Klaus Ebnet *Editor*

Cell Polarity 2

Role in Development and Disease

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ISBN 978-3-319-14465-8 ISBN 978-3-319-14466-5 (eBook)
DOI 10.1007/978-3-319-14466-5

Library of Congress Control Number: 00703242

Springer Cham Heidelberg New York Dordrecht London

Printed on acid-free paper

Springer International Publishing AG Switzerland is part of Springer Science+Business Media (www.springer.com)

Preface

Cell polarity refers to an asymmetric distribution of proteins, lipids, or RNA in the cell. Most animal cells are polarized. In many cell types, polarity is morphologically visible. Neurons, for example, contain a single, long axon and multiple, short dendrites. Migrating fibroblasts protrude multiple lamellipodia selectively at the leading edge. In other cases, cell polarity manifests itself at the molecular and functional level. Stem cells divide asymmetrically by unequally partitioning cell fate determinants into the two daughter cells thus generating daughter cells with different cell fates. Epithelial cells contain two different membrane compartments characterized by a different molecular composition and different functions. Cellular polarization is a highly dynamic process, and the ability of individual cells to polarize is required for the early development of the zygote and for the generation of functional organs. It is not surprising that a loss of the ability of cells to develop and/or maintain a polarized state results in developmental defects and promotes tumor formation. Given the many facets of cellular polarization in different cell types and tissues, it is a central question how this diversity is generated at the molecular level. Remarkably, the molecular diversity is much smaller than expected. It turned out that a small set of polarity proteins, identified in *C. elegans* and conserved in all metazoans, acts as a hub to regulate cell polarity in many different contexts. Specificity in polarization is achieved by dynamic interactions of this molecular hub with other signaling complexes and the intersection between polarity-regulating pathways with other signaling networks.

This book on cell polarity is designed to provide a state-of-the-art overview on the most relevant aspects of cell polarity. It covers the relevant model organisms for the analysis of cell polarity including *C. elegans*, *Drosophila*, lower vertebrates, and Mammalia. In the first volume, it describes the molecular tools that are used to generate cell polarity (Vol. 1, Part I) and introduces various aspects of cell polarity and the mode of their regulation (Vol. 1, Part II). In addition, the first volume underscores the influence of cell-cell adhesion to the generation of cell polarity in different types of cellular interactions (Vol. 1, Part III), and it illustrates the role of polarized protein trafficking during the establishment of apicobasal polarity in

epithelial cells (Vol. 1, Part IV). The second volume of the book has a major focus on physiological and pathophysiological aspects of cell polarity. It describes processes of polarization during early development in various organisms (Vol. 2, Part I), and it illustrates the impact of cell polarity on the asymmetric division of stem cells (Vol. 2, Part II). Furthermore, it describes the important role of cell polarity for tissue homeostasis (Vol. 2, Part III), and it provides examples of how pathogens target cell polarity signaling pathways for their own benefits (Vol. 2, Part IV).

This is the first book that describes cell polarity in a variety of cell types, tissues, and organisms. In its entity, it provides a comprehensive overview on the universal biological phenomenon of cell polarity, and it illustrates a principle of evolution, i.e., the invention of core mechanisms and their adaptation to new functions to generate diversity and higher complexity. The book is of interest for both basic and applied research, for researchers at all levels, for lecturers, and for clinicians.

Münster, Germany Klaus Ebnet

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Part I
Cell Polarity During Development

Chapter 1
Cell Polarity in One-Cell *C. elegans* Embryos: Ensuring an Accurate and Precise Spatial Axis During Development

Martin Mikl and Carrie R. Cowan

Abstract Cell polarity in one-cell *C. elegans* embryos guides an asymmetric cell division that starts the resulting cells along different developmental paths. Cell polarization starts in response to a signal from the centrosomes, triggering a cell-wide reorganization of the cortex. Functionally, the most notable change is the establishment of two mutually exclusive, antagonistic cortical domains, each composed of distinct PAR proteins. PAR proteins are a diverse but generally conserved group of polarity regulators that exert their polarizing effects through different downstream components. In one-cell *C. elegans* embryos, PAR polarity dictates the asymmetric segregation of fate determinants in the cytoplasm and controls the position of the cleavage furrow, allowing for unequal cell division. *C. elegans* embryos have been informative in identifying cell polarity factors, largely because embryo development is invariant and thus deviations from normal are easy to detect. It is becoming increasingly apparent that the invariance of *C. elegans* polarization results from parallel pathways and backup mechanisms that ensure robustness when components vary or the system is compromised. Recent work now points to a significant role of protein homeostasis in the accuracy and precision of polarity establishment in *C. elegans*, raising questions about the regulatory circuits that underlie this robustness.

Keywords Centrosome • Fate determinant • PAR protein • Protein homeostasis • Robustness • Symmetry breaking

1.1 Introduction

Cell polarity allows for the spatial organization of cellular functions. It is a prerequisite for asymmetric cell division, cell migration, cell adhesion, unidirectional growth, polarized uptake or secretion, and cellular differentiation. Cell polarity creates regional specializations within a cell and guides the formation of

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K. Ebnet (ed.), *Cell Polarity 2*, DOI 10.1007/978-3-319-14466-5_1

specialized structures, such as axons or cilia. It can either be a transient feature of a cell for a specific purpose—for instance, polarized transport during asymmetric division in yeast—or constitute a permanent intracellular compartmentalization, as in the specification of neuronal processes. Cell polarity also informs a higher organizational level, including tissue patterning, organ formation, and early embryonic development. Organisms start as single-celled zygotes, and from this one cell, all the different tissues will arise through asymmetric cell fate decisions guided by cell polarity (Betschinger and Knoblich 2004).

Cell polarization involves three general steps:

- Symmetry breaking: an external cue from the environment or an internal, spontaneous signal determines when and where a cell polarizes.
- Cortical reorganization: the cell cortex is partitioned into two domains in response to the break in symmetry, a process involving cytoskeletal reorganization and the asymmetric distribution of polarity determinants.
- Functional polarization: individual molecules, complexes, or compartments that will control the downstream polarized processes distribute asymmetrically according to the cortical polarity.

Polarized cells are found in every developmental context. There are many common principles but also important cell type-specific differences (St Johnston and Ahringer 2010). *S. cerevisiae* polarize the cytoskeleton and membrane trafficking machinery in response to an internal or a spontaneous signal, leading to asymmetric growth and division or budding (Slaughter et al. 2009). The cell membrane of epithelial cells is divided into distinct compartments: an apical domain, which communicates with the external environment, and a basolateral domain, specialized for contacts with the extracellular matrix and polarized transport (Nelson 2009; Orlando and Guo 2009). Neurons spatially separate inputs and outputs by specifying cellular compartments for either function: the axon for sending and dendrites for receiving signals (Tahirovic and Bradke 2009). In oocytes and embryos, cell polarity allows for the specification of individual cell fates (Munro and Bowerman 2009; Prehoda 2009; Roth and Lynch 2009).

1.2 Cell Polarity in *C. elegans*

1.2.1 C. elegans *as a Model System*

Cell polarity has been extensively studied in *C. elegans*, a well-established metazoan model for developmental biology, neuroscience, gene expression, and aging. The technical advantages that make *C. elegans* such an attractive model system include its optical transparency, rapid life cycle, and easy maintenance in the laboratory (Brenner 1974). Interfering with gene function is simple due to the large collection of mutants, genetic tools, and efficient gene knockdown using

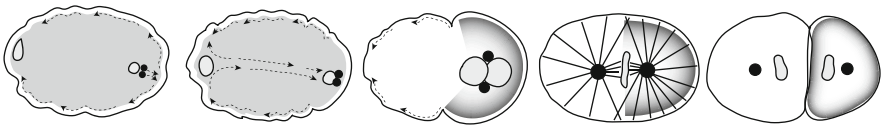

Fig. 1.1 Cell polarization and asymmetric cell division in *C. elegans* embryos. Cartoons depict approximately 25 min from polarity establishment (*left*) to cleavage of the one-cell embryo (*right*). Cortex: *black outline*; nuclei: *light gray blobs*; centrosomes: *black dots*; microtubules: *black lines*; cytoplasmic fate determinant: *diffuse*, *graded gray*; cortical and cytoplasmic flows: *dotted arrows*

RNAi (Fire et al. 1998). Especially in the context of development, *C. elegans* has proven to be an outstanding model system because of its invariant development, including the fully characterized cell lineage (Deppe et al. 1978; Sulston et al. 1983). Investigations of cell polarity, taking advantage of the stereotyped sequence of events that underlie polarization, have allowed for the discovery of many key players in this process that have proven to be conserved throughout evolution.

C. elegans has a short life cycle whose exact duration depends on the temperature. Under optimal conditions, the generation time is approximately 3 days. After hatching, the worm goes through four larval stages before reaching adulthood. *C. elegans* is a self-fertilizing hermaphrodite, resulting in genetically identical progeny. Sperm production occurs in the fourth larval stage, followed by a switch to oocyte production. The reproductive system of the worm consists of the somatic gonadal sheath and the germline cells, together forming two U-shaped gonad arms, which are connected to the shared uterus through spermathecae. The germline stem cells are located at the distal tip of the syncytial gonad arm. Germ cells then pass through a mitotic zone before entering meiosis and arresting in prophase I. Shortly before arrest, the diakinesis-stage nucleus migrates distally within the oocyte cytoplasm to lie near the plasma membrane, facilitating polar body extrusion after the meiotic divisions (Fabritius et al. 2011). Meiosis resumes after fertilization, which occurs inside the worm as oocytes pass through the sperm storage organ, the spermatheca (Greenstein 2005). Because of the gonad architecture, fertilization usually occurs at the side of the oocyte that passes through the spermatheca first (Goldstein and Hird 1996), opposite the displaced female nucleus, giving rise to the stereotypic maternal-paternal axis of one-cell embryos (Fig. 1.1).

