental colitis.

In addition to its role as a PtdSer receptor, BAI1 mediates engulfment of gram-negative bacteria [76]. The TSR region of BAI1 directly interacts with the LPS inner core of gram-negative bacteria. BAI1-mediated engulfment of bacteria by Mφs through this interaction induced production of the pro-inflammatory cytokine TNF [76]. Furthermore, a recent study revealed that BAI1 mediates reactive oxygen species (ROS) production and bacterial clearance in vivo [84]. BAI1-deficient mice showed impaired bacterial clearance and were more susceptible to peritoneal infection. These studies suggest that BAI1 can act as a pattern recognition receptor that mediates bacterial clearance through microbicidal activity and local inflammation (Fig. 2c). An interesting question that arises from these observations is how the signaling downstream of BAI1 remains anti-inflammatory during apoptotic cell engulfment while being pro-inflammatory during bacterial engulfment; whether this is mediated through other receptors that interact with BAI1 during apoptotic cell recognition or a different set of intracellular signaling molecules that might be attracted to the tail of BAI1 during these recognition events remains to be determined.

Besides its immune-related activities, BAI1 possesses other unique functions. As a PtdSer receptor, BAI1 induces myoblast fusion into multinucleated myofibers in response to apoptotic or PtdSer-exposing myoblasts [85]. Moreover, BAI1 regulates spinogenesis and synaptogenesis [86]. Finally, in a study of BAI1-deficient mice, BAI1 was found to regulate spatial learning and memory as well as synaptic plasticity [87].

3.2 BAI2 (ADGRB2) and BAI3 (ADGRB3)

BAI2 (human chromosome location at 1p35) and BAI3 (human chromosome location at 6q12) were identified as homologs of BAI1 [88]. Although expression patterns are not well characterized, BAI2 and BAI3 are found in many tissues, including the brain, heart, skeletal muscle, intestine, and thymus [70, 72]. Both receptors possess four TSR motifs, and although immune-related functions have not been reported yet, BAI3 possesses the motif necessary for binding ELMO proteins that allows BAI1 to engulf apoptotic cells [89]. Moreover, similar to BAI1, BAI3 has been implicated in skeletal myoblast fusion and muscle fiber formation [90].

4 ADGRG Subfamily

The ADGRG subfamily member GPR56 is one of the best-studied aGPCRs, well known for its causal involvement in neuronal and cortical development [91]. The gene encoding GPR56 clusters with the genes for GPR97 and GPR114 on chromosome 16q21, suggesting a common evolutionary origin. In contrast to GPR56, which is expressed in many different cell types, very little is known about GPR97 and GPR114, which both seem to have a rather restricted cellular distribution [92]. All three genes are expressed in leukocytes (Fig. 1).

4.1 GPR56 (ADGRG1)

GPR56 has been strongly linked with development. HSPCs in the mouse embryo and adult bone marrow abundantly express GPR56, but levels substantially decrease as cells differentiate. Recent studies have explored the role of GPR56 in HSPCs. Solaimani-Kartalaei et al. found a critical role of GPR56 in the formation of hematopoietic clusters during endothelial to hematopoietic cell transition [93]. In contrast, GPR56 deficiency does not impair HSPC maintenance or function during steady-state or myeloablative stress-induced hematopoiesis, and GPR56-deficient cells respond normally to physiological and pharmacological mobilization signals, despite the reported role of this aGPCR as a regulator of cell adhesion and migration in neuronal cells [94]. Thus, GPR56 expression is required for generating the first hematopoietic stem cells, but largely dispensable for steady-state and regenerative hematopoiesis.

Flow cytometric analysis with newly generated mAbs detected the presence of GPR56 in human cytotoxic NK and T lymphocytes, including $CD8^+$, $CD4^+$, and γδ T cells [95, 96]. Primary infection with cytomegalovirus, which generates a vast population of $CD8^+$ T cells with an effector phenotype, induced a strong increase in GPR56 expression in virus-specific $CD8^+$ T cells that remained detectable during latency. In NK-92 cells, ectopic expression of GPR56 inhibits spontaneous and SDF-1-stimulated cell migration [96]. Investigation of NK cells from polymicrogyria patients with a null mutation in the GPR56 gene and NK-92 cells overexpressing GPR56 revealed that GPR56 suppresses the production of inflammatory cytokines and cytolytic proteins, degranulation, and target cell killing [97]. GPR56 pursues this activity by associating with the tetraspanin CD81. Expression of GPR56 was triggered by Hobit, a homolog of the transcription factor Blimp-1, and declined upon cell activation. Thus, GPR56 controls natural cytotoxicity displayed by NK cells in order to protect the body against harmful viruses and neoplasms (Fig. 2d). Circulating T and NK cells in the mouse do not express GPR56, which may be due to the different way they acquire cytotoxic capacity.

4.2 GPR97 (PB99, ADGRG3) and GPR114 (ADGRG5)

A microarray study of mature human leukocyte populations detected GPR97 transcripts in neutrophils and eosinophils [96]. Moreover, GPR97 gene expression that was found in murine pre-B cells and thymocytes, but not in mature B and T cells, suggested a role in early lymphoid development. Investigation of independently raised knockout mice revealed no necessity of GPR97 for B- or T-cell development [98], but implied an essential role in follicular versus marginal zone B-lymphocyte fate decision [99]. In spite of its expression in eosinophilic granulocytes, GPR97 was found to be dispensable for inflammation in ovalbumin-induced asthmatic mice [100]. GPR114 is another aGPCR transcribed in lymphoid and myeloid cells [96]; yet, as for GPR97, expression needs to be confirmed at the protein level.

5 Biological and Translational Implications

aGPCRs have been implicated in the development and function of HSPCs, monocytes, Mφs, DCs, granulocytes, lymphocytes, and, thus, most lineages of hematopoietic cells. Nevertheless, it has not been clear for quite some time whether these noncanonical GPCRs regulate cellular homeostasis and activation or whether they also control specific immune functions. Studies on the role of BAI1 in Mφs and GPR56 in cytotoxic lymphocytes imply that aGPCRs indeed regulate innate, and possibly also adaptive, effector functions. Thereby, these studies paved the way for further exploring the biological role of these receptors using novel genetic models and pharmacological tools. As a prime example, BAI1 facilitates clearance of apoptotic cells by Mφs and intestinal epithelial cells and modulates the inflammatory status within tissue.

EGF-TM7 subfamily aGPCRs figure prominently in the biology of polymorphonuclear granulocytes, an aspect that has been overlooked in the beginning, due to the prominent presence of the EMR1 ortholog F4/80 on Mφs in the mouse and the upregulation of CD97 during lymphocyte activation. Legrand et al. recently showed that an afucosylated mAb directed against EMR1 efficiently eradicates eosinophils through Ab-dependent cell-mediated cytotoxicity, resulting in long-term in vivo depletion of eosinophils in monkeys and, thus, providing a treatment option for eosinophilic disorders [19]. Similarly, mAbs to mouse CD97 eliminate neutrophils (and possible also eosinophils) in an Fc receptor-dependent manner, thereby ameliorating various inflammatory conditions [64]. These studies imply that biologicals targeting aGPCRs may be useful in treating immune cell-related diseases.

Association of aGPCRs with hematopoietic malignancies has not been investigated systematically so far, despite their role in solid tumors (see [101]). A recent study reported the association of CD97 expression with internal tandem duplications within the juxtamembrane region of the FMS-like tyrosine kinase receptor FLT3 (FLT3-ITD) in patients with acute myeloid leukemia (AML),

which is associated with an aggressive clinical phenotype [102]. CD97 knockdown resulted in reduced cell adhesion and trans-well migration in vitro. Moreover, GPR56 expression identifies primary human AML cells with high repopulating potential in vivo [103]. Elevated expression of GPR56 was found in AML cells with high ecotropic viral integration site-1 expression, a refractory type of the disease with a poor prognosis. Knockdown of GPR56 expression decreased the cellular adhesion ability through inactivation of RhoA signaling, resulting in a reduction of cellular growth rates and enhanced apoptosis [104].

Despite an increasing number of studies that have linked aGPCRs with immune function(s), main uncertainties persist. For example, though many of the protein folds found in the NTF of aGPCRs are involved in cell–cell interactions, evidence is scarce that aGPCRs contribute to the communication between immune cells. Further, it is not clear to what extent aGPCR autoproteolysis and signaling facilitate immune functions. Moreover, functional redundancy between members of the EGF-TM7 subfamily and the GPR56/GPR97/GPR114 cluster seems possible and warrants further investigation. Related to this, the aGPCR signature of immune cells need to be unraveled comprehensively. Exploring these issues eventually will improve our understanding of the biology of aGPCRs and, thereby, may widen the canon of GPCR activities in the immune system.

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Heart Development, Angiogenesis, and Blood-Brain Barrier Function Is Modulated by Adhesion GPCRs

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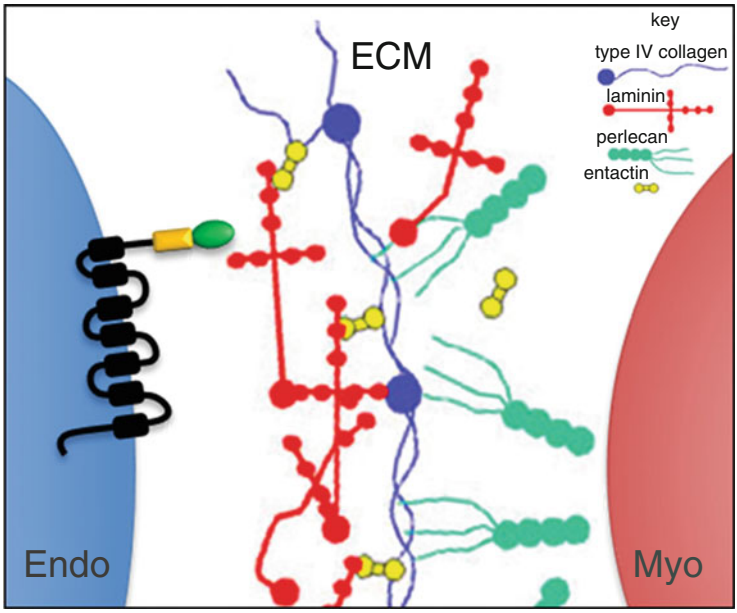
Graphical Abstract



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Contents

Abstract

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Keywords

Heart development • Trabeculation • Blood-brain-barrier • Cardiovascular • Angiogenesis • Endothelium • Adhesion GPCRs

1 Introduction

The cardiovascular system in adult organisms forms a network of interconnected endothelial cells, supported by mural cells and displaying a high degree of hierarchy: arteries emerging from the heart ramify into arterioles and then capillaries, which return to the venous systems through venules and veins. The cardiovascular system allows blood circulation, which in turn is essential for hemostasis through gas diffusion, nutrient distribution, and cell trafficking. In this chapter, we have summarized the current knowledge on how adhesion GPCRs (aGPCRs) impact heart development, followed by their role in modulating vascular angiogenesis.

2 Adhesion GPCRs in Cardiac Development

2.1 Overview of Cardiac Development

The mammalian heart is the first organ to develop in the embryo. It evolves from a simple tubular structure, the linear heart tube, into a highly complex four-chambered organ (Fig. 1). The cells destined to form the linear heart are symmetrically arrayed in two groups: the primary (PHF) and the secondary (SHF) heart field (Fig. 1a) which together form the precardiac mesoderm or cardiac crescent (Fig. 1b). Later, the cells of the PHF initiate to differentiate into the endocardial and myocardial lineages and fuse in the embryonal midline forming the primitive linear heart tube (Fig. 1c) [2]. At this stage, the heart is formed by only two cell layers, the myocardium and endocardium, separated by an extracellular matrix (ECM) called cardiac jelly. Subsequently, the heart tube loops to the right, and

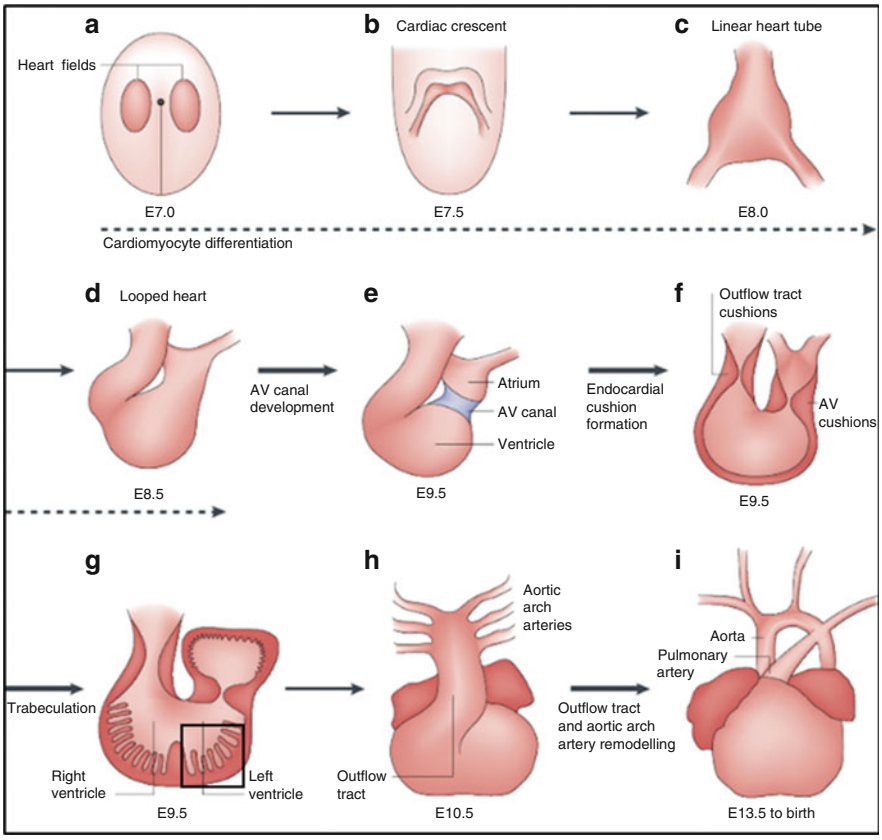

Fig. 1 Overview of cardiac development. Representation of the key events in the development of the murine heart starting from embryonic day 7 (E7) to embryonic day 13.5 (E13.5). Trabeculation appears at E9.5 at the basis of the ventricular walls. Figure adapted from [1]

cells of the SHF are added culminating in the formation of the embryonal heart (Fig. 1d, e) with different distinguishable cardiac regions: the inflow tract (IFT), embryonal atrium, atrioventricular canal (AVC), embryonal ventricle, and outflow tract (OFT). Each of these regions has a differential pattern of gene expression as well as different functional characteristics. Later on in development, the AVC and OFT present endocardial cushions on their inner surface, which are focal expansions of the cardiac jelly (Fig. 1f). The endocardial cells that line the AVC and OFT cushions undergo epithelial to mesenchymal transition (EMT) to populate the cushions with mesenchymal cells initiating the transformation into the AV and OFT valves. Signals exchanged between specialized myocardium and cushion endocardium are required for endocardial EMT [3]. Subsequently, trabeculae appear during the ventricular chamber development and maturation (Figs. 1g and 2), which consist of highly organized cardiomyocyte muscular protrusions of the ventricular wall, lined by a single layer of endocardial cells [2, 4].

2.2 Regulation of Cardiac Development by Adhesion GPCRs

Currently, six aGPCRs have been detected in the endocardium and/or myocardium of the heart. Among these are three members of the subfamily I. ADGRL1 (latrophilin-1) has been detected via Northern blot analysis during rat heart development with an intense expression at postnatal day 3. Yet, the cellular origin of ADGRL1 expression as well as its function remains unknown [5]. ADGRL2 (latrophilin-2) has been shown to be expressed in the AVC and to be required for EMT [6]. In situ hybridizations revealed that ADGRL2 is expressed in the mesoderm leaving the primitive streak in chicken. At later stages ADGRL2 is expressed in the endocardium as well as the myocardium during cushion formation and subsequently in the mesenchymal cells populating the AVC cushion. Knockdown experiments in explant cultures demonstrated that ADGRL2 mediates cell-cell separation and is required for the population of the cardiac cushion with mesenchymal cells. ADGRL4 (ELTD1) has been detected by Northern blot, RT-PCR, and in situ hybridization analyses in rat hearts. Northern blot analyses of isolated cardiomyocytes from E16 to 12-week-old rats and fetal vs. human heart tissue revealed that the expression levels of ADGRL4 increase with age, which was verified by RT-PCR for rats. In situ hybridization of 2-week-old rats demonstrated that ADGRL4 is expressed in cardiomyocytes and vascular smooth muscle cells [7]. Patients undergoing heart transplant due to heart failure show a decrease in ADGRL4 protein expression. Moreover mice with ADGRL4 knockout exhibit a more severe induced hypertrophy, suggesting ADGRL4 as a target in the treatment of hypertrophic cardiomyopathies [8].

Of the subfamilies II and VI, only the members ADGRE5 (CD97) and ADGRF5 (Gpr116) have been detected in the adult mouse heart. Studies utilizing a reporter mouse as well as antibody staining revealed expression of ADGRE5 in cardiomyocytes [9], while the expression of ADGRF5 was detected by RT-PCR utilizing RNA from whole hearts [10]. The function of both adhesion GPCRs in the heart is unknown.

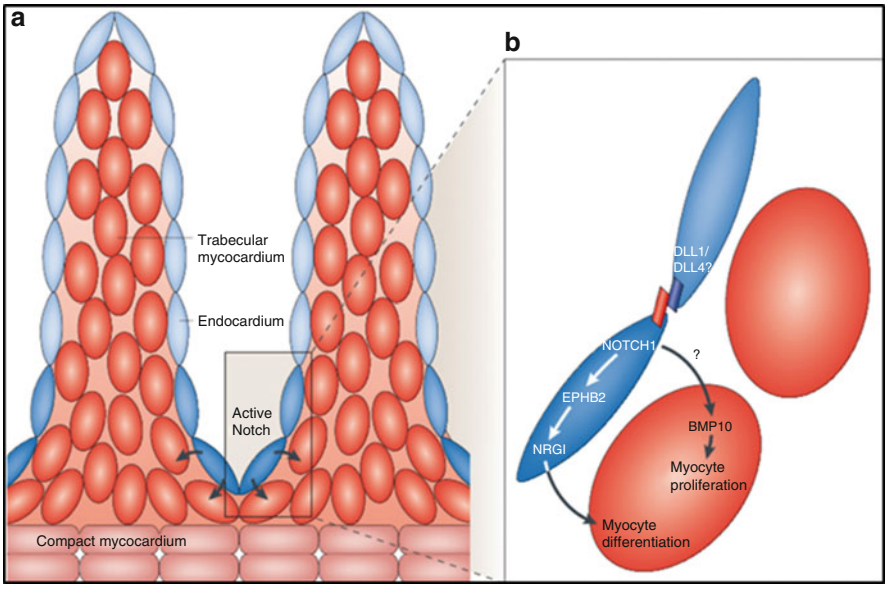

Fig. 2 Ventricular trabeculation. (**a**) The myocardial cells of the trabeculae (*red oval* shaped) are outlined by the endocardial cells (*blue oval* shaped) arranged in a single-cell layer. (**b**) The main pathways involved in heart trabeculation. Figure adapted from [1]

Finally, it has been demonstrated that the subfamily VIII member ADGRG6 (Gpr126) is required for trabeculation during zebrafish and mouse heart development [11–13]. Communication between myocardium and endocardium is essential for the proper formation of the trabeculae even though the genetic regulatory network behind this is not well known. Among key molecules with a well-established role in trabeculation are NRG1/ErbB2/ErbB4 [14–16], EphrinB2/EphB4 [17, 18], and BMP10 [19] (Fig. 2). Lately also the Notch signaling network has been shown to regulate trabeculation via the expression of EphrinB2, NRG1, and BMP10 [20]. Notch1 activity is restricted to the trabecular endocardium and has been shown to be an upstream regulator of ADGRG6. The analysis of the Notch1-ADGRG6 relationship has confirmed ADGRG6 among the key regulatory elements of trabeculation [21].

Interestingly, pathways required for trabeculation, such as NRG1/ErbB [22] and Notch [23], are also involved in another developmental process that is known to be dependent on ADGRG6, namely, Schwann cell differentiation and myelination of peripheral nerves [24] (see also [25] on this topic). Moreover, the ECM, which is known to be required for trabeculation [26–28], plays an important role in the function of ADGRG6. For example, it has been shown that the ECM proteins laminin and type IV collagen, which are known to be crucial for the regulation of Schwann cell function [29], are in vitro activating ligands of ADGRG6 [30, 31]. These data suggest that the interaction of ADGRG6 with the ECM might also underlie trabeculation (Fig. 3).

Collectively, several members of the aGPCR family are expressed in the heart in cardiomyocytes and/or endocardial cells, and at least two members are required for

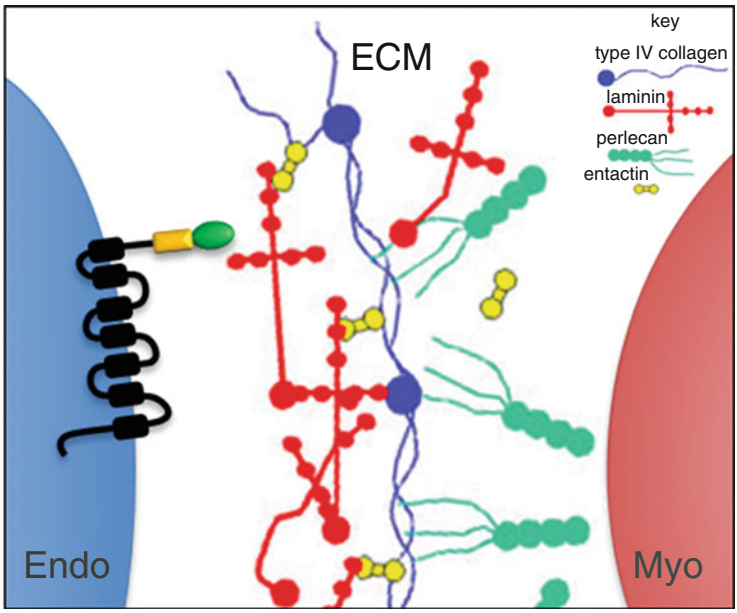

Fig. 3 Extracellular matrix and trabeculation. Between the endocardial (Endo) and the myocardial cells (Myo) lies the extracellular matrix (ECM) mainly composed of type IV collagen and laminin. The figure suggests a putative interaction between ADGRG6 expressed in the endocardial cells and the ECM

proper early heart development. However, the downstream targets of these aGPCRs as well as the cellular functions controlled by them remain elusive.