1.2.2 Polarization of the C. elegans *Zygote*

1.2.2.1 Symmetry Breaking

The unfertilized oocyte does not possess any inherent asymmetries that influence establishment of cell polarity in the embryo (Goldstein and Hird 1996). Newly fertilized zygotes are unpolarized and establish the anterior-posterior polarity axis about 30 min after fertilization (Fig. 1.1). Prior to polarity establishment, the

actomyosin cortical network undergoes stochastic cycles of contraction and relaxation throughout the cortex (Munro et al. 2004). When meiosis II is complete and the embryo enters the first mitotic cell cycle, cortical contractions stop in a small (~5 μm^2) region (Bienkowska and Cowan 2012), marking the nascent posterior pole of the cell. This noncontractile posterior domain expands at the expense of the contractile cortex (Munro et al. 2004), which, by default, is the anterior. Expansion of the posterior domain generates anteriorly directed cortical flow and posteriorly directed flow of the internal cytoplasm (Hird and White 1993). The molecular nature of the cue that initiates this cortical reorganization is largely unknown, but several studies have shown that the sperm-derived centrosomes specify the symmetry-breaking site (Bienkowska and Cowan 2012; Cowan and Hyman 2004a, 2006; Cuenca et al. 2003; Goldstein and Hird 1996; Hamill et al. 2002; O'Connell et al. 2001; Wallenfang and Seydoux 2000). Centrosomes are thought to harbor regulatory signals, such as the Rho GAP CYK-4 (Jenkins et al. 2006), that could change the contractility of the actomyosin cortex in the local region around the centrosomes. The GAP activity of CYK-4, however, is not required for symmetry breaking (Tse et al. 2012), suggesting other cues must contribute to the local downregulation of actomyosin contractility. It has also been observed that the Rho GEF ECT-2 disappears from the symmetry-breaking site (Motegi and Sugimoto 2006; Munro et al. 2004), which could induce a change in the actomyosin cortex that would cause local cortical rearrangement (Cowan and Hyman 2004b; Munro et al. 2004) but would be unlikely to produce large-scale cortical flow (Mayer et al. 2010).

The position of the centrosome at the time of polarity establishment determines where on the cell cortex contractions will cease and where the posterior pole will form (Bienkowska and Cowan 2012; Cowan and Hyman 2004a; Cuenca et al. 2003; Goldstein and Hird 1996). In wild-type embryos, centrosomes are typically within a few micrometers of the cortex at the time symmetry breaking becomes apparent (Bienkowska and Cowan 2012). Centrosome position, however, does not appear to be regulated. Centrosomes can initiate symmetry breaking from any position within the cell. The site of symmetry breaking corresponds to the site on the cortex closest to the centrosomes. The polarity axis organizes around the symmetry-breaking site, regardless of where symmetry breaking occurs.

1.2.2.2 Cortical Polarity

Contractile asymmetry is a hallmark of polarization in *C. elegans* embryos. The physical changes in cortical activity initiate the asymmetric distribution of cortical effector molecules, which will then guide the polarized distribution of cell fate determinants and asymmetric division (Fig. 1.1). At the core of these processes is a group of so-called PAR proteins. The PAR proteins were originally identified in *C. elegans* as *par*titioning-defective mutants in a screen that directly assayed cell fate determination in the early embryo, taking advantage of the highly stereotyped cell division patterns and lineage architecture of *C. elegans* (Kemphues et al. 1988).

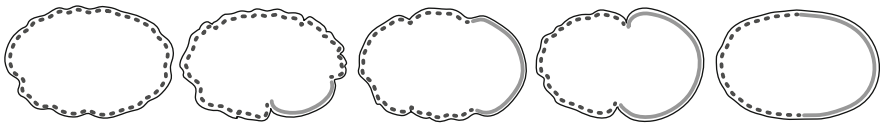

Fig. 1.2 PAR polarization in one-cell *C. elegans* embryos. Cartoons depict approximately 15 min from initiation of polarity (*left*) to the steady state (*right*). Cortex: *black outline*; aPARs: *dotted dark gray line*; pPARs: *light gray line*. Posteriorization—between the second and third embryos—orients the polarity axis to the long axis of the eggshell (not indicated)

The PAR proteins are central to the polarization of the embryo and help define cell polarity through their own asymmetric localization (Fig. 1.2) (Boyd et al. 1996; Etemad-Moghadam and Guo 1995; Guo and Kemphues 1995; Watts et al. 1996). PAR proteins have been shown to be involved in many polarized cell types across many metazoan species (Goldstein and Macara 2007; Knoblich 2010). In particular, investigations of *Drosophila* oocyte polarity and neuroblast asymmetric cell division identified roles for homologs of all of the PAR proteins—with the exception of PAR-2—and contributed substantially to the molecular understanding of how PAR proteins are restricted to domains and how the reciprocal domains may antagonize each other (Benton and St Johnston 2003b; Betschinger et al. 2003; Doerflinger et al. 2003; Parton et al. 2011; Petronczki and Knoblich 2001; Shulman et al. 2000; Tomancak et al. 2000).

Initially after fertilization, the embryo is unpolarized and the anterior PAR complex (aPARs), consisting of the PDZ domain containing proteins PAR-3 and PAR-6 and the serine/threonine kinase PKC-3, is distributed all over the cortex (Fig. 1.2). After symmetry is broken, the aPARs are displaced from the symmetry-breaking site by the cortical flows generated by the asymmetric properties of the actomyosin cortex, as PAR-6 moves away from the posterior pole with the same speed as myosin foci (Munro et al. 2004) and shows the properties of advective transport by the flowing cortex (Goehring et al. 2011b). The RING domain protein PAR-2 and, subsequently, the MARK-family serine/threonine kinase PAR-1 then localize to the posterior cortex, forming two antagonistic cortical domains and defining the anterior-posterior axis. Around the time of nuclear envelope breakdown in the first mitotic cell cycle, the cortical areas occupied by aPARs and pPARs are almost identical in size. The two mutually exclusive PAR domains are maintained throughout the cell cycle. PAR polarity at the cortex controls multiple downstream processes to direct asymmetric cell division and the adoption of different cell fates.

1.2.2.3 Functional Polarization

The PAR domains at the cortex control a number of asymmetrically distributed processes that will contribute to the differences between the anterior and posterior blastomeres after cell division (Fig. 1.1). The anterior and posterior blastomeres are unequally sized, with the anterior almost twice the volume of the posterior. This size asymmetry is important for enabling the correct cell-cell interactions later in

development. The difference in size results from asymmetric displacement of the mitotic spindle and the spindle's subsequent role in determining where the cell will cleave. During anaphase, the spindle moves toward the posterior cortex due to PAR protein-dependent differences in the pulling forces acting on astral microtubules (Grill et al. 2001). The midpoint of the spindle axis specifies the position along the anterior-posterior axis at which the cytokinetic ring forms (Bringmann and Hyman 2005). The posterior displacement of the spindle results in a posteriorly positioned cytokinesis furrow, thus determining the physical asymmetry of division.

The anterior and posterior blastomeres also differ with respect to cell fate. This is already apparent at the two-cell stage, when the cells show different patterns of gene expression and differences in cell cycle timing (Kemphues et al. 1988; Kirby et al. 1990; Mello et al. 1996). The differences in gene expression appear to be regulated largely by differential translation of a common population of mRNAs (Seydoux and Fire 1994), arising from the unequal distribution of several RNA-binding proteins that regulate translation of associated mRNAs (Bowerman et al. 1993; Guedes and Priess 1997; Mango et al. 1994; Mello et al. 1992; Ogura 2003; Tabara et al. 1999; Tenenhaus et al. 2001). The segregation of these RNA-binding proteins to the anterior or posterior is therefore a decisive step in controlling gene expression. The PAR proteins provide the spatial coordinates for the asymmetric partitioning of these regulatory molecules (Bowerman et al. 1997).

How do the cortical PAR proteins control the distribution of fate determinants diffusing in the cytoplasm? The best studied of these RNA-binding proteins with respect to localization is MEX-5, a tandem zinc-finger protein similar to the human tristetraprolin protein family (Pagano et al. 2007). In one-cell embryos, MEX-5 starts off uniformly distributed throughout the cell but establishes a concentration gradient along the anterior-posterior axis within about 10 min after entry into the first mitotic cell cycle (Cuenca et al. 2003). The higher concentration of MEX-5 in the anterior ensures its unequal inheritance during division. MEX-5 diffuses freely in the cytoplasm (Daniels et al. 2010). The gradient forms in response to spatial changes in MEX-5's diffusion rate, which results from a change in MEX-5 phosphorylation by a cytoplasmic gradient of PAR-1 in the posterior (Griffin et al. 2011). The protein phosphatase 2A LET-92 is uniformly localized and counteracts PAR-1-dependent phosphorylation and thereby returns MEX-5 to its slow-diffusing state. Although the molecular basis for the change in MEX-5 diffusion is not entirely clear, binding to mRNA substrates appears to contribute to slow the rate. The mechanism by which the PAR-1 gradient is established has not been studied but might arise from changes in PAR-1 diffusion through its interactions with the posterior cortex.

1.2.2.4 Par Phenotypes

Mutations in the PAR proteins affect cell polarity in characteristic ways. They do not seem to interfere with the signal to break symmetry, as contractile polarity can be established in Par mutants (Kirby et al. 1990). In *par-2* mutants, the posterior

cortex is cleared of aPARs due to the cortical flows generated by the polarized actomyosin cortex, but this initial asymmetry cannot be maintained (Boyd et al. 1996; Cuenca et al. 2003). As contractility is downregulated later in the cell cycle, the aPARs move back into the posterior side and ultimately occupy the entire cortex. *par-1* mutants do not show a failure to maintain a posterior domain (Boyd et al. 1996), but the asymmetric distribution of cell fate determinants is impaired (Guo and Kemphues 1995). This places PAR-1 as a link to the downstream effects of PAR polarity. Mutations in aPARs lead to a failure to exclude PAR-2 from the cortex, and consequently, PAR-2 occupies the whole embryo cortex even before symmetry breaking (Etemad-Moghadam and Guo 1995; Tabuse et al. 1998; Watts et al. 1996). The Par mutants were found to affect all asymmetric cell divisions in embryos, equalizing the size of the resulting blastomeres, synchronizing subsequent divisions, and coordinating orientation of the cell division planes (Kirby et al. 1990). The Par mutant phenotypes indicate the gene products to be master regulators of cell polarity and asymmetric cell division in *C. elegans*.