3 Adhesion GPCRs in Angiogenesis

3.1 Adhesion GPCRs and Endothelium Heterogeneity

Endothelial cells line the inner side of the entire blood vessel tree. Several layers of specialization maintain a high diversity in between the different vascular beds. First is the aforementioned hierarchy of vessels, from large arterial vessels to capillaries, and back to the venous system. Overimposed on this global architecture, vascular beds acquire local properties in the vicinity of each organ or depending on hemodynamic conditions. Hence, a mix of transmitted differentiation and microenvironment-induced changes leads to a very high heterogeneity in between each vascular bed, increased again by the salt and pepper pattern of expression of certain genes. Among the most conspicuous differences lies the nature of the junctions connecting endothelial cells to each other and the presence of openings like fenestrations. As a consequence, the number of specific, pan-vascular genes is rather limited, and as explained thereafter, no aGPCR seems to be expressed at a high level on the whole endothelium [32, 33].

3.2 Adhesion GPCRs Expression in Vascular Beds

Analysis of microarray data revealed that 58 genes are broadly and specifically enriched in microvascular endothelium [34]. Correlating with the known endothelial markers (VEGFR2, PECAM), two aGPCRs appeared highly expressed in vascular endothelium: ADGRL4 and ADGRF5 are, respectively, on the third and the fifth position of this ranking, suggesting they might be broadly expressed as pan-vascular genes. However, this study was not based on a profiling of vascular beds in all organs.

A more detailed analysis came from the profiling of organ-specific vascular beds, established by Shahin Rafii's team [35]. For the majority of aGPCRs, the expression over the different vascular beds is homogenous. However, 13 of them show a peak of expression in a discrete number of organ vasculature, summarized in the following chart (analyzed by Dr. Liqun He):

		Testis	Spleen	Muscle	Brain	Lung	Heart	Glome-rulus	Muscle	Liver
I	ADGRL3(Lphn3)				■	■				
	ADGRL2(Lphn2)					■		■		
	ADGRL1(Lphn1)					■				
	ADGRL4(Eltd1)			■	■	■	■	■		
II	ADGRE1(EMR1, F4/80)									■
	ADGRE4(EMR4)									■
	ADGRE5(CD97)					■				■
III	ADGRA2 (Gpr124)				■					
	ADGRA3 (Gpr125)					■				
VI	ADGFR5 (Gpr116)			■	■	■	■	■		
VIII	ADGRG6 (Gpr126)				■	■				■
	ADGRG3(Gpr97)								■	
	ADGRG1(Gpr56)			■			■			

Each peak of expression might indicate that the aGPCRs intervene in the acquisition or maintenance of the organ-specific phenotype of vascular beds, like the lung (ADGRE5, ADGRA3, ADGRL1), the liver [ADGRE1 (EMR1, F4/80) and ADGRE4 (EMR4)], the brain [ADGRA2 (Gpr124)], or the bone marrow [ADGRG3 (Gpr97)]. Those results also suggest that ADGRL4 as well as ADGRF5 to some lesser extent might be somewhat less expressed in the vasculature of liver, bone marrow, spleen, and testis, rather than fully pan-vascular.

The recent progresses in single-cell profiling are progressively adding up a complementary parameter: are some aGPCRs expressed *exclusively* in the endothelial cells of a given organ? This requires establishing the transcriptome profiles of each cell type from a given organ.

The brain cortex of adult mice has been to date the subject of two intensive single-cell profiling, from Ben Barres and Sten Linnarsson laboratories [36, 37]. They revealed that 13 aGPCRs are exclusive to a specific cell type of the cortex, among which, two exclusively and abundantly expressed in the cortical endothelium (ADGRF5 and ADGRL4), in line with profiling of endothelial cells from the retina in p8 Tie2-Cre/GFP mice [38]. Besides, a group of aGPCR is abundant in the endothelium but co-expressed at lower levels by additional cell types: ADGRL2 (astrocytes, microglia, neuron), ADGRE5 (microglia, oligodendrocytes precursor cells, astrocytes), ADGRA2 (oligodendrocytes precursor cells), ADGRG6 (astrocytes, neurons), and ADGRG3 (neuron) [36].

Profiling of other organs is still less advanced, even if significant progress has been done in mapping the lung endothelium proteome [39].

Taken together, those expression profiles suggest a group of 13 genes with potential impact on endothelial functions:

- ADGRL4 and ADGRF5 possess the higher vascular expression, especially in the brain, lung, heart, and kidney endothelium.
- ADGRA2, ADGRG6 in the brain.
- ADGRL3 (Lphn3), ADGRL2, ADGRL1, ADGRG3, ADGRG1 (Gpr56), ADGRE5, ADGRE1, ADGRE4, and ADGRA3 have peaks of expression in a restricted subset of organ-specific vascular beds.

Extensive transcriptomic analysis of lymphatic vessels is still missing, yet the available data are promising when it comes to expression of aGPCRs. For instance, comparison in between freshly sorted lymphatic endothelial cells and blood vascular endothelial cells from mouse intestines showed that the lymphatic vessels expressed more ADGRG3, ADGRA2, and ADGRC1 (Celsr1) than their vascular counterpart and on the contrary seem to repress ADGRL2 and ADGRG1 [40].

As described below, to this list should be added aGPCRs with a steady expression, regulating key ubiquitous functions such as control of planar cell polarity, which might kick in on discrete spatio-temporal window in vascular development (see ADGRC1 below), as well as aGPCRs whose expression is induced very transiently, under normal or challenging conditions (see Sect. 3.4). Such identification requires in-depth profiling of organ-specific vascular beds, covering various developmental time points. For instance, very little is known about differential expression of aGPCRs upon arterial-venous differentiation, even if profiling studies suggest that arteries express more ADGRE5 and ADGRG1 but less ADGRA2, ADGRD1(Gpr133), and ADGRG6 than their venous counterpart in the umbilical cord [41, 42].

3.3 Adhesion GPCRs Knockouts in Physiological Vasculature

3.3.1 Formation of the Initial Vascular Network

Genes regulating critical, nonredundant events in vascular development lead to temporal specific lethality when knocked down in mice models. They pinpoint the sequence of morphologic events that lead to a fully functional vascular network. Two successive processes shape the vascular network. First, a primary vascular plexus is formed by the fusion of endothelial precursors, the angioblasts, at different sites of vascularization, among which the yolk sac, at E7.5. Mice lacking VEGFR2, a receptor for the key angiogenic factor VEGF, die around E8.5/E9.5 when those angioblasts fail to form [43]. In the brain, the angioblast migrates to create the perineural vascular plexus that covers the surface of the brain (E7.5–E8.5) [44].

Later, starting at about E9, capillaries will emerge from those primitive vessels through proliferation and migration of endothelial cells or splitting of preexisting vessels. Together those processes are known as angiogenesis. In the brain still, intracerebral vessels sprout from this network and dive gradually deeper into the neuroectodermal tissue toward the subventricular zone at E9.5. Animals in which this cerebral sprouting angiogenesis is impaired are severely affected, such as the (VEGF flox/flox; nestin Cre) mouse strain which dies at about E13.5 [45].

3.3.2 Maturation of the Vascular Network

Newly formed vessels mature and acquire location-specific differentiation. In certain organs, the vascular maturation might largely expand over the postnatal period. In the brain, vessels establish the blood-brain barrier (BBB) by tightening of the inter-endothelial junctions, downregulation of a number of transporters, and expression of specific markers such as Glut-1. The mouse BBB becomes functional at embryonic day 15.5 (E15.5) [46]. However, angiogenesis and parallel maturation persist at the postnatal stage [47], and therefore late lethality might occur such as in the Claudin-5-deficient mice [48]. Another example is the dorsal aorta, which forms very early on E8.5. Alteration in its maturation can lead to ectopic branching and glomeruloid vascular malformations, such as in the *S1pr1*$^{-/-}$ embryos (E11.5–E12.5), leading to rapid demise and synchronous death of the embryos between E13.5 and E14 [49].

3.3.3 Early Lethality Associated with Vascular Deficiency in Adhesion GPCRs Knockouts

However, in mice, only 3 genes out of the 13 aGPCRs defined above are associated with early lethality. In the case of ADGRA2, developed below, central nervous system hemorrhages result in lethality starting at E15.5 and preventing the survival of any mutant beyond E17.5 [50]. ADGRL2 mutant mice might also die according to a very similar temporal window (European Mouse Mutant Archive, unpublished results). Finally, ADGRG6 knockout mice are not viable, but the mutants die over a period ranging from late embryonic stages to early postnatal days (p8–p16, [24]). In the case of ADGRL2 and ADGRG6, a potential vascular phenotype has not been

described yet, and ADGRG6 knockout is affected by a very severe demyelination phenotype.

The rest of the genes do not affect viability of the mice upon genetic inactivation. In particular, ADGRL4 and ADGRF5, the broadest expressed aGPCRs in endothelial cells, are perfectly viable [51] as well as ADGRL1 [52], ADGRL3 [53], ADGRG3 [54], ADGRG1 [55], ADGRE1 [56], and ADGRA3 [57], while the viability of the ADGRE4 knockout mouse is still unknown.

3.3.4 Adhesion GPCRs Regulating Blood-Brain Barrier

ADGRA2 (GPR124) is specifically expressed in the vessels of the brain and spinal cord, as well as within the arteries located outside the CNS. It is also expressed in the embryonic epithelium of the lung and esophagus and becomes exclusively vascular in adult tissues, with endothelial expression in CNS including the brain and retina and pericytic expression in the brain and peripheric organs. Global deletion of ADGRA2 or its endothelial-specific deletion in mouse results in embryonic lethality at E15.5. Vascular fronts fail to invade the neuroepithelium in the forebrain and spinal cord of the knockout animals at E11, forming abnormal tufts and profound CNS hemorrhages. Interestingly, this defect in migration and patterning is also accompanied by a defect in maturation of the vessels. Plasmalemma vesicle-associated protein, normally downregulated upon BBB induction, is maintained in ADGRA2-deficient vessels, whereas Glut-1, a marker of mature cerebral blood vessel, is absent. The absence of maturation of the blood vessels was functionally demonstrated as large deposits of extravascular fibrin surrounding glomeruloid tufts, and more importantly, biotin injected in living embryos was detected in both intra- and extravascular regions in the forebrain and ventral spinal cord, evidencing vascular leakage and hence a dysfunctional BBB. Overexpression of ADGRA2 in endothelial cells in culture enhanced their barrier properties, but interestingly overexpression in mouse leads to a progressive brain-specific vascular phenotype characterized by areas of hypervascularity. These lesions resemble venous angiomas, are restricted to the cortex, and frequently accompanied by calcifications [50, 58, 59].