1.2.2.5 Molecular Functions of PAR Proteins

How do PAR proteins function? Although their common name suggests a homogenous group of proteins, they have very different molecular functions and subcellular localizations. Not all of the proteins identified in the original *C. elegans* partitioning-defective screen showed an asymmetric localization: in contrast to asymmetrically distributed cortical PAR-1, PAR-2, PAR-3, and PAR-6 discussed above, PAR-4 and PAR-5 are distributed uniformly, both in the cytoplasm and at the cell cortex (Morton et al. 2002; Watts et al. 2000). Cytoplasmic PAR-1 plays an important role in cell fate determination (Griffin et al. 2011) in addition to its function at the cortex.

The cortical PAR proteins fall into two groups, anterior and posterior PARs (Fig. 1.2). PAR-3 and PAR-6 are PDZ domain-containing proteins and, as such, provide a scaffold for the serine/threonine kinase aPKC/PKC-3. PAR-3 probably oligomerizes and recruits PAR-6 and PKC-3 (Benton and St Johnston 2003a; Dawes and Munro 2011; Li et al. 2010). In addition, efficient access of the aPAR complex to the cortex is dependent on CDC-42 (Aceto et al. 2006; Schonegg and Hyman 2006). aPKC can phosphorylate PAR-2 in vitro, preventing it from localizing to the membrane (Hao et al. 2006). Once a posterior domain has formed, PAR-2 binds to and recruits PAR-1 to the cortex, and PAR-1, in turn, phosphorylates PAR-3 to prevent it and the aPAR complex from localizing to the same cortical area (Motegi et al. 2011). PAR-2's molecular role in this process is largely unknown although it appears to bind to lipids directly, probably mediated by electrostatic interactions between a basic domain of PAR-2 and the negatively charged phospholipids. PAR-2's affinity for phospholipids is reduced by phosphorylation by PKC-3 and by phospho-mimicking mutations, whereas a non-phosphorylatable version of PAR-2 localizes all over the cortex (Hao et al. 2006; Motegi et al. 2011). PAR-2 may provide a physical link to the plasma

membrane to recruit PAR-1 to the posterior domain. PAR-1, PAR-2, and PAR-3 all contain domains predicted to interact directly with phospholipids (Motegi and Seydoux 2013), and this also seems to be the case in *Drosophila* (Krahn et al. 2010), suggesting lipid binding could be of general importance in the PAR network. PAR-2's RING domain has been shown to be required for access to the cortex (Hao et al. 2006), although the molecular role of this domain in cortical localization is not clear. Binding to the actomyosin cortex might play an additional role in PAR protein localization, since disruption of actin filaments prevents cortical localization of PAR-3 and PAR-2 (Boyd et al. 1996; Severson et al. 2002).

In general, PAR localization to the cortex appears to be a dynamic process, since PAR-2 and PAR-6 exchange readily between the cytoplasm and the cortex and diffuse laterally on the cortex (Goehring et al. 2010, 2011a). No diffusion barrier exists between the two domains. Extensive mixing is prevented by reciprocal inhibitory interactions that lower the affinity of the respective other complex for the cortex.

The roles of the PAR proteins that do not localize asymmetrically are less well understood. PAR-4 is an LKB-family serine/threonine kinase (Watts et al. 2000). PAR-4 has an indirect role in PAR polarity, as it appears to control actomyosin contractility (Chartier et al. 2011), which feeds into cell polarization but is dispensible for PAR polarity itself (as discussed below). Motegi and Seydoux (Motegi and Seydoux 2013) hypothesize that PAR-4 could also have a role in aPAR and pPAR mutual exclusion, since the *Drosophila* and mammalian homolog LKB1 can phosphorylate DmPar1 (Lizcano et al. 2004). This could, however, also be linked to its role in contractility, which is also affected by DmPar1. In general, DmPar4 resembles a weak DmPar1 mutant (St Johnston and Ahringer 2010).

PAR-5 is a 14-3-3 protein and is thus thought to recognize phosphorylated substrates. 14-3-3 proteins participate in numerous cellular processes (Denison et al. 2011; Freeman and Morrison 2011; Gardino and Yaffe 2011; Kleppe et al. 2011; Obsil 2011; Steinacker et al. 2011; Zhao et al. 2011), and *C. elegans* PAR-5 has been associated with cell cycle control, DNA damage response, chromosome stability, nuclear export, and endocytosis (Aristizábal-Corrales et al. 2012; Lo et al. 2004; Winter et al. 2012). In *C. elegans*, its role in polarity is not well understood. In *Drosophila*, DmPar5 recognizes DmPar3/bazooka that has been phosphorylated by DmPar1, leading to disassembly of the aPAR complex and its removal from the cortex (Benton and St Johnston 2003b; Benton et al. 2002). A similar function has been proposed for PAR-5 based on yeast-2-hybrid experiments and phenotypic similarity with *par-2* mutants but has not been demonstrated in vivo (Benton et al. 2002; Morton et al. 2002).

Cell polarity is essential for the development of the worm. Loss of PAR proteins or other crucial players in the polarization of the *C. elegans* zygote leads to a loss of spatial information in the zygote and embryonic lethality. Because of the importance of cell polarity, however, it arguably should be able to accommodate a certain degree of genetic and environmental variability that may produce fluctuations in protein levels, for example. Recent reports indicate that the *C. elegans* polarity

network is a system with many backup mechanisms, ensuring robust polarization even when individual elements are compromised.

1.2.3 Robustness of Polarization

Cell polarization in *C. elegans* embryo is a highly robust process, refractive to numerous genetic perturbations and accommodating stochastic variations in many cellular mechanisms. Wild-type embryos grown at varying temperatures or particular timing mutants undergo the stereotypic pattern of asymmetric cell divisions that characterize *C. elegans* embryogenesis, despite an almost twofold difference in the rate of development (Skiba and Schierenberg 1992). Several mutants have been identified that change the normal path of cell polarization—for instance, *math-33* (McCloskey and Kemphues 2012) and the gene of unknown function F26H9.6 (Schenk et al. 2010)—but nonetheless produce normal embryos that grow into fertile adults. Variations in the amount of actomyosin contractility are tolerated and have no detectable effects on the resulting cell polarity (Rose et al. 1995).

Robustness in development, or canalization, was defined by Waddington in 1942 as the consistency of a phenotype despite the genetic diversity that must necessarily exist in a population and based on the observation "that the wild type of an organism, that is to say, the form which occurs in Nature under the influence of natural selection, is much less variable in appearance than the majority of the mutant races" (Waddington 1942). Given that phenotypes different than the norm could be selected for under specific environmental or experimental regimes, he reasoned that the underlying, genetic diversity must always be present but not expressed, by selecting for mechanisms that minimize the effect of that variation on the phenotype. How is this diversity—genetic or stochastic—suppressed at the level of the phenotype? How is a phenotype buffered against differences during development? How are dysfunctions of individual components compensated?

Research on the establishment of cell polarity and asymmetric cell division in the *C. elegans* zygote has led to the identification of numerous essential molecules. Apart from these required genes, enhancer and suppressor screens identified many other contributors to polarization, which mainly confer robustness and only become essential in a sensitized background (Fievet et al. 2013; Labbe et al. 2006; Morton et al. 2012). Analyses of genetic interactions showed that for several aspects of the polarization process, parallel pathways or backup mechanisms seem to exist that ensure polarization even in the presence of small perturbations of the system.

1.2.3.1 Symmetry Breaking and Polarity Establishment

The centrosome-cortex signaling mechanism that breaks symmetry is robust against variations in centrosome position within the cell (Bienkowska and Cowan 2012; Houk et al. 2012). Centrosomes can break symmetry from the cell interior,

suggesting the signal can travel over distances of several microns. The cytoplasmic flows resulting from symmetry breaking move the centrosome toward the cortex, giving rise to the stereotypic centrosome-cortex apposition accompanying polarization in *C. elegans* embryos. However, assuming the symmetry-breaking signal diffuses radially from the centrosomes, multiple breaks in symmetry at the cortex would be expected, perhaps temporally separated according to centrosome proximity. Even when centrosomes are in the middle of the embryo, polarization initiates at only one site. In wild-type embryos, a transient PAR-2 domain is sometimes found at the meiotic (anterior) pole, probably induced by the meiotic spindle (Cowan and Hyman 2004b; Cuenca et al. 2003; Tsai and Ahringer 2007; Wallenfang and Seydoux 2000). This meiotic PAR-2 domain is lost as the centrosomal domain expands, dependent on centrosome-induced flows (Hamill et al. 2002). Thus additional breaks in symmetry and/or their subsequent expansion into a polarity axis appear to be inhibited once the centrosome-dependent polarity axis is initiated.

Negative feedback mechanisms preventing secondary axis formation in *C. elegans* have not been described. Inhibition of multiple responses to a diffusing centrosomal signal might act through the changes in the actomyosin cortex that occur during the initial break in symmetry. The cortex, once cortical flow has begun, may no longer be competent to receive the centrosomal signal. Cortical tension and contractility differ in unpolarized and polarized embryos (Mayer et al. 2010), although these changes may take several minutes after symmetry breaking to become relevant. In migrating neutrophils, plasma membrane tension prevents a secondary polarity axis from forming (Houk et al. 2012). However, *C. elegans* embryos with two centrosomes, due to double fertilization, break symmetry at two sites and not necessarily at the same time (D. Bienkowska and C.R. Cowan, unpublished observations), suggesting the cortex remains competent for symmetry breaking after polarization is initiated. Inhibition might rely on the centrosome-cortex signaling mechanism. For instance, symmetry breaking might require a limiting factor, such as PAR-2 (Goehring and Hyman 2012). Symmetry-breaking signals at different sites would compete for the factor until one would stochastically win and initiate polarization. Such competition occurs in *S. cerevisiae* during polarized growth, when limiting amounts of a Cdc42-activating complex prevent multiple polarity axes from stabilizing (Howell et al. 2012) and was suggested to underlie the disappearance of the meiotic PAR-2 domain in *C. elegans* embryos (Motegi and Seydoux 2013). Additionally, global negative regulators might inhibit polarization throughout the cell cortex. The Aurora A family kinase AIR-1 may play such a role, as AIR-1 depletion often leads to embryos with two breaks in symmetry despite the presence of a single polarizing cue (Noatynska et al. 2010; Schumacher et al. 1998) (S. Sanegre Sans and C.R. Cowan, manuscript in preparation).

The signal from the centrosome triggers a local downregulation of the actomyosin cortex and breaks the initial symmetry of the zygote (Munro et al. 2004). Severe disruption of the actomyosin cortex—by pharmacological inhibition of actin polymerization or in strong non-muscle myosin loss-of-function conditions—

prevents polarity establishment (Guo and Kemphues 1996; Hill and Strome 1988, 1990; Motegi and Sugimoto 2006; Schonegg and Hyman 2006). However, contractility per se is dispensible in this pathway: partial depletion of non-muscle myosin or disruption of the RhoGTPase cascade that regulates myosin activity (*ect-2*, *mlc-4*) eliminates detectable cortical contractility and cortical flows but does not prevent PAR polarity from being established (Motegi et al. 2011; Zonies et al. 2010). PAR-2 was shown to interact with microtubules in vitro, and microtubules and PKC-3 compete for PAR-2 binding (Motegi et al. 2011). In the contractility-independent pathway, centrosomal microtubules are thought to protect PAR-2 from phosphorylation by PKC-3 and thereby allow PAR-2 to bind to the membrane even when PKC-3 occupies the same region. Once PAR-2 has bound to the membrane, PAR symmetry is broken and mutual antagonism between the anterior and posterior PAR domains may be sufficient to generate the cell polarity axis (Goehring et al. 2011a). Polarity establishment through this contractility-independent pathway is delayed relative to wild-type embryos (Zonies et al. 2010), which initially rely on the contractility-dependent pathway to remove inhibitory PKC-3. Around the time of symmetry breaking, the centrosome matures, which includes the recruitment of pericentriolar material and the nucleation of microtubules (Cowan and Hyman 2004a, 2006). Sufficient centrosomal microtubules might need to be recruited before they can exert their protective function and allow PAR-2 to overcome PKC-3 activity, which might explain the observed delay in the appearance of a PAR-2 domain.

Embryos may employ the contractility-dependent pathway to ensure a rapid response upon entry to the cell cycle to allow sufficient time for cellular polarization to be established before the cell divides. Symmetry breaking in the absence of contractility—relying on microtubule-dependent clustering of PAR-2—is less efficient than the contractility-dependent pathway but provides a secondary mechanism to ensure polarization of the embryo. In a wild-type situation, both contractility-dependent and contractility-independent pathways might act together to optimize the efficiency of symmetry breaking and at the same time ensure the singularity of the polarization axis through the common dependence on a single cellular structure, the centrosome.

Symmetry breaking is spatially coordinated with the position of the centrosome but is randomly positioned within the one-cell zygote. The embryo is ellipsoid due to the physical constraints of the eggshell and often possesses a "maternal-paternal" axis along the long axis of the eggshell due to the normally opposing positions of the female and male pronuclei (Fig. 1.1). Because the mitotic spindle will orient along the long axis during cell division, and because PAR polarity positions cell fate information, correct asymmetric cell division requires that the spindle axis and polarity axis are aligned (Tsou et al. 2003). This is achieved, in part, by PAR polarity-dependent control of spindle position, specifically the posterior-directed movement of the spindle during anaphase. However, the tendency for the spindle to align with the long axis imposed by the eggshell overrides PAR polarity. Thus the consistent alignment of the polarity axis with the long axis of the eggshell is required for the fidelity of asymmetric cell division.

The PAR polarity axis aligns to the long axis of the eggshell in a process referred to as posteriorization (Fig. 1.2) (Cowan and Hyman 2004b; Rappleye et al. 2002). Regardless of where on the cell cortex the pPAR domain initiates, the embryo rotates within the eggshell until the symmetry-breaking site is located at the pole of the long axis. Posteriorization depends on actomyosin contractility. In embryos in which contractions are absent or reduced (*nop-1*(-) or *mlc-4*(*partial loss-of-function*) embryos), the PAR polarity axis does not align with the long axis of the eggshell (Shelton et al. 1999; Tse et al. 2012). The mechanics underlying the posteriorization movement have not been established. Cortical and cytoplasmic flows might generate convective currents within the embryo to drive rotation. Alternatively, the pseudocleavage furrow may cause rotation as it ingresses, minimizing the diameter of the ring as it contracts. Through posteriorization, all embryos achieve the same outcome from their diverse initial states.

1.2.3.2 Polarity Maintenance

The existence of a backup system to ensure polarization of the embryo and a correction mechanism to align the polarity axis with the long axis of the embryo underscores the robustness of polarization. In addition, during polarity maintenance, backup systems buffer against variation and ensure the propagation of cell polarity. The canonical PAR pathway contains numerous feedback loops and inhibitory interactions that allow for PAR polarity to reach a stable state. Mutual inhibitory relationships constantly lead to a refinement of the domain boundary and prevent mixing of the domains (Goehring et al. 2011a, b). In addition, positive feedback loops in both domains seem to contribute to the stability of the domains. On the anterior side, interactions between PAR-6 and CDC-42 are required for accumulation and maintenance of PAR-6 at the cortex and consequently for the correct size of the anterior domain (Aceto et al. 2006; Schonegg and Hyman 2006). In addition, the GTPase dynamin acts in the maintenance of the anterior domain (Nakayama et al. 2009). Under the control of PAR-6, dynamin regulates endocytosis and thereby PAR-6, CDC-42, and RHO-1 accumulation at the anterior cortex. On the posterior side, auto-recruitment of PAR-2 and consequently more PAR-1 helps to maintain the pPAR domain (Motegi et al. 2011). In addition to cortical flows during symmetry breaking, later flows dependent on CDC-42 help the expansion of an initially small PAR-2 domain (Zonies et al. 2010).

PAR-2 is thought to be central to maintaining the posterior domain. Severe *par-2* loss-of-function mutants or depletion of PAR-2 lead to the spreading of aPARs into the posterior cortex and a loss of polarity (Boyd et al. 1996). Several backup mechanisms exist to ensure robust polarization. Recently discovered parallel pathways can compensate for mild impairments in PAR-2 function. The *C. elegans* homolog of *Drosophila lethal* (*2*) *giant larvae* (*lgl*), LGL-1, seems to contribute to the robustness of the posterior domain (Beatty et al. 2013; Hoege et al. 2010). Depletion of LGL-1 in a weak *par-2* mutant leads to synthetic lethality, potentially due to upregulation of PAR-6 (Beatty et al. 2013). Conversely, overexpression of

LGL-1 can rescue even a more severe PAR-2 depletion, indicating that LGL-1 can take over the function of PAR-2 (Beatty et al. 2013; Hoege et al. 2010). In addition, the CDC-42-inactivating protein CHIN-1 contributes to the asymmetric distribution of aPARs, as it can maintain polarity independently of PAR-2 and LGL-1 (Beatty et al. 2013; Kumfer et al. 2010).

Another mechanism acting during the final step in asymmetric cell division further ensures precise cell polarity. Here, the asymmetric segregation of the PAR domains into the nascent daughter cells is controlled. In wild-type embryos, the anterior and posterior PAR domains each occupy half of the cortex, but the position where the cleavage furrow will form is shifted slightly toward the posterior. Despite the fact that the PAR domain boundary is quite close to the site of furrow ingression (displaced ~5 % embryo length), the domain boundary undergoes a slight shift to match the furrow position precisely (Schenk et al. 2010). The shift is more marked in mutants that have altered PAR domain sizes, in which the domain boundary is shifted anteriorly or posteriorly prior to entry into mitosis. This correction mechanism, while negligible in wild-type embryos, accommodates significant variation in PAR domain size to allow correct asymmetric cell division.

The existence of parallel pathways to maintain correct cell polarity and of a mechanism to coordinate the polarity boundary with the position of cell division raises the question of whether more variation in PAR domain size might exist in embryos developing in the wild, where they will experience fluctuations in temperature, metabolism, or gene expression. How is PAR domain size controlled in wild-type embryos and is it robust to variation?

Recently, it has been shown that manipulating the relative amounts of cortical PAR proteins—for instance, by overexpression of PAR-2 or by partial depletion of PAR-6—can influence the size of the domains (Fig. 1.3). Goehring et al. (Goehring et al. 2011b) explain this by limiting amounts of aPARs and pPARs that normally constrain the expansion of the posterior domain. Redundancy and feedback loops can buffer the polarity system from imprecision in the aPAR/pPAR balance (Motegi and Seydoux 2013). Although the amounts of cortical PAR proteins influence the size of the domains, this cannot account for the precise regulation of PAR domain sizes in the embryo, as a decrease in PAR-6 by 40 % or an increase by 25 % allowed normal polarization (Beatty et al. 2013; Pacquelet et al. 2008). Additionally, the changes in domain size upon increase or decrease in PAR-2 or PAR-6 levels are more modest than the change in overall protein levels would predict (Goehring et al. 2011b). Finally, domain size depends on the mechanism of symmetry breaking and is not the same in the absence of centrosome-induced flows, despite equivalent levels of PAR proteins (Motegi and Seydoux 2013). Several mechanisms influence PAR domain size. Fluctuations in these inputs must be buffered to ensure robust polarization and the accuracy and precision of domain sizes observed in wild-type embryos.