Mechanistic investigations revealed how ADGRA2 functions as a WNT7A-/WNT7B-specific costimulator of β-catenin signaling in brain endothelium, both in mouse and zebrafish. ADGRA2$^{-/-}$ CNS angiogenesis defects can be rescued by artificially activating canonical Wnt signaling in the endothelium. ADGRA2 activity seems critical only in the tip cells leafing the sprouts, as the restoration of single wild-type tip cells in mosaic Wnt/β-catenin-deficient perineural vessels is sufficient to initiate the formation of CNS vessels. ADGRA2 could therefore ensure a high signal-to-noise ratio for WNT7a and WNT7b induced, in a context where signal strength of the Wnt pathway is close to the minimal threshold required for normal development and function [60–62].

As indicated before, Adgrf5 is expressed at high levels in various vascular beds and especially in the CNS vasculature. Genetic ablation in the mouse showed an anatomically normal and largely functional vascular network, but a disrupted blood-brain barrier. At 1.5 months of age, cerebral vascular leakage could be evidenced

by tracer accumulation in the brain parenchyma. This leakage is maintained over age and phenocopied by an endothelial-specific deletion of ADGRF5, suggesting a cell-autonomous defect in late maturation or maintenance of the cerebral endothelium [51].

3.3.5 Regulation of Valve Formation by Adhesion GPCRs

The ADGRC1 case also shows that important aspects of the vascular role of aGPCRs might still be unknown. ADGRC1 is a critical determinant of planar cell polarity. Its complete knockout is partially lethal and leads to various defects, but its specific knockout in lymphatic vessels leads to abnormal organization of the valves in mesenteric vessels, at E18.5. This suggests that aGPCRs mediating general functions like planar cell polarity might control vascular phenotypes, if deleted in a tissue-specific fashion [63].

3.4 Adhesion GPCRs in Challenged Vasculature

In this last part more elusive functions regulated by aGPCRs in the endothelium but that were not evidenced during development will be reviewed: a pathological context is necessary to reveal this function or an in vitro model necessary to assess a fine mechanistic aspect, and in both cases it remains to be verified if those functions are regulated by the studied aGPCR during vascular development, as cultured cells might lack important cues derived from the in vivo microenvironment.

At least five aGPCRs have been subjected to mechanistic studies in the endothelium:

ADGRA2, whose role has been extensively studied in vivo, is also demonstrating peculiar properties in vitro: its expression is induced upon capillary morphogenesis in cultured venous endothelial cells, after which its N-terminal part is cleaved, through thrombin activity, and then mediates cell survival by bridging glycosaminoglycans of the ECM to integrin $\alpha_V\beta_3$ [64–66].

In a parallel fashion, the extracellular domain of ADGRG3 can be used as a substrate for cultured venous endothelial cells, via an interaction with integrin α5β1 and αvβ3. It can also act as a chemoattractant when used in migration assays [67].

The extracellular fragment of ADGRB1 (BAI1) on the contrary acts as an inhibitor for the migration of dermal microvascular cells [68].

In the case of ADGRL4, silencing in venous endothelial cells had a slight effect on cell adhesion and no effect on endothelial migration in 2D but reduced quite dramatically the migration in a 3D beads assay. This inhibition might be paralleled with results in zebrafish, where the ADGRL4 morphants displayed blocking of intersegmental vessels, never reaching the dorsal position observed in wild type [69].

In lymphatic endothelial cells, silencing of ADGRG3 leads to dichotomous effects on the migration, with an enhanced collective cell migration and cell adhesion and reduced single-cell migration. In this system, ADGRG3 tilts the

Cdc42/RhoA balance, leading to F-actin redistribution and focal adhesion rearrangements [40].

The role of aGPCRs in tumor endothelium has been tackled mostly through orthotropic models (see also [70]).

ADGRE5 is induced in the human metastatic breast cancer cell line MDA-MB-231 [71], as well as elevated in various types of carcinomas. 3T3-derived tumor cells expressing no ADGRE5 or permanently transfected with ADGRE5 isoforms were injected into mice and used to demonstrate a greater vessel density in the tumors derived from ADGRE5-expressing cells, suggesting that ADGRE5 expression on the surface of tumor cells might stimulate angiogenesis [67]. On the opposite, ADGRA2 silencing in human endothelial cells inhibited angiogenic vessel formation and tumor growth in mouse xenograft [72].

ADGRB1 has been studied extensively in relation to tumor. While present in normal tissues, its expression is reduced or lost in cerebral or colorectal tumors [73, 74]. Its expression is controlled by p53, and it gets cleaved to release its 120 kDa extracellular domain, named vasculostatin, upon action of a proprotein convertase/MMP-14 cascade (Kaur et al. 2005 [75]). ADGRB1 or vasculostatin expression limits tumor growth and vascularization after human pancreatic adenocarcinoma cell line injection, murine renal cell carcinoma, gliomas, and in matrigel plug assay [76–78].

As mentioned before, ADGRL4 is a broad marker of the endothelium. Its involvement in the angiogenic response of tumors has also been solidly demonstrated: first, it was identified as part of a vascular cluster of genes expressed in vivo in human tumor samples derived from different cancer types (neck squamous cell carcinomas, breast cancers, and clear cell renal cell carcinomas). This cluster was first validated by studying modulation of the signature genes in response to antiangiogenic treatments in preclinical models. Second, detection of ADGRL4 by immunohistochemistry of tissue microarrays containing primary human tumor samples (head and neck, renal, colorectal, and ovarian cancer samples) confirmed an increased expression of the aGPCR in tumor vessels. Finally, the role of ADGRL4 in tumor was studied by injecting siRNA incorporated into chitosan nanoparticles into an orthotropic model of ovarian carcinoma and a colorectal cancer model. This resulted in a significant decrease in tumor weight, associated with a reduced metastatic dissemination and a steep increase in survival [69].

It would be interesting to see if some of these results hold true in mouse models harboring oncogenic lesions, in which the cooptation of the endogenous microenvironment reflects the patient situation more closely.

Finally, a number of challenging situations, often related to pathological context, might unravel unexpected roles for aGPCRs, following the example of ADGRF5, which upon genetic inactivation completely reverses the pathological neovascularization in the oxygen-induced retinopathy model [51]. In this perspective, a number of expression data suggest modulation of aGPCRs in the endothelium upon challenge:

– Shear stress: ADGRA2 and ADGRG6 expression is elevated upon shear stress [79, 80].
– Ischemia: ADGRB2 (BAI2) was discovered in the brain upon ischemic challenge [81].
– Hypoxia: ADGRE5, ADGRC2 (Celsr2), ADGRG5 (Gpr114), and ADGRG1 are induced, while ADGRD1, ADGRB3 (BAI3), and ADGRG7 (Gpr128) are repressed [82].
– Inflammation/cytokine exposure: ADGRL4, ADGRE2 (EMR2), ADGRF4 (Gpr115), ADGRB1, and ADGRG5 are induced, while ADGRL1, ADGRL2, ADGRE5, and ADGRD2 (Gpr144) are downregulated [83–85].
– Cirrhosis: ADGRA3 and ADGRC1 expression is induced, while ADGRL1, ADGFR5, and ADGRL4 are repressed [86].

4 Conclusions

Cardiac and vascular functions of aGPCRs are still largely unknown. Deletion of two aGPCRs, ADGRG6 and ADGRA2, has resulted in a severe cardiac and brain vascular phenotype, respectively. While the observed phenotypes of aGPCRs knockout mice demonstrate the importance of aGPCRs in the cardiovascular field, the ligands or mechanisms activating aGPCRs as well as their downstream signaling pathways during heart development, angiogenesis and in blood-brain barrier function remain elusive.

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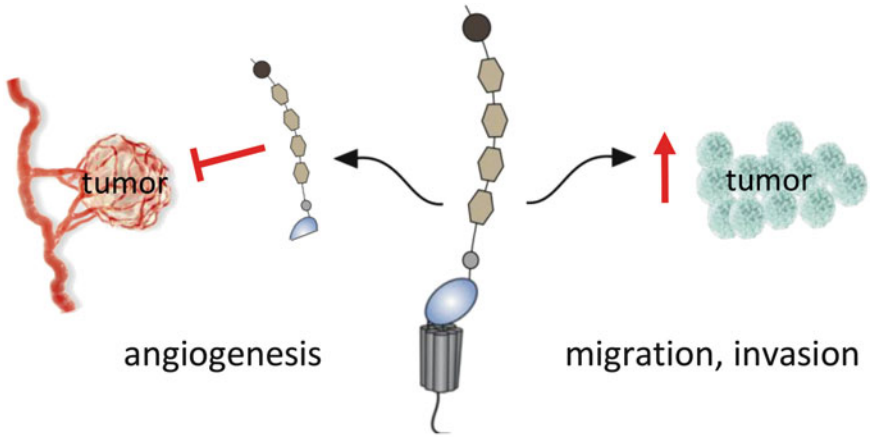
Adhesion GPCRs in Tumorigenesis

Gabriela Aust, Dan Zhu, Erwin G. Van Meir, and Lei Xu

Graphical Abstract



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Contents

Abstract

Alterations in the homeostasis of several adhesion GPCRs (aGPCRs) have been observed in cancer. The main cellular functions regulated by aGPCRs are cell adhesion, migration, polarity, and guidance, which are all highly relevant to tumor cell biology. Expression of aGPCRs can be induced, increased, decreased, or silenced in the tumor or in stromal cells of the tumor microenvironment, including fibroblasts and endothelial and/or immune cells. For example, ADGRE5 (CD97) and ADGRG1 (GPR56) show increased expression in many cancers, and initial functional studies suggest that both are relevant for tumor cell migration and invasion. aGPCRs can also impact the regulation of angiogenesis by releasing soluble fragments following the cleavage of their extracellular domain (ECD) at the conserved GPCR-proteolytic site (GPS) or other more distal cleavage sites as typical for the ADGRB (BAI) family. Interrogation of in silico cancer databases suggests alterations in other aGPCR members and provides the impetus for further exploration of their potential role in cancer. Integration of knowledge on the expression, regulation, and function of aGPCRs in tumorigenesis is currently spurring the first preclinical studies to examine the potential of aGPCR or the related pathways as therapeutic targets.