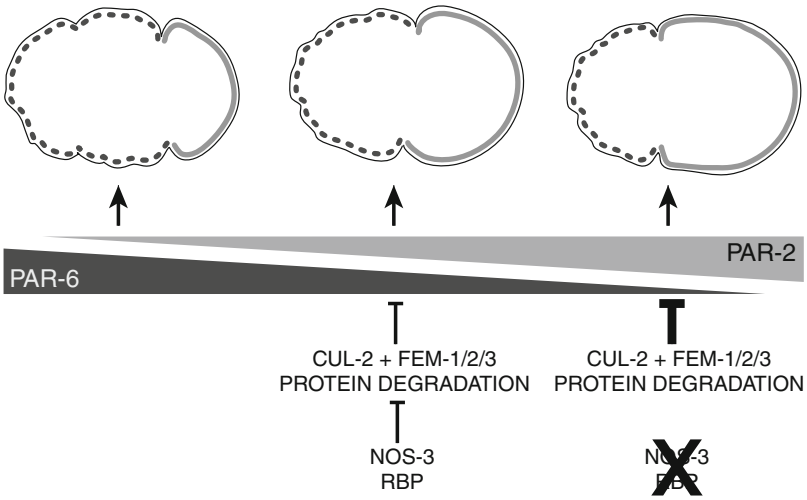

Fig. 1.3 Protein amounts control size. Relative levels of the aPAR PAR-6 (*dotted dark gray line*) and pPAR PAR-2 (light gray line) can change PAR domain sizes. PAR protein levels are regulated by posttranscriptional and posttranslational mechanisms, such as those depicted

1.2.3.3 Transduction of Cortical Polarity

The functional outcome of PAR polarity at the cortex is the spatial compartmentalization of several cellular processes taking place inside the cell. Cortical polarity is translated into cytoplasmic gradients of fate determinants in order to specify cell fate and into asymmetric pulling forces on astral microtubules to generate a physically asymmetric division. As for PAR polarity establishment and maintenance, recent work makes clear that the transduction of cortical polarity likewise relies on redundancy and tight control of protein levels to achieve these robust outcomes. For instance, the pulling forces acting on the mitotic spindle are supplied far in excess of what is required for posterior displacement (Pecreaux et al. 2006). The resulting higher forces lead to posterior spindle pole oscillations, characteristic of asymmetric cell division *C. elegans*. Reducing the number of force generators—active dynein motors—prevents oscillations with no consequence for asymmetric cell division. Backup, in this case, is provided by an oversupply of the relevant molecule.

The number of force generators available during asymmetric cell division has changed during evolution. Embryos from the closely related worm *C. briggsae* undergo asymmetric cell division according to the same mechanistic framework used by *C. elegans* embryos (Brauchle et al. 2009). *C. briggsae* embryos show reduced amplitude of posterior spindle pole oscillations, suggesting the number of active force generators may be less than in *C. elegans*. Through a comparative approach, Delattre and colleagues (Riche et al. 2013) found that *C. briggsae* embryos have lower levels of the dynein regulator GPR on the cortex compared

to *C. elegans* embryos. In *C. elegans*, GPR protein is made from two almost identical loci, *gpr-1* and *gpr-2*, while in *C. briggsae*, there is a single *gpr* locus. In another closely related worm, *C. remanei*, there is likewise a single *gpr* locus (www.wormbase.org). Thus it appears that *C. elegans* may have retained both products of a gene duplication that provided increased GPR levels and robust spindle displacement. Whether other nematodes never underwent the duplication or instead sacrificed the extra copy because of increased protein production costs is unclear. The alternative strategies in *C. elegans* and *C. briggsae*, however, may suggest that there are both benefits and potential costs associated with robustness.

Redundancy with respect to cell fate decisions appears to be encoded in the molecular mechanism by which cytoplasmic fate determinants are segregated in one-cell embryos. MEX-5 responds to a gradient of PAR-1 to achieve asymmetry. The cytoplasmic fate determinant and RNA-binding protein PIE-1, in contrast, appears to rely on the intersection of multiple protein gradients to achieve its enrichment in the posterior (A.U. Goeppert and C.R. Cowan, manuscript in submission). PIE-1 localization to the posterior depends on the MEX-5 gradient (Cuenca et al. 2003) and that of another RNA-binding protein, MEX-1. Loss of MEX-1 does not completely prevent PIE-1 gradient formation but delays it and results in more variation in the shape of the PIE-1 gradient. Loss of MEX-5 largely abolishes the PIE-1 gradient. Thus, as for the gradients that pattern gene expression in early *Drosophila* embryogenesis, combinatorial gradients appear to refine positional information for cell fate determinant segregation in one-cell *C. elegans* embryos. This added level of control may protect fate decision-making from stochastic variations.

The concentrations of fate determinants influence gradient formation in one-cell embryos and fate decisions later in development and are actively regulated. The *zi*nc-*f*inger-interacting protein ZIF-1 was found to control PIE-1 degradation in somatic blastomeres after the 4-cell stage (DeRenzo et al. 2003). ZIF-1 appears to act as an adapter to target proteins for degradation by the proteasome. ZIF-1 also controls PIE-1 levels in the gonad and one-cell embryos in a manner consistent with a role in degradation: loss of ZIF-1 leads to elevated concentrations of PIE-1 (A.U. Goeppert and C.R. Cowan, manuscript in submission). The higher concentration of PIE-1 in *zif-1* mutant embryos facilitates PIE-1 gradient formation, allowing faster establishment and reducing variability among embryos. Although this more efficient gradient formation would seem to be advantageous, the presence of ZIF-1 prevents it, even though ZIF-1 itself is nonessential under normal growth conditions. In the case of PIE-1, a precise concentration may be more critical than accurate segregation. As with PAR polarity establishment, backup mechanisms exist to ensure PIE-1 is not present in somatic cells.

In *C. elegans* early embryos, robustness appears to come both from the activity of multiple pathways that ensure the same end point and tight regulation within individual pathways. Precise control of protein levels appears to dictate multiple aspects of cell polarization.

1.3 Control of Protein Levels in the Germline and Early Embryo

Recent discoveries make clear that protein concentrations control several steps in cell polarization and asymmetric cell division in one-cell *C. elegans* embryos. PAR domain size is set by PAR protein amounts (Goehring et al. 2011b). Posteriorization and cortical flows are sensitive to the degree of myosin contractility (Rose et al. 1995). Spindle pole size is proportional to the total amount of the centrosomal protein SPD-2 (Decker et al. 2011). Spindle oscillations are controlled by cortical GPR levels (Pecreaux et al. 2006; Riche et al. 2013). Gradients of cytoplasmic fate determinants form through concentration-dependent mechanisms (Griffin et al. 2011), and the concentrations of fate determinants appear to be precisely maintained (DeRenzo et al. 2003). Defining the mechanisms of protein homeostasis is key to understanding robustness of this developmental system.

Protein homeostasis is regulated by two processes in the early embryo before zygotic transcription is activated: the translation rate of maternally supplied transcripts and the degradation rate of proteins in the zygote. The sensitivity of cell polarity to the precise amounts of particular molecules indicates that the system may be able to sense concentration and modulate production or degradation accordingly. Keeping the abundance of a polarity protein at a precise level could rely on feedback networks in which, for instance, the amount of a protein regulates its own translation or degradation: high protein suppresses translation or promotes degradation, low protein activates translation or inhibits degradation, and optimal protein maintains translation or degradation at a steady state. The amount of protein may be directly coupled to regulation, for instance, if the protein is a translational regulator, or more often, indirectly coupled. In the early embryo, examples of molecular feedback circuits that would achieve either direct or indirect translational or degradation control are poorly understood. While some of the many RNA-binding proteins (RBPs) important for fate determination in the embryo may control their own expression, this has not been demonstrated. Further, understanding a mechanism for indirect coupling of protein amount to translational or degradation control, as would be necessary for the PAR proteins, raises many questions. For instance, can the embryo measure PAR domain size and actively adjust PAR protein concentrations to compensate for bigger or smaller domains? If so, how are cortical PAR proteins measured relative to total PAR protein? If not, when does the embryo or oocyte assess PAR protein concentration? How do PAR proteins control the regulators of *par* mRNA translation or PAR protein degradation? Before we can approach these questions, we first need an understanding of gene regulation in the early *C. elegans* embryo.

1.3.1 *Principles of Gene Regulation in the* C. elegans *Germline*

The first decision in gene expression happens at the transcriptional level, where multiple factors contribute to deciding if a gene is transcribed into mRNA. In many cases, quantitative regulation occurs at this stage, and the rate of transcription can influence protein levels. The one-cell embryo, however, is transcriptionally inactive and zygotic transcription is thought to begin at the 4-cell stage (Seydoux and Fire 1994). When zygotic transcription begins in 4-cell embryos, transcription in the asymmetrically dividing germline P lineage remains repressed by the transcription factor PIE-1 (Seydoux et al. 1996; Tenenhaus et al. 1998). PIE-1 is exclusively inherited by the cells of the germline lineage during asymmetric cell division, under control of PAR polarity (Cuenca et al. 2003). This suggests that polarity establishment and asymmetric cell division are controlled by mRNAs and proteins made in the germline of the mother. In the adult germline, transcription is regulated globally, initially repressed, and then derepressed to allow for germ cell proliferation (Nakamura et al. 2010). Most of the regulatory mechanisms in the germline appear to be mediated by RBPs (Kershner et al. 2013; Lee 2006; Nousch and Eckmann 2013), suggesting posttranscriptional mechanisms. A large proportion of the *C. elegans* genome—over 4 %—appears to encode RBPs (Tamburino et al. 2013). In the germline, the default state of transcription is "on" and expression is actively repressed posttranscriptionally (Nakamura et al. 2010). This regulatory mechanism is exemplified by the observation that loss of two RBPs acting as translational repressors, GLD-1 and MEX-3, causes germ cells to overproliferate and adopt somatic cell fates, as if activating the embryonic program (Ciosk et al. 2006). The PUF domain proteins FBF-1 and FBF-2 are required for the maintenance of germ cell progenitors (Crittenden et al. 2002) and the switch from spermatogenesis to oogenesis (Zhang et al. 1997). RBPs in the germline have two functions: repressing mRNAs controlling differentiation and refining the temporal and spatial expression of germline mRNAs (Reinke 2008).

Why does regulation occur at the level of translation rather than transcription? One possible reason could be that the germline genome must maintain a transcriptionally competent state to facilitate totipotency, and it may be impossible to simultaneously repress transcription in this particular chromatin environment (Nakamura et al. 2010; Seydoux and Braun 2006). In addition, hundreds of maternally provided mRNAs are necessary for early embryogenesis and thus must be transcribed in the germline but prevented from being translated. RNA regulators thus control the translational potential of mRNA expression. The short duration of oogenesis and early embryogenesis in *C. elegans* could be an additional reason why the worm relies heavily on posttranscriptional mechanisms rather than de novo transcription, especially compared with other organisms (Nousch and Eckmann 2013).

Cell type-specific regulation of gene expression and consequently protein amount is determined largely through sequences in the 3′ untranslated region

(3′UTR). A study investigated the contribution of transcriptional—promoter—versus translational—3′UTR—control for the establishment of specific expression patterns in the gonad (Merritt et al. 2008). Fusion constructs of GFP::histone H2B under the control of either a potentially ubiquitous promoter and a 3′UTR with a defined expression pattern or vice versa exhibited expression patterns dependent on the 3′UTR used—with the exception of sperm cells, possibly because most RNA is discarded during spermatogenesis (Reinke 2008).

1.3.2 Modes of Translational Regulation

Translational regulators generally modulate the affinity of an mRNA for the ribosomal machinery. The different mechanisms involved differ in their specificity and are certainly not mutually exclusive. Indeed multiple RBPs often act together to ensure the correct expression of a given mRNA at a certain developmental time point (e.g., Oldenbroek et al. 2012, 2013). The features of the mRNA that control translation are the 5′cap, the poly(A) tail, and the 5′ and 3′UTR (Rhoads 2006). A translatable mRNA molecule forms a loop by bringing together the cap structure and the 3′ end (Fig. 1.4). Translation initiation is mediated by the cap-binding protein eukaryotic initiation factor 4E (eIF4E) and counteracted by eIF4E-binding proteins. *C. elegans* has five eIF4E paralogs, which seem to act on different, often functionally related sets of mRNAs. Examples for translational regulation in oocytes and early embryos involving cap-dependent translational regulation are *zif-1* and *mei-1*, which are both targets of regulation by eIF4E and its binding partner (Guven-Ozkan et al. 2008; Li et al. 2009). Inhibition of eIF4E-dependent translation contributes to the oocyte-to-embryo transition (*mei-1*, Li et al. 2009) and germline inheritance of the cell fate determinant PIE-1 in the early embryo (*zif-1*, Guven-Ozkan et al. 2008).

Although a poly(A) tail is added co-transcriptionally, several cytoplasmic mechanisms exist to affect the length of the poly(A) tail posttranscriptionally and thereby influence mRNA stability and translation. Partial depletion of LET-711, a member of the deadenylation complex, affects spindle positioning in the first embryonic division (DeBella et al. 2006). The *C. elegans* cytoplasmic poly(A) polymerases GLD-2 and GLD-4, which add a poly(A) tail to deadenylated, translationally repressed mRNAs and can thereby reactivate them, have been implicated in many germline functions, from germ cell fate decisions to early embryogenesis (Kim et al. 2010; Schmid et al. 2009; Wang et al. 2002). In general, regulation of poly (A) tail length might be a common mechanism in the germline for the exact control of protein amounts (Nousch and Eckmann 2013), and many developmentally regulated mRNAs show complex poly(A) tail dynamics (for instance, Ahringer et al. 1992). The consequences of poly(A) tail length are mediated by cytoplasmic poly(A)-binding protein (PABP), which facilitates looping of the mRNA and translation initiation (Fig. 1.4). A longer poly(A) tail can bind more copies of the PABP, thereby increasing the effect. PAB-1, a *C. elegans* PABP, is essential for

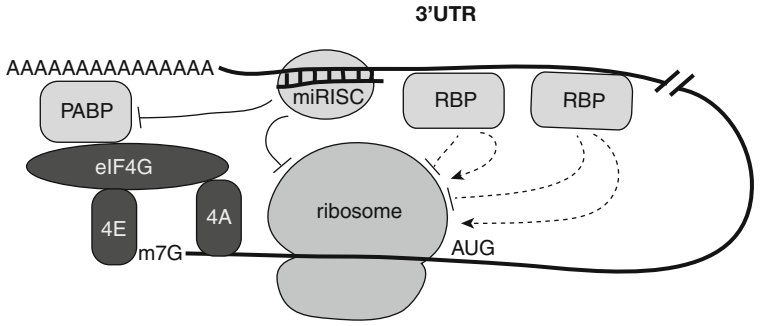

Fig. 1.4 Translational control by the 3′UTR. Translation initiation is facilitated by circularization of the mRNA. The 3′ poly(A) tail loops back to the 5′ m7G cap structure, mediated by PABP and initiation factors (eIF4G, 4E, 4A), to recruit the ribosome and initiate scanning. Different elements in the 3′UTR, such as sites for miRNAs or RBPs, can exert negative or positive effects on translation by influencing initiation factor assembly or ribosome recruitment or function. The use of alternative polyadenylation sites or splicing of the 3′UTR could select for or against inclusion of such sites

germline development, demonstrating the importance of this mechanism in the gonad (Ciosk et al. 2004).

A widely employed mechanism for the fine-tuning of protein levels in many developmental decisions is miRNA-mediated regulation (Fig. 1.4), although so far there are no known miRNAs directly controlling germline or early embryo development in *C. elegans*. Loss of core components of the miRNA pathway, for instance, Drosha or Dicer, leads to sterility, indicating an involvement of miRNAs in essential processes (Denli et al. 2004; Knight and Bass 2001).

The largest group of mRNA regulators involved in germline development and early embryogenesis consists of RBPs that belong to several highly conserved protein families (Lee 2006). RBPs bind mRNAs, preferentially in the 3′UTR, with varying sequence specificity and affect translational efficiency and mRNA stability (Fig. 1.4). In *C. elegans*, the contribution of the 5′UTR tends to be less than in other species, probably due to the removal of the endogenous 5′UTR during trans-splicing, the process of segmenting a polycistronic transcript (Nousch and Eckmann 2013). Numerous examples have been reported where binding of an RBP ensures the correct spatiotemporal control of translation of a target mRNA and is decisive for cell fate specification, discussed below.

The most prominent RBP known to be crucial for many aspects of germline development is the KH domain protein GLD-1 (Francis et al. 1995). It shows high affinity for RNA and recognizes a seven-nucleotide sequence in the 3′UTR (Wright et al. 2011). GLD-1's mRNA targets are involved in many biological processes: it has been implicated in the maintenance of germ cell totipotency and inhibition of premature differentiation (Ciosk et al. 2006), in preventing mitotic entry and the initiation of an embryo-like transcriptional program by inhibiting cyclin E *cye-1* mRNA (Biedermann et al. 2009), and in defining the early embryonic expression of the Notch receptor GLP-1 (Marin and Evans 2003). In general, GLD-1 acts as a

translational repressor (Jungkamp et al. 2011), but recently, it was shown to also stabilize a subset of its mRNA targets (Scheckel et al. 2012), potentially ensuring sufficient mRNA for a later stage when they become derepressed. This finding also underscores the potentially manifold consequences of RBP-mRNA interactions.

Two other KH domain proteins have also been shown to affect early embryogenesis, MEX-3 and GLD-3. MEX-3 acts as a translational repressor and prevents the premature accumulation of the embryonic cell fate determinant PAL-1 in oocytes (Draper et al. 1996; Hunter and Kenyon 1996). Together with the CCCH zinc-finger protein MEX-6 and the RNA recognition motif protein SPN-4, MEX-3 helps restrict embryonic PAL-1 expression to posterior blastomeres (Huang et al. 2002). GLD-3, in contrast, has a lower binding specificity and is thought to act as a translational enhancer, acting on *fem-3* mRNA to promote sperm fate by counteracting repression by the PUF domain protein FBF-1/2 (Eckmann et al. 2002).

The PUF domain proteins constitute another group of translational regulators, mainly repressing translation and functioning in many processes in the germline and the embryo (Lublin and Evans 2007; Mainpal et al. 2011; Merritt and Seydoux 2010). PUF-8, one of the more extensively studied PUF family members in *C. elegans*, appears to act redundantly with other posttranscriptional regulators in the germline (Ariz et al. 2009; Pushpa et al. 2013), suggesting this family may fine-tune regulation by providing an additional level of control.

In 3′UTR-mediated translational regulation, not only the binding affinity of an RBP can be regulated but also the presence of the binding site in the 3′UTR. Alternative 3′UTR selection, through alternative splicing or alternative polyadenylation site selection, can influence the translation of an mRNA by creating or removing binding sites for regulators like RBPs and miRNAs. Over 40 % of *C. elegans* genes have alternative 3′UTRs, although only a small fraction of these involve alternative splicing (Mangone et al. 2010). Research on alternative splicing mainly focused on qualitative differences (Zahler 2005), in which the resulting protein is changed, but the existence of alternative 3′UTR splicing events suggests that splicing may also be used for the quantitative regulation of gene expression.

In addition to controlling protein production, control over protein degradation can be regulated to influence overall abundance. Protein degradation through the proteasomal pathway is signaled by substrate ubiquitination (Kipreos 2005). Three E3 ubiquitin ligases have been identified as being required for embryogenesis (Moore and Boyd 2004), one of which is the key polarity protein PAR-2 (Levitan et al. 1994), raising the possibility of polarity-dependent control of protein homeostasis in the early *C. elegans* embryo. The E3 ligase adapter ZIF-1 regulates degradation of cell fate determinants in the somatic cells of the embryo (DeRenzo et al. 2003). Whether ZIF-1 has the same function in one-cell embryos and specifically in cell polarity remains untested.

1.3.3 Controlling the Abundance of Polarity-Relevant Factors in the Early Embryo

More and more data point to the importance of tight control of protein levels in cell polarization. There is, however, limited information about the mechanisms controlling the abundance of the factors that establish polarity in the early embryo. How are polarity protein levels controlled? Stochastic fluctuations in protein levels need to be accommodated or corrected, but the cell may also require different protein levels depending on environmental or developmental challenges. In order for robust protein homeostasis to be achieved, polarity protein levels would not be measured by concentration alone but rather through a feedback mechanism based on the function of the protein or its localization.

What mechanisms might contribute to protein homeostasis during cell polarization? Manipulating the relative amounts of aPARs and pPARs leads to a shift in the size of the PAR domains, indicating that limiting cytoplasmic pools control the extent of PAR-2 domain extension (Goehring et al. 2011b). The amounts of PAR proteins must be tightly controlled to ensure accurate cell polarity. PAR-6 levels are controlled by a combination of posttranscriptional and posttranslational regulation (Fig. 1.3) (Pacquelet et al. 2008). The RBP NOS-3 is a negative regulator of PAR-6 levels, most likely through its repression of the cullin CUL-2, which in turn and together with the adaptor FEM-1 targets PAR-6 for degradation. Loss of NOS-3 leads to more CUL-2 and thus less PAR-6, which rescues a mutant with reduced PAR-2, potentially by restoring the balance between aPAR and pPAR levels. The other members of the aPAR complex, PKC-3 and PAR-3, are not targets of CUL-2 (Pacquelet et al. 2008), suggesting PAR-6 might be the limiting factor in the anterior. Depletion of CUL-2 alone leads to an extended PAR-2 domain (Liu et al. 2004), which is consistent with an imbalance in anterior-posterior PAR balance but not with the increased PAR-6 levels, as would be expected and would be predicted to expand the PAR-6 domain. CUL-2 may thus have additional targets in the PAR network.