Keywords

Tumor cell migration • Tumor invasion • Metastasis • Tumor angiogenesis • Tumor therapy

Abbreviations

CCLE	Cancer Cell Line Encyclopedia
CTF	C-terminal fragment
ECD	Extracellular domain
ECM	Extracellular matrix
GAIN	GPCR autoproteolysis inducing
GPS	GPCR-proteolytic site
NTF	N-terminal fragment
TG2	Tissue transglutaminase
TSR	Thrombospondin repeat

1 Introduction

Cancer arises through the sequential accumulation of driver gene mutations which ultimately confer a growth advantage upon the cells in which they have occurred, leading to tumor formation through a clonal selection process. An important cancer hallmark is the ability of cancer cells to acquire invasive and metastatic properties which is associated with progression to systemic disease and reduced survival. Invasion and metastasis require tumor cell detachment and migration, extravasation into the circulation, and subsequent seeding at distant sites accompanied by altered extracellular matrix (ECM) turnover and initiation of tumor neo-angiogenesis. Each of these steps involves direct tumor cell-cell and tumor cell-microenvironment interactions, including stromal elements such as tumor-associated fibroblasts, endothelial cells, and tumor-infiltrating immune cells as well as the ECM. aGPCRs regulate cell adhesion, migration, polarity, and guidance; cellular functions that are also required for tumorigenesis. Characteristic for the aGPCRs is the large ECD with multiple adhesive folds, which is coupled to a seven-span transmembrane (7TM) domain and an intracellular domain (ICD) (see also [1–4]). Many aGPCRs are cleaved at the GPS in the juxtamembrane region which is part of and regulated by a GPCR autoproteolysis-inducing (GAIN) domain and results in an N- (NTF) and a C-terminal fragment (CTF), which remain associated at the cell surface (discussed in depth in [3, 5]). The various possible signaling scenarios resulting from this bipartite structure have been reviewed recently [6, 7] and in [8–10].

2 Software for the In Silico Interrogation of Cancer-Related Databases

A number of online resources provide user-friendly software for the rapid interrogation of large-scale cancer genomics data sets, and this in silico data mining provides useful starting information on aGPCR expression or mutation in a variety of cancer samples and cell lines.

1. The Cancer Cell Line Encyclopedia (CCLE) (www.broadinstitute.org/ccle) contains detailed genetic characterization for more than 1000 human cancer cell lines [11].
2. The cBioPortal for Cancer Genomics (www.cbioportal.org) provides analysis tools to query, analyze, and visualize large-scale cancer genomics data sets, including The Cancer Genome Atlas (TCGA) [12, 13].
3. The Human Protein Atlas (HPA) database (www.proteinatlas.org) contains high-resolution images showing the spatial distribution of proteins in dozens of normal and malignant tissues [14]. Note: the specificity of the used paraffin-suitable antibodies is not verified in depth.
4. The Catalogue of Somatic Mutations in Cancer (COSMIC) (cancer.sanger.ac.uk/cosmic) is designed to store and display somatic mutation information.
5. The SurvExpress is a biomarker validation tool and database for cancer gene expression with survival analysis [15].

3 Identification of Adhesion GPCRs in Cancer

In the last two decades, it has become apparent that changes in aGPCRs occur in cancer, either at the gene or protein expression level. Studies addressing the relevance of these changes to the tumorigenesis process are currently limited. CD97 was the first aGPCR identified to be correlated to cancer [16]. While it is not expressed in normal thyrocytes and well-differentiated papillary and in part follicular thyroid cancers, the protein was found to be elevated in anaplastic carcinomas. In the following years other aGPCRs such as GPR56 were identified to be cancer related [17]. Non-biased screens such as mRNA microarrays, genome-wide association (GWA) studies, or proteome analyses confirmed previously identified cancer-related changes in aGPCRs [18], showed alterations in new tumor entities [19], or identified new aGPCRs involved in cancer [20]. Recently, a whole-genome sequencing (WGS) approach in 441 human breast, lung, ovarian, and prostate cancers identified the aGPCRs *ADGRB3 (BAI3)* and *ADGRL3 (LPHN3)* among 77 significantly mutated genes [21]. Further studies are warranted to determine whether these mutations are functionally significant to cancer development or progression or simply passenger mutations.

4 Alterations in Adhesion GPCR Expression in Tumors Compared to Normal Tissue

aGPCRs may be induced, increased, decreased, or silenced in tumor compared to the corresponding normal cells. A quick way to determine whether a particular aGPCR may be present in tumor cells is to interrogate the CCLE [11]. This database shows high levels of mRNA for ADGRA3 (GPR125), ADGRB2 (BAI2), ADGRC2 (CELSR2) and ADGRC3 (CELSR3), CD97, GPR56, ADGRG6 (GPR126), ADGRL1 (LPHN1), and ADGRL2 (LPHN2) genes in hundreds of cell lines derived from nearly all tumor entities (note: the normal corresponding cells are not included in the database). The mRNA of other aGPCRs is absent in these cell lines, including many members of the ADGRF and ADGRG families. Whether expression in cultured cell lines reflects expression in tumor cells in vivo needs to be addressed in each individual aGPCR and tumor type.

There is also evidence for changes in posttranslational modification of aGPCRs associated with cancer, which could have functional consequences. This was first shown for CD97. In normal smooth and skeletal muscle cells, the non-modified CD97 protein core is present, whereas in their malignant counterparts, i.e., in leiomyosarcoma and rhabdomyosarcoma cells, CD97 is found N-glycosylated [66, 67]. It is well known that glycosylation patterns are modified in cancer [88, 89]; therefore, it is important to determine whether such changes are relevant to the tumorigenic process for each individual aGPCR. N-glycosylation of CD97 is mapped to the adhesive EGF-like domains and is needed for the binding of the interaction partner CD55 [67], which is consistent with CD97 and CD55 often being co-expressed in cancer [53, 54, 90–93].

Regulation of aGPCR expression in stromal cells, such as tumor-associated fibroblasts and endothelial or tumor-infiltrating immune cells, as shown for ADGRA2 (GPR124) and all BAIs, may also support or suppress tumor progression. For example, BAI1 has been shown to play a role as an engulfment receptor on macrophages that can recognize apoptotic cells through binding of membrane-exposed phosphatidylserine groups to BAI1's extracellular thrombospondin repeats (TSRs) [94]. Variation in BAI1 expression on macrophages or possibly tumor cells may change the dynamics of dying tumor cell clearance from tumors, related inflammation, and cancer metabolism.

5 Soluble Adhesion GPCR and Cleavage Fragments in Tumorigenesis

As a direct consequence of the autocatalytic cleavage of aGPCRs at the GPS, as well as through (additional) independent proteolysis events within their ECD, the NTF or smaller fragments may be released from the receptor and appear in body fluids. The existence of circulating soluble NTFs is reported for GPR124 [33], BAI1 [69, 85], BAI2 [95], CD97 [96], ADGRF5 (GPR116) [97], GPR126 [98], and LPHN1 [99].

Theoretically, soluble NTFs could engage with interaction partners over large distances far away from the site at which they were released and thereby modulate heterotypic tumor-stromal interactions that may be relevant to tumorigenesis. This has been shown experimentally for the NTFs of the various isoforms of CD97, which differ in the number of EGF-like domains in their ECDs. Soluble CD97 stimulates tumor angiogenesis in vitro and in vivo through binding of integrins and chondroitin sulfate, a constituent of the ECM, in an isoform-specific manner [100]. Soluble CD97 is found at sites of inflammation [96, 101, 102], most likely released from CD97-positive immune cells, but not in body fluids of patients suffering from CD97-positive tumors [48]. These data suggest either that unknown cell-specific mechanisms control the release of the NTF or that an additional independent proteolysis event, restricted to immune cells, is necessary for this release.

In contrast to the pro-tumorigenic activity of CD97 NTFs, the NTFs of BAI1 have anti-angiogenic and anti-tumorigenic activities [69, 70] as further discussed below.

6 Biological Functions of Adhesion GPCRs in Cancer

6.1 Proliferation, Apoptosis, and Cell Cycle Regulation

Little is known about the role of aGPCRs in tumor growth, either through alteration in tumor cell division or cell death mechanisms including apoptosis. A few aGPCRs have been shown to promote cell proliferation and/or prevent apoptosis, although their role in signaling is not clarified. Downregulation of ADGRL4 (ELTD1) by microRNA-139-5p leads to an inhibition of glioblastoma cell proliferation in vitro [103]. CD97 protects fibrosarcoma HT1080 and cervical HeLa cancer cell lines from serum starvation- and staurosporine-induced intrinsic apoptosis [104].

Preliminary studies examining the effect of GPR56 on in vitro tumor cell proliferation and apoptosis have yielded contradictory results and may reflect the different cancer types and studies performed. One study reported that the knockdown of GPR56 expression induces a transient activation of apoptosis in several cancer cell lines, suggesting that GPR56 may be important for cell survival and have pro-tumorigenic functions [32]. This pro-apoptotic response could be overcome, as this research group was able to generate stable cell lines with GPR56 knockdown. These clones show a reduction in anchorage-independent growth in vitro, and some also have decreased tumor growth in vivo [32]. Similar observations were reported in the EVI-1^{high} leukemia cell line, in which GPR56 knockdown induced apoptosis although its effects on leukemia progression in vivo were not explored [22]. This pro-growth function of GPR56 contrasts with observations by another group in metastatic melanoma (A375P and MC-1) cell lines. This study suggests that GPR56 is necessary for the subcutaneous growth of these cells in mice, but does not affect their in vitro proliferation [60, 105]. The mechanisms linking GPR56 to in vivo tumor growth are found to depend on

regulation of proper ECM deposition by the melanoma cells [60]. A larger panel of cell lines as well as a combination of in vitro and in vivo studies will be needed to fully understand the function of GPR56 during cancer progression.

6.2 Cell Adhesion and Interaction with ECM

Some aGPCRs have been implicated in regulating tumor cell adhesion, but potential mechanisms of these effects have not been investigated in depth. Knocking down *Gpr125* in murine myeloid sarcoma lines that harbor $KRAS^{G12C}$ and MLL/AF10(OM-LZ) oncogenes leads to reduced myeloid sarcoma burden in mice, and a decrease in cell adhesion in culture, possibly contributing to their reduced tumorigenicity [106].

ECM proteins are large peptides that assemble into highly ordered structures modulating various aspects of cell behavior, including cell adhesion, migration, survival, and proliferation, which are key factors during tumorigenesis [107]. GPR56 NTF was discovered to bind tissue transglutaminase (TG2) [105] and collagen III [108]. It was reported that GPR56 internalizes TG2 from the surface of melanoma cells, resulting in defects in ECM deposition in melanomas [60]. This defect may contribute to the inhibitory function of GPR56 on melanoma growth and metastasis. Knocking down GPR56 inhibits the adhesion of EVI-1^{high} leukemia cells to ECM [22], and granule cells from the rostral cerebellum of $Gpr56^{-/-}$ mice are defective in adhering to fibronectin and laminin [109]. The adhesion effect of GPR56 could be blocked by its NTF: purified recombinant GPR56 NTF inhibits adhesion of glioma cells to fibronectin [82]. Taken together, the above studies point to a promoting role of GPR56 in cell-ECM adhesion, but whether this is a shared mechanism among different tumor cells and whether it contributes to the function of GPR56 in cancer progression remain to be investigated.