In the posterior, the *C. elegans* homologs of the *Drosophila* tumor suppressor Brat, *ncl-1* and *nhl-2*, show genetic interactions with *par-2*, as RNAi depletion leads to rescue of a weak *par-2* mutant (Hyenne et al. 2008). In contrast to NOS-3, the rescue of the Par phenotype by *ncl-1* and *nhl-2* depletion is not mediated by an effect on PAR-6 protein levels, indicating that CeBRATs might contribute to PAR-2 protein levels directly. The mammalian ortholog of Brat, TRIM32, has been shown to function in both posttranscriptional, through miRNA activation (Schwamborn et al. 2009), and posttranslational, through an E3 ligase domain (Kudryashova et al. 2005), regulation. Regulation of *par* translation itself likely contributes to maintaining correct protein homeostasis, although these mechanisms have not been identified. *par* mRNAs contain 3′UTRs that are much longer than average and encode alternative isoforms (UTRome.org; Mangone et al. 2010), thereby offering a platform for RNA-based regulation of expression. The

significance of these mechanisms for the fidelity of cell polarity in *C. elegans* remains to be determined.

Most known mechanisms regulating PAR protein levels involve protein degradation rather than translational control. The deubiquitination machinery and protein degradation have been shown to be important for symmetry breaking (McCloskey and Kemphues 2012) and may be involved in ensuring optimal protein levels for accurate polarization. Degradation-mediated feedback has been shown to control *Drosophila* wing planar polarity, where tight regulation of Dishevelled and Flamingo protein levels by ubiquitination specifically at cell-cell junctions is required for optimal asymmetric localization and robust polarity (Strutt et al. 2013). Too much of either protein leads to an excess growth of polarized domains, which reduces overall asymmetry; too low protein concentrations cause less efficient clustering of polarity complexes. A small perturbation of the asymmetric distribution by too much or too little of the polarity proteins might not lead to an immediate severe defect, but tight regulation of their abundance confers robustness to tissue polarity—a scenario that likely resembles the situation in the *C. elegans* embryo.

More work will define the molecular mechanism that regulate protein homeostasis and thereby contribute to the robustness of cell polarization in one-cell *C. elegans* embryos. Sophisticated genome editing (Friedland et al. 2013; Tzur et al. 2013) and precise single-cell measurements (Hashimshony et al. 2012) can now complement genome-wide approaches (Fievet et al. 2013; Labbe et al. 2006; Morton et al. 2012) to identify the feedback systems and backup pathways that allow for accurate and precise polarization during development, even in the face of environmental and genetic variation.

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Chapter 2
Establishment and Maintenance of Cell Polarity in the *C. elegans* Intestine

Olaf Bossinger, Tobias Wiesenfahrt, and Michael Hoffmann

Abstract *C. elegans* provides a powerful in vivo model system in which to study epithelial apicobasal polarity during embryonic, larval, and adult development. Specifically, the assembly of adherens junctions and their role in tissue morphogenesis and organogenesis have been analyzed in great detail. In most *C. elegans* epithelia, junctional proteins localize to the multiplex apical junction (CeAJ), a single electron-dense structure that acts as a hub to integrate the barrier/fence and adhesive functions of different types of junctions found in vertebrates and *Drosophila* (e.g., tight and adherens junctions, desmosomes, septate junction).

Two core components of the CeAJ are the HMP-1/α-catenin–HMP-2/β-catenin–HMR-1/E-cadherin complex (CCC) and the DLG-1/Discs large–AJM-1 complex (DAC). The apically localized PAR-3–PAR-6–PKC-3 complex and the basolaterally localized regulator LET-413/Scribble both mediate the formation and maturation of the CeAJ, whereas LET-413 additionally maintains the polarization of *C. elegans* epithelia in the embryo. Starting in late embryogenesis and advancing in larval development, polarized trafficking and the lipid composition of the plasma membrane come more into focus with regard to the maintenance of epithelial cell polarity (e.g., in the intestine, a *simple* epithelial tube made of only 20 cells). Remarkably, the function of most embryonic epithelial polarity key players is still crucial for the de novo formation of epithelial tubes (e.g., the spermatheca) but seems dispensable for the maintenance of their apicobasal polarity during *C. elegans* postembryonic development.

The CeAJ promotes robust adhesion between epithelial cells and thus provides mechanical resistance for physical strains. However, in contrast to vertebrates and *Drosophila*, the CCC is not essential for general cell adhesion. In the *C. elegans*

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K. Ebnet (ed.), *Cell Polarity 2*, DOI 10.1007/978-3-319-14466-5_2

embryonic intestine, at least two adhesion systems, including HMR-1/E-cadherin and SAX-7/L1CAM, associated linker proteins (e.g., the DAC), and cytoskeletal organizers (e.g., ERM-1/ezrin–radixin–moesin, IFO-1, also referred to as TTM-4), act redundantly to mediate adhesion at the intestinal CeAJ.

In this chapter, we will first focus on the general aspects of intestinal development in *C. elegans* including specification, cell proliferation, and basic anatomy. We then discuss the establishment of the apicoluminal membrane domain (ALMD), the assembly of the CeAJ, and the formation of the lumen and the brush border. Next, we look at adhesion systems and cytoskeletal organizers that operate at the CeAJ and in the subapical cytoplasm to equip the lumen with a high degree of mechanical resilience and to ensure the integrity of the intestinal tube. Finally, we consider mechanisms that drive the expansion of the ALMD and maintain the apicobasal polarity of the intestine during the *C. elegans*' life cycle.

Keywords *C. elegans* intestine anatomy • Junctional assembly • Lumen/brush border formation • Polarity establishment • Polarity maintenance • Proliferation

2.1 General Aspects of Intestinal Development in *C. elegans*

Nematodes are an extremely diverse and species-rich phylum. Roundworms inhabit virtually all available habitats on earth. The assumption that embryogenesis shows little variation within the phylum Nematoda is based on the observation that the early cell lineage in *C. elegans* (Sulston et al. 1983) is similar to the pattern found in *Ascaris* and other nematodes. In both species, five somatic founder cells (AB, E, MS, C, D; Fig. 2.1a–c) and a primordial germ cell (P4, Fig. 2.1c) are born through a series of stem cell-like asymmetric divisions. However, the analysis of a larger variety of species from different clades of the phylogenetic tree (Blaxter 2011) demonstrated that prominent variations in the crucial steps of embryogenesis exist among representatives of this phylum (Schierenberg 2006). While different cell patterns also exist to form an intestine in nematodes (Houthoofd et al. 2006), these evolutionary modifications seem to have no effect on the ultimate design of the embryonic intestine, a bilateral, symmetric, epithelial tube of only 20 cells (Fig. 2.2a). The intestine is one of the few cell lineages in *C. elegans* (Fig. 2.1j) where a reasonable transcriptional regulatory hierarchy can be proposed that controls development throughout the life cycle (Table 2.1), beginning with maternally derived factors in the cytoplasm of the zygote (e.g., SKN-1/Nrf and POP-1/TCF/LEF), progressing through a small number of zygotic GATA-type transcription factors (END-1 and END-3), and ending with a further set of GATA-type transcription factors (ELT-2, Fig. 2.2a, b, and ELT-7) that drive differentiation and function (Kormish et al. 2010; Maduro 2010; McGhee 2007; McGhee 2013; Maduro 2009).