Several other aGPCRs such as ADGRE2 (EMR2) and GPR126 have also been shown to interact with the ECM [110–112], but the impact of these interactions on cancer has not been explored.

6.3 Cell Migration and Invasion

Among all aGPCRs, CD97 has been the most studied in the context of cell migration and invasion (see also [113] on this topic). Its association with tumor invasion is reported in several studies. It is upregulated at the invasive tumor front [42, 48, 91], and its high expression correlates with lymph node invasion [48, 54], advanced tumor stages [48, 53, 54, 91, 93], and patient survival [53, 77, 93]. The positive effects of CD97 on tumor invasion are verified in experimental models both in vitro and in vivo [49, 58]. Consistent with its function in promoting

migration and invasion, CD97 is reported to enhance trans-well cell migration toward fetal calf serum in 15 different colorectal cancer cell lines [48] and toward fetal calf serum and lysophosphatidic acid (LPA) in AML cells (MV4-1 cell line) [29]. Consistently, directed cell migration and invasion stimulated by fetal calf serum are decreased following CD97 knockdown in a prostate cancer cell line (DU145) [58]. Conflicting results have been published on the effect of CD97 in a fibrosarcoma cell line (HT1080) on in vitro migration and in vivo formation of tumors and metastases: in one study CD97 overexpression increases migration in vitro and induces earlier tumor growth [49], whereas another study found the opposite [114].

Other aGPCRs have also been reported to regulate cancer cell migration. GPR56 inhibits cell migration toward stromal cell-derived factor 1 in leukemia cell lines [22], and GPR116 promotes cell migration and invasion toward serum in a breast cancer cell line (MDA-MB-231) through the Gαq-p63RhoGEF-RhoA/Rac1 pathway [65].

6.4 Angiogenesis

Several aGPCRs are found to regulate tumor angiogenesis, the process through which tumors elicit vessel formation that is critical for their sustained growth (see also [115] on this topic). The most studied one is BAI1, which inhibits angiogenesis in glioblastomas, the most malignant primary intracranial brain tumor which is incurable [69, 70, 116, 117] as well as other cancer types [36, 37, 118, 119]. The ECD of BAIs contains TSRs, and some TSRs are known to negatively regulate angiogenesis [120, 121]. Cleavage of the BAI1 ECD leads to the release of two fragments, dubbed vasculostatin-40 (Vstat40) and vasculostatin-120 (Vstat120) due to their molecular size (Fig. 1), and both were shown to suppress blood vessel formation in a variety of in vitro and in vivo assays [69, 70, 116]. When overexpressed, GPR56 can also inhibit tumor angiogenesis in melanoma, probably by blocking the secretion of vascular endothelial growth factor (VEGF) [61].

A number of aGPCRs also stimulate neovascularization in tumors. GPR124 was described first as a tumor endothelial marker (TEM5) [125]. ELTD1 is identified as part of a core angiogenic signature composed of genes whose expression jointly correlates with that of several well-recognized angiogenesis and/or endothelial cells "seed" genes, in more than 1000 human primary tumors, and is subsequently found to be essential for angiogenesis and tumor growth [34]. The NTF of CD97 promotes angiogenesis via binding to integrin α5β1 and αvβ3 and elicits a chemotactic response in endothelial cells leading to their recruitment to the tumor [100].

7 Families of Adhesion GPCR in Tumorigenesis

Table 1 gives an overview on published studies of aGPCRs in human tumors.

7.1 ADGRA

GPR124 was originally identified as an endothelial marker that is upregulated during tumor angiogenesis [126]. A soluble GPR124 fragment, probably the NTF, is shed from *GPR124*-transfected endothelial cells. Further proteolytic processing creates a protein subunit that mediates endothelial survival and subsequent tumor angiogenesis via interactions with glycosaminoglycans and the integrin αvβ3 [33]. Only cell lines derived from Ewing sarcoma, chondrosarcoma, and osteosarcoma strongly express GPR124 [11]. microRNA miR-138-5p, found to be decreased in gefitinib-resistant non-small cell lung cancer (NSCLC) cell lines (PC9GR, H1975), directly targets GPR124. Thus, downregulation of GPR124 is discussed as a therapeutic approach to overcome NSCLC gefitinib resistance [127]. Knockdown of *Gpr125* reduces tumor cell aggregation and diminishes myeloid sarcoma formation induced by transplantation of immortalized acute myeloid leukemia [106].

7.2 ADGRB

The BAI1–3 proteins are predominantly expressed in the brain [116, 128]. The human *ADGRB1* (*BAI1*) gene is located on chromosome 8q24, and BAI1 is the most studied member in this aGPCR subfamily. BAI1 contains several well-defined protein modules in the N-terminus such as an integrin-binding Arg-Gly-Asp (RGD) motif followed by five TSRs, a hormone binding domain and a GAIN domain with a GPS (Fig. 1) [85]. BAI1 can be cleaved at the GPS releasing a soluble, 120 kDa anti-angiogenic NTF called vasculostatin (Vstat120), which was reported to suppress the proliferation of endothelial cells by blocking αvβ5 integrin [129], reduce the migration of cultured microvascular endothelial cells in a CD36-dependent manner [70], and inhibit angiogenesis and glioma growth in vivo [69, 71, 72, 116, 130]. Subsequently, a novel more distal proteolytic processing event was identified in BAI1, and it generates a more abundant cleaved NTF containing only the RGD and the first TSR (Vstat40). This fragment was also able to inhibit angiogenesis in vitro and in vivo [71]. At the other end of the protein, the C-terminus of BAI1 contains an intracellular proline-rich region (PRR) and a terminal PDZ-binding domain (Fig. 1), which associates with a number of intracellular signaling and scaffolding proteins [131, 132], but the role of these signaling events in cancer has not been studied to date. BAI1 also plays an important role in myogenesis, synaptic plasticity, and phagocytosis [124], but it is unclear whether any of these functions can also intersect with cancer.

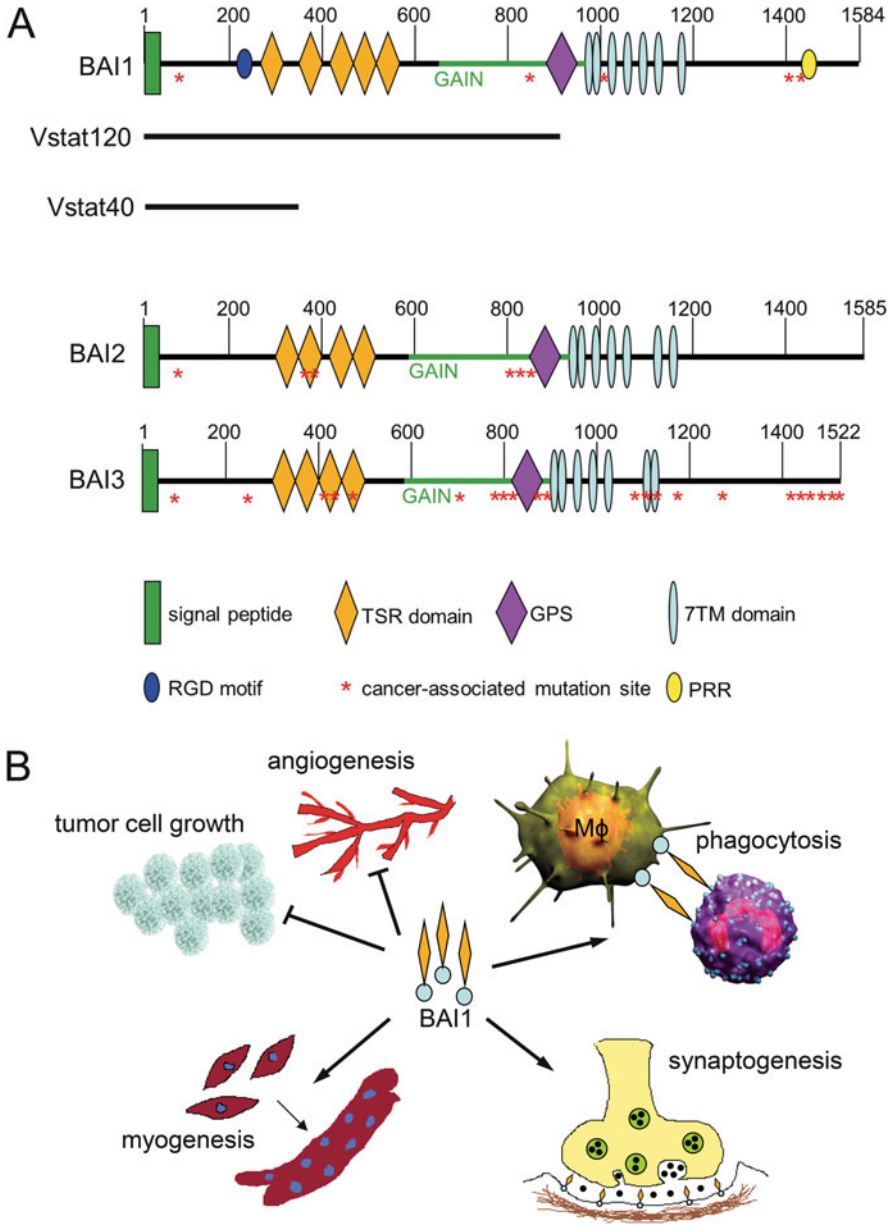

Fig. 1 Structure and functions of BAIs (ADGRBs). (**a**) Schematic of the three BAI proteins, representing the major known structural and functional features as well as known proteolysis events that generate either Vstat120 [69] or Vstat40 [71], and cancer-associated somatic mutations [21, 122]. Abbreviations: *TSR* thrombospondin type 1 repeat, *GAIN* GPCR autoproteolysis-inducing domain [123], *GPS* GPCR-proteolytic site, *7TM* seven-transmembrane region, *RGD* Arg-Gly-Asp integrin-binding motif, *PRR* proline-rich region. (**b**) Multiple functions of BAI1:

BAI1 mRNA levels are consistently downregulated in primary glioma specimens and cell lines [72, 73], and in brain metastases from lung adenocarcinoma [37]. Pulmonary adenocarcinomas, gastric, and colorectal cancers also show reduced BAI1 expression compared with normal tissue [36, 37, 44, 46]. A recent study also suggests that BAI1 expression is significantly reduced in breast cancer and correlates with poorer patient survival [62].

The human *BAI2* and *BAI3* genes were discovered as a result of their sequence identity with BAI1 [128] and are localized on chromosomes 1p35 and 6q12, respectively. The amino acid sequence is highly conserved in all three members; however, BAI1 contains five TSRs, while BAI2 or BAI3 each contains only four. In addition, the extracellular RGD motif and the intracellular proline-rich region (PRR), a domain known to interact with Src homology 3 (SH3) and WW domain-containing proteins [133], are unique to BAI1. Unlike BAI1, the expression of BAI2 or BAI3 is not silenced in glioma cells [72]. Moreover, recent studies determined higher BAI2 expression in tumor metastasis and advanced stages [134, 135].

Importantly, recent discoveries have shown that the BAI genes are silenced and/or undergo somatic mutations in several cancers (Fig. 1), including the lung, breast, ovarian, and brain [21, 72, 122], suggesting that eliminating their function might be required for tumor formation. Whether the identified mutations alter BAI1 function and may contribute to tumor formation has not been investigated to date. The function of BAI2 and BAI3 in tumorigenesis needs to be further investigated.

7.3 ADGRC

Although the role of ADGRCs (CELSRs) as key components in planar cell polarity (PCP) was clarified elegantly in knockout mice, our knowledge on their expression and function in human malignancies is rather limited. Unbiased screening approaches for cancer-related proteins often scored *CELSRs* as interesting hits, but most of them await further validation as to their role in cancer. *CELSR1* is upregulated in B cells of patients with chronic lymphocytic leukemia (CLL) [25]; *CELSR1* and *CELSR3* are found to be present in gastrointestinal and brain tumors, respectively [47]; and *Celsr1* is overexpressed in Graffi murine leukemia virus (MuLV)-induced hematologic malignancies with a lymphoid subtype [24]. Gene expression profiling of mantle cell lymphoma shows that *CELSR1* is downregulated in the non-nodal form compared to other lymphomas [27], and *CELSR1* expression is increased in pure ductal breast carcinomas in situ (DCIS) compared to such

Fig. 1 (continued) BAI1 inhibits tumor cell growth in part by inhibiting angiogenesis. Moreover, its TSRs interact with exposed phosphatidylserine at the outer leaflet on apoptotic cells and elicit the engulfment in macrophages (Mϕ). BAI1 also promotes myogenesis. Deficiency of BAI1 promotes PSD-95 degradation at the synapses and induces enhanced long-term potentiation. Figure adapted from [124]

Table 1 Expression of adhesion GPCRs in tumors

System	Tissue	Tumor	ADGRA2 GPR124	ADGRB1 BAI1	ADGRB2 BAI2	ADGRB3 BAI3	ADGRC1 CELSR1	ADGRC3 CELSR3	ADGRE2 EMR2	ADGRE3 EMR3	ADGRE5 CD97	ADGRF5 GPR116	ADGRG1 GPR56	ADGRG2 GPR64	ADGRG4 GPR112	ADGRL1 LPHN1	ADGRL4 ELTD1
Hematopoietic		Leukemia											[22, 23]				
		B-cell leukemia					[24]										
		Chronic lymphocytic leukemia (CLL)					[25]										
		Acute lymphatic leukemia (ALL)									[19, 26]						
		Mantle cell lymphoma (MCL)					[27]										
		Acute myeloid leukemia (AML)									[28], [29]		[30]				
		Other leukemias											[31]				
Cardiovascular	Heart												[32]				
	Endothelial cells		[33]														[34, 35]
Respiratory	Lung	Non-small cell lung cancer (NSCLC)		[36, 37]	[21]	[38]						[20]	[32, 39]			[40]	
		Small cell lung cancer (SCLC)				[41]											
Digestive	Esophagus										[42]		[43]				
	Stomach	Gastric cancer		[44]							[42]		[45]				
	Large bowel	Colorectal		[46]			[47]				[48, 49]		[32]				
	Neuroendocrine	Ileal carcinoid													[50]		
	Liver	Hepatocellular carcinoma					[51]						[52]				
	Gall bladder, bile duct	Gall bladder carcinoma									[53]						
	Pancreas	Pancreatic cancer									[42, 54]		[32, 55]				
Urinary	Kidney	Wilms' tumor (pediatric)															
		Renal cancer											[32, 56]				
Endocrine	Thyroid	Thyroid cancer									[16, 57]						

Reproductive	Prostate	Prostate cancer									[58]						
	Ovary	Ovarian carcinoma			[21]	[21]							[32]				
	Uterus	Cervical											[32]				
Skin	Skin	Skin tumors														[59]	
		Melanoma											[32, 60, 61]				
	Mammary	Breast cancer, invasive ductal		[62]	[21]		[63]		[64]			[65]	[32]				
Musculoskeletal	Skeletal muscle	Rhabdomyosarcoma									[66]						
	Smooth muscle	Leiomyosarcoma									[67]						
	Bone related	Ewing sarcoma												[68]			
Brain/nervous system	Neurons, glia	Brain tumors		[37]				[47]					[32]				
		Glioblastoma multiforme (GBM)		[69–73]					[74, 75]	[76]	[18, 21, 77–81]		[82, 83]				[84]
		Malignant glioma		[85, 86]	[72]	[72]							[82]				
		Astrocytoma											[82]				
		Medulloblastoma												[87]			

Most studies were performed at the protein level. Only aGPCRs with published data are included. The direction of regulation is not indicated because of the partly complex investigated scenarios

tumors with invasive components [63]. Moreover, the gene locus of *CELSR1* is hypermethylated in hepatocellular carcinoma (HCC) compared to control tissue [51].

7.4 ADGRE

ADGRE1–3 (EMR1–3) expression is restricted to leukocytes. Thus, almost only cell lines derived from hematologic malignancies express them [11]. EMR2 is not or rarely expressed in gastric, pancreatic, esophageal, and colorectal carcinomas [42, 136]. Here, only a subpopulation of tumor-infiltrating macrophages is strongly EMR2 positive. Aberrant expression of EMR2 protein is reported in breast carcinomas. It is associated with poor patient survival [64]. *EMR2* is found upregulated in glioblastoma, in particular in the mesenchymal subtype [74], and is associated with poor overall survival and an invasive phenotype [75].

EMR3 is also found to be increased in glioblastoma and associated with poor survival [76]. In colon cancer EMR3 is restricted to tumor-infiltrating immune cells [137].

CD97 is the only ADGRE family member whose expression is not restricted to immune cells. In human, its expression varies in cells of epithelial and mesenchymal origin from negative, as in keratinocytes and thyrocytes, to low in enterocytes, and high in pneumocytes and leukocytes. Cancer cell lines are almost all moderately or strongly *CD97* positive, suggesting that, compared to normal tissues, CD97 is frequently induced and/or increased in the corresponding malignancies. Low *CD97* levels are found only in cell lines derived from neuroblastomas. Small cell lung cancer (SCLC) cell lines are nearly all CD97 negative, while non-small cell lung cancer (NSCLC) cell lines are strongly CD97 positive, a fact that could be helpful in discriminant analysis on lung cancers.

In thyroid cancer, CD97 is induced and expression levels correlate with malignant progression [16, 57], i.e., only few papillary and follicular thyroid carcinomas are CD97 positive, whereas most anaplastic cancers strongly express CD97. In normal human intestinal epithelial cells, CD97 resides at low levels in lateral cell contacts, whereas in colorectal cancer, the molecule is increased. In part, this upregulation parallels its new cellular location within the cytoplasm [48, 49]. Overexpression of CD97 in scattered tumor cells is observed at the tumor invasion front [48]. The presence of these CD97-positive scattered tumor cells correlates with higher tumor stage and higher lymphatic vessel infiltration, both prognostic factors in colorectal cancer. CD97 is also upregulated in gastric cancer [42]. Further supporting an oncogenic role, in mouse models of colorectal and gastric cancer, CD97 supports local tumor growth and promoted metastatic spread [49, 138].

In gall bladder carcinoma, CD97 expression is an independent risk factor for overall survival [53]. CD97 is highly expressed in tumor cells of poorly differentiated pancreatic ductal adenocarcinoma and in tumor-infiltrating leukocytes in chronic pancreatitis samples, but not on normal pancreatic epithelial cells [139]. In a study of

37 pancreatic cancer patients, CD97 correlates with aggressiveness and was associated with prognosis [54]. In esophageal cancer CD97 is among the 19 genes with promoter hypomethylation and upregulation [140].

CD97 mediates invasion in prostate cancer cells, at least in part, by associating with lysophosphatidic acid receptor 1 (LPAR1), leading to enhanced LPA-dependent RHO and extracellular signal-regulated kinase (ERK) activation. Consistent with its role in invasion, depletion of CD97 in prostate cancer cells (PC3 cell line) results in decreased bone metastasis without affecting subcutaneous tumor growth. CD97 and LPAR1 are significantly co-expressed in clinical prostate cancer specimens.

CD97 is highly expressed in glioblastoma (WHO grade IV) and has prognostic significance in two independent cohorts of 187 and 539 glioblastoma patients, respectively [18, 77]. CD97 is also identified as a potential biomarker for glioma-initiating cells through an in vivo phage display screen [78], suggesting it represents a potential new therapeutic target [77]. In contrast, CD97 expression is minimal in WHO grade II and III astrocytomas [79]. Experimental data suggest that CD97 regulates tumor cell invasion in glioblastomas. Decreasing CD97 by siRNA reduces migration and invasion, but not proliferation in two glioblastoma cell lines [77]. Mass spectroscopy-based proteomic analysis showed that CD97 is enriched in membrane fractions of invadopodia, actin-rich protrusions, of invasive glioblastoma cells [18]. The mechanism underlying CD97-mediated promotion of invasion of glioblastoma cells is under investigation. Suppression of Wilms' tumor gene product WT1 by siRNA led to a decrease in the invasiveness of glioblastoma cell lines paralleled by a suppression of *CD97* RNA [80].

Further evidence for a role of CD97 in tumorigenesis comes from screening studies demonstrating that it is a direct target of the tumor suppressor microRNA-126 in the breast cancer cell line MDA-MB-231 [141].

Additionally, evaluation of CD97 expression in leukemia is highly informative. Gene expression studies and characterization of the leukemia cell surface proteome identified CD97 as a marker for minimal residual disease in acute lymphoblastic leukemia (ALL) [19]. CD97 expression also accounts for the most informative differences between normal and malignant cells in ALL [26]. CD97 has further been identified as a leukemic stem cell marker in acute myeloid leukemia (AML) [28]. In AML, expression levels of CD97 are associated with internal tandem duplications within the juxtamembrane region of the FMS-like tyrosine kinase receptor FLT3 (LFT3-ITD) [29].

Whether the function of CD97 in tumorigenesis could be modulated by other interaction partners such as LPAR1 is unknown. CD55 [142], chondroitin sulfate B [110], $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins [100], and CD90 [143] bind distinct sites within the NTF of CD97 at immune cells; nothing is known whether these interaction partners also bind CD97 in tumors. A few articles have been published on co-expression of CD55 and CD97 in solid tumors but not on the resulting functional consequence [53, 54, 91–93].

7.5 ADGRF

GPR116, highly enriched in fetal and adult lung [144–146], is part of a gene expression signature that differentiates adenocarcinoma of lung and breast origin in effusions [20]. Knockdown of *GPR116* suppresses migration and invasion, whereas ectopic expression enhances invasion of the breast cancer cell line MDA-MB-231 [65]. GPR116 promotes constitutively breast cancer metastasis via the Gαq-p63RhoGEF-Rho GTPase pathway [65]. Its expression is significantly correlated with breast tumor progression, recurrence, and poor prognosis.

7.6 ADGRG

Among the ADGRG members, GPR56 is the most studied in the context of cancer progression. It was reported in 1999 to be downregulated in highly metastatic melanoma cell lines compared with poorly metastatic lines [17]. Its inverse correlation with metastatic potential was evaluated in more depth later in an experimental metastasis model which shows the downregulation of GPR56 in several highly metastatic melanoma derivatives compared with their poorly metastatic parental line (A375P). Re-expression of GPR56 inhibits the growth and metastasis of melanoma cells [105], supporting a tumor suppressor role for GPR56 in melanoma. Mechanistic studies in one of the metastatic derivatives (MC-1) revealed that GPR56 overexpression inhibits the activation of PKCα, resulting in the suppression of VEGF secretion from melanoma cells, leading to angiogenesis inhibition [61]. In contrast to the full-length receptor, expression of the CTF of GPR56 or deletion of the serine threonine proline (STP)-rich segment (ΔSTP-GPR56) leads to enhanced activation of PKCα, VEGF secretion, and angiogenesis. These opposing functions of GPR56 and its CTF indicate that GPR56 might exist in different activation states, although the signaling mechanisms of GPR56 in cancer have not been elucidated. The NTF of GPR56 binds to tissue transglutaminase (TG2) [105] and collagen III [108]. The interaction between GPR56 and collagen III in cancer progression is not reported. TG2 is a cross-linking enzyme in the ECM and thought to play pleiotropic roles in cancer progression [147]. The interaction of GPR56 with TG2 in melanoma growth was evaluated recently in a xenograft model using the immunodeficient $Tg2^{-/-}$ mice [60]. TG2-knockdown melanomas growing in these mice are depleted of TG2 in both cancer cells and stroma and found to be much smaller than the control tumors growing in $Tg2^{+/+}$ mice, arguing that TG2 promotes melanoma growth. This tumor-promoting function is abolished by GPR56 overexpression, probably via receptor-mediated TG2 internalization from the cell surface [60]. Whether GPR56 functions similarly in metastasis and in other cancer types remains to be determined.

In contrast to the situation in melanoma, GPR56 is often found upregulated in other cancer types relative to the corresponding normal tissues [32, 43, 148, 149], so perhaps its function in cancer is cell of origin and/or stage specific. Knocking down of *GPR56* induces transient apoptosis and reduces the in vitro growth of several

cancer cell lines derived from colon (HCT116), melanoma (M14), and cervix (HeLa). Notwithstanding this pro-apoptotic response, the authors were able to derive stable GPR56 knockdown in all the cancer cell lines they tested, including those from the cervix (HeLa), colon (HCT116), melanoma (A2058 and M14), ovary (OVCAR3 and OVCAR8), prostate (PC3), pancreas (AsPC1), and lung (NCI-H460). A reduction in anchorage-independent growth in vitro was observed in all the knockdown lines, and decreased tumor growth in vivo was observed in the knockdown of A2058, PC3, and HCT116 cell lines [32]. This study suggests that GPR56 plays a tumor-promoting role in certain cancer cells. Similar pro-proliferation effects of GPR56 are observed in EVI1^{high} leukemia cell lines AML1 and HNT34, in which knocking down of *GPR56* enriches cells at the subG1 phase of the cell cycle and induces apoptosis [22].

The discrepancies among the above studies need to be followed up in future investigations. It is possible that GPR56 has both pro- and anti-growth functions during cancer progression, for example, via distinct binding partners. In fact, in a recent study [60], knocking down of both GPR56 and TG2 leads to a much more severe reduction in subcutaneous growth of MC-1 cells than *TG2*-knockdown alone, suggesting that GPR56 may have tumor-promoting functions in MC-1 cells in the absence of TG2. This effect of GPR56 was not observed in the WM115 melanoma cell line, pointing again to the complexity of GPR56 function in cancer.

Very recently, GPR56 was identified as a novel and stable marker for leukemic subpopulations with high repopulating capacity, a key feature attributed to leukemia stem cells, for the majority of acute myeloid leukemia (AML) samples [30]. High GPR56 expression was significantly associated with high-risk genetic subgroups and poor outcome.

The second member of the ADGRG group, *GPR64*, was found upregulated in Ewing sarcomas (ES) compared with normal tissues and other sarcomas [68]. *Gpr64* knockdown in Ewing sarcoma lines led to a reduction in tumor growth and metastasis. This tumor-promoting function of Gpr64 was mediated by the induction of placental growth factor (PGF) and matrix metalloproteinase 1 (MMP1) [68]. The mRNA of GPR64 was detected at high levels in cell lines from prostate cancer, non-small cell lung cancer (NSCLC), and melanomas and at moderate to low levels in cell lines from brain, ovary, breast, and colon cancers [68]. It was also identified as a marker for a subgroup of medulloblastomas characterized by overactive WNT signaling [87]. *GPR64* silencing in the highly motile cancer cell lines Hs578T and MDA-MB-231 resulted in a reduction of cell adhesion and migration [150].

ADGRG4 (GPR112) was identified as a marker for neuroendocrine carcinoma cells [151] and was predicted to be one of the candidate genes for targeted therapy of ileal carcinoids [50].

None of the other ADGRG members (ADGRG3, GPR97; ADGRG5, GPR114; GPR126; ADGRG7, GPR128) have been reported to affect tumorigenesis or be dysregulated in cancer samples.

7.7 ADGRL

The CCLE predicts high expression of LPHN1 and LPHN2 in many tumors [11]. A gene identification study in breast tumors led to *Latrophilin-1* [59]. Indeed, a number of breast tumor cell lines apparently overexpress the gene [152]. An invasion-associated four-gene signature including *Latrophilin-1* obtained from the NCI-60 cell line panel has significant prediction in non-small cell lung cancer (NSCLC) [40]. A genome-wide screen between a recurrent muscle-invasive cisplatin-resistant urothelial bladder carcinoma and its adjacent non-tumor tissue found cancer-associated alternative splicing with differential exon usage for *LPHN2* [153]. Linkage studies have revealed numerous *Latrophilin-3* loss-of-function mutations in breast, lung, ovarian, and prostate cancers [21].

ELTD1 is a regulator of physiological and tumor angiogenesis in vitro and in vivo [34]. ELTD1 showed higher expression on endothelium in peritumoral vessels compared to vessels of matched normal tissues [34]. Silencing of *ELTD1* in human ovarian and colorectal cancer xenografts implanted in mice inhibited tumor growth [34]. The transcriptome of the microvasculature associated with glioblastoma identified *ETLD1* as 1 of 95 upregulated genes [35]. Indeed, ELTD1 can be used as a vascular biomarker in glioblastoma [84]. It displays higher expression in high-grade compared with low-grade gliomas. miR-139-5p suppresses glioma cell proliferation by targeting *ELTD1* and regulating the cell cycle [103].

7.8 ADGRV

To identify molecular biomarkers associated with low-grade glioma-associated epileptic seizures, one of the initial symptoms of this tumor, RNA sequence data were collected [154]. A lower expression level of *ADGRV1 (VLGR1)* was found in patients with epileptic seizures compared to seizure-free patients and was confirmed by quantitative RT-PCR.

8 Tumor Therapy and Adhesion GPCRs

Accumulating evidence of the direct involvement of aGPCRs in tumor pathogenesis provides a solid foundation for their further study as potential therapeutic targets. Some aGPCRs are silenced with tumor formation and appear to have tumor suppressor activity (BAI1), while others are overexpressed in cancer and may act as oncogenic factors (CD97, GPR116, ELTD1).

The observation that BAI1 is epigenetically silenced in malignant glioma [72, 155], along with the recent findings of multiple somatic point mutations in the BAI1–3s in several cancers [21, 122], suggests that tumorigenesis may select for BAI1 silencing or inactivation. Importantly, exogenous restoration of BAI1 expression reduces growth and vascularization of tumors derived from gliomas and

pancreatic and renal cell carcinomas [36, 119, 156, 157]. Taken together, these studies suggest that BAI1 fulfills the criteria for a *bona fide* tumor suppressor, and there is significant potential for BAI1 and its extracellular fragments as therapies for the treatment of human cancers. *BAI1* is silenced in glioblastoma multiforme due to methylation of a CpG island in the gene regulatory region, which leads to binding of methyl-CpG-binding domain protein 2 (MBD2) and transition to a suppressive chromatin conformation [72]. Treatment of glioma cells with 5-aza-2-′-deoxycytidine (5-Aza-dC) or knockdown of *MBD2* by shRNA resulted in reactivation of *BAI1* expression and restoration of BAI1 functional activity in that it conferred potent anti-angiogenic activity to glioma cell conditioned media in vitro and in vivo [72]. These findings have therapeutic implications since inhibiting MBD2 could offer a strategy to reactivate BAI1 expression and suppress tumor growth. Sequence-specific antisense inhibitors of MBD2 have been shown to inhibit both anchorage-independent growth of human cancer cell lines in vitro and the growth of human tumor xenografts in vivo [158, 159]. At present, epigenetic approaches in cancer therapy have focused primarily on inhibitors of the DNA methyltransferases and histone modifiers (e.g., HDACs). Thus, targeting MBD2 to reactivate BAI1 may represent a novel promising cancer therapeutic intervention.

In contrast to BAI1, some aGPCRs are overexpressed in tumors. Expression of CD97 was found in human thyroid carcinomas, but not in the corresponding normal thyrocytes [16, 57]. In human thyroid cancer cell lines, CD97 depletion reduced Rho-GTP and decreased lysophosphatidic acid (LPA)-stimulated invasion [57]. Similarly, GPR116 protein expression correlates with clinical progression stages in breast cancer and peaks in tumors with distant metastases [20, 65]. Knockdown of GPR116 in highly metastatic breast cancer cells (MDA-MB-231) suppressed cell migration, invasion, and metastasis in vivo [65]. These studies suggest that modulation of aGPCR expression in tumors may be effective therapeutics. A recent study identified ELTD1 as a novel angiogenesis regulator [34, 35, 84]. ELTD1 is upregulated in tumor endothelial cells and is a good prognostic marker. Moreover, targeting *Eltd1* blocks tumor angiogenesis and substantially inhibits tumor growth in vivo [34]. The function of GPR56 in different cancer types was found to differ, with both pro- and anti-tumorigenic properties, so the therapeutic implication of targeting GPR56 in cancer in general awaits careful evaluation. ADGRG4 (GPR112) was reported to serve as a marker for neuroendocrine carcinoma cells [151] and proposed a therapeutic target for treating ileal carcinoids [50].

In summary, alterations in the expression pattern of different aGPCRs have been documented in several cancers, suggesting that they may potentially serve as biomarkers of disease or therapeutic targets. Undoubtedly, the interest in the therapeutic targeting of the aGPCR family will continue to grow in the upcoming years, but first requires a more comprehensive characterization of their fundamental biological function.

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