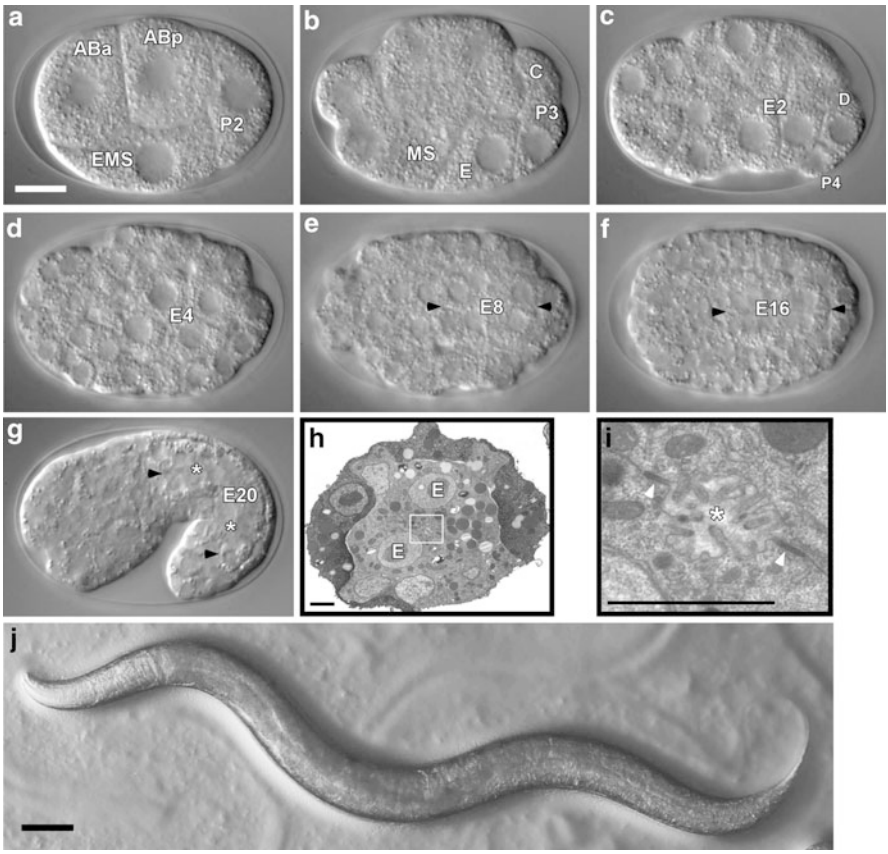

Fig. 2.1 *C. elegans* embryogenesis and development of the intestine (E lineage, the midgut, or endoderm). (**a**–**c**) Generation of five somatic founder cells (AB, MS, E, C, and D) and the primordial germ cell P4. (**a**) 4-cell stage; (**b**) 12-cell stage: the intestinal precursor cell, the E cell, is born at 35 min (Table 2.1) past the 2-cell stage; and (**c**) 26-cell stage/gastrulation (60 min): the two intestinal precursor cells (E2) migrate into the embryo. (**d**–**f**) Four (E4, 110 min), eight (E8, 160 min), and sixteen (E16, 260 min) intestinal precursor cells are born. (**g**) Morphogenesis phase (420 min): "tadpole" stage (E20 intestine). (**h**, **i**) Ultrastructure of the E20 intestine. Electron micrograph of the cross section through one intestinal ring (nuclei of E cells marked) in a "comma" embryo (Table 2.1). Microvilli (*boxed* area zoomed in) project into the nascent lumen (*white asterisk*), which is sealed by the CeAJ (*white arrowheads*). (**j**) Adult *C. elegans* hermaphrodite crawling on agar plate with *E. coli* as food source. Nomarski DIC optics (**a**–**g**), TEM micrographs (**h**, **i**; chitinase treated, osmium only; photo courtesy of Richard Durbin), and micrograph taken from dissecting scope (**j**, Nikon AZ100M, Canon EOS 6D). *Black arrowheads* (**e**–**g**) and *white asterisks* (**g**, **i**) indicate the anterior and posterior borders of the intestinal primordium and the developing lumen, respectively. The pharynx and hindgut (**g**) are to the left of the anterior and the posterior intestinal borders, respectively. Orientation (**a**–**g**, **j**): anterior (*left*), dorsal (*top*). Bars: 10 μm (**a**), 2 μm (**h**, **i**), 50 μm (**j**)

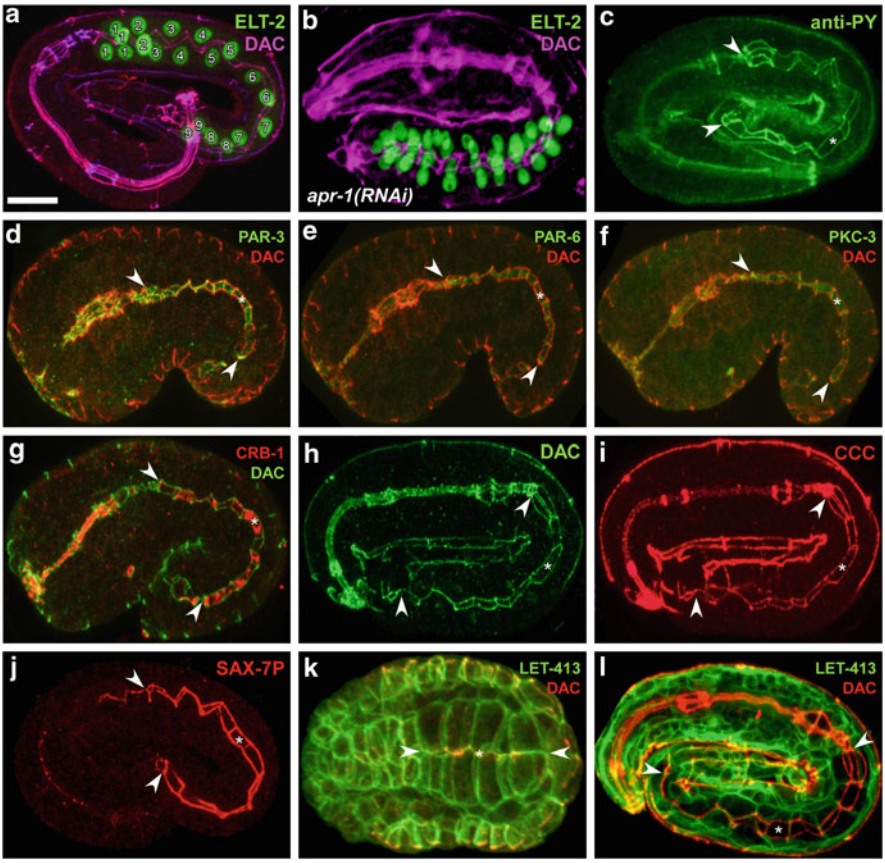

Fig. 2.2 Distribution of intestinal markers during *C. elegans* embryogenesis. (**a**, **b**) Wild-type embryo (fourfold stage, Table 2.1) and *apr-1*(*RNAi*) embryo (threefold stage) stained against the nuclear ELT-2 GATA-factor (anti-GFP fluorescence) and the junctional DLG-1/Discs large–AJM-1 complex (DAC, merged anti-DLG-1 and anti-AJM-1 fluorescences). The GFP fluorescence and corresponding *numbers* (**a**) indicate the position of intestinal nuclei within crescent-shaped cells forming nine rings (the so-called ints: int1, four cells, and ints 2–9, each two cells). Note the increased number of intestinal cells (~40) after RNAi by feeding against the *apr-1* gene (**b**). (**c**) In the wild-type embryo (fourfold stage), phosphotyrosine (PY) epitopes become enriched at the *C. elegans* apical junction (CeAJ), which is consistent with the results of studies in vertebrates and insects (Müller and Wieschaus 1996; Takata and Singer 1988). (**d–g**) In mid-morphogenesis ("comma" stage, Table 2.1), the PAR-3–PAR-6–aPKC complex (*green*) and the Crumbs protein (CRB-1, *red*) localize at the apicoluminal membrane domain (ALMD), which is sealed by the CeAJ (anti-AJM-1 (**d–f**, *red*) and anti-DLG-1 (**g**, *green*) fluorescences of the DAC), to separate from the basolateral membrane. (**h–j**) Junctional distribution of the DAC (*green*, anti-DLG-1 fluorescence), the HMP-1/α-catenin–HMP-2/β-catenin–HMR-1/E-cadherin complex (CCC, *red*, anti-HMP-1 fluorescence), and phosphorylated SAX-7/L1CAM (threefold stage). (**k**, **l**) Localization of cortical LET-413/Scribble (*green*, anti-GFP fluorescence) and the DAC (*red*, anti-AJM-1 fluorescence). In the "lima bean" stage (**k**), both proteins colocate at the ALMD of the intestine (note *orange* color). In the threefold stage (**l**), LET-413 is predominantly expressed basolaterally in *C. elegans* epithelia. (**a–l**) Immunofluorescence images showing confocal projections of the developing alimentary tract of *C. elegans* embryos after MeOH/acetone fixation. *White arrowheads* and *white asterisks* (**c–l**) indicate the anterior and posterior borders of the intestinal primordium and the developing lumen, respectively. The pharynx and hindgut (**d–j**) are to the

2.1.1 Basic Anatomy and Development of the Intestine

The *C. elegans* digestive tract is composed of a variety of tissues and cell types (Altun and Hall 2009c; White 1988; Bird and Bird 1991; Kormish et al. 2010). It forms an epithelial tube running inside the cylindrical body wall, is placed parallel to the gonad, and is separated from both by the pseudocoelom, a fluid-filled body cavity. The alimentary system can be subdivided into the foregut (stomodeum; buccal cavity and pharynx; Altun and Hall 2009d; Mango 2007), the midgut (intestine; Altun and Hall 2009b; McGhee 2007), and the hindgut (proctodeum; Altun and Hall 2009a) and is composed of only 127 cells (Sulston et al. 1983; Schnabel et al. 1997). In comparison to human digestive tracts, it lacks both an intestine-sheathing innervated muscle layer and a regenerating stem cell population. In *C. elegans*, the ingested *E. coli* bacteria flow through the digestive tract by the muscular pumping of the pharynx at the anterior end (Albertson and Thomson 1976; Mango 2007) (Mango 2009), and the waste material is discarded (Zhao and Schafer 2013; Wang et al. 2013; defecation cycle: Rae et al. 2012) through the opening of the anus at the posterior end by a coordinated action between body wall muscles and the muscles controlling the anus. Despite apparent differences in comparison to *Drosophila* and vertebrates, the basic biochemistry and cell biology of *C. elegans* intestinal cells have many of the same fundamental features as intestinal cells in these more complex systems: a striking apicobasal polarity; a prominent apical junctional belt; apical microvilli with rootlets extending into the terminal web region, both absorption and secretion; and a shared function as the place of the primary response to environmental stress.

Developmentally, the midgut/intestine derives clonally from the E lineage (Fig. 2.1), whereas the foregut and hindgut have a mixed lineage from ectodermal and mesodermal origins. The cell division sequence of the E blastomere has been described previously (Deppe et al. 1978; Schnabel et al. 1997; Leung et al. 1999; Sulston et al. 1983). We refer to the E cells collectively as the intestine (E lineage, the midgut, or endoderm) and indicate specific stages of the intestine according to the number of E cells present: E2, E4, E8, E16, E18, or E20 (Fig. 2.1). The E cell is born on the ventral surface of the 8-cell embryo where it divides along the a/p axis (Fig. 2.1b, c). During gastrulation, the E2 cells migrate into the interior of the embryo, where they divide l/r (Fig. 2.1c, d). The E4 and E8 cells (Fig. 2.1d, e) mostly divide a/p and some d/v. Hence, the E16 intestine (Fig. 2.1f) is made up of a dorsal layer of 10 cells (5 × 2 l/r pairs) and ventral layer of 6 cells (3 × 2 l/r pairs). The anterior- and posteriormost pairs undergo an additional d/v and a/p division, respectively, to finally generate the E20 intestine (Fig. 2.1g). In two distinct intercalation events, one in the E16 intestine and another in the E20 intestine, cell pairs of the ventral layer intercalate into the dorsal layer (Hoffmann et al. 2010;

Fig. 2.2 (continued) left of the anterior and posterior intestinal borders, respectively. Orientation: anterior (*left*), dorsal (*top*). Bar: 10 μm

Table 2.1 Timeline depicting the selected landmarks of *C. elegans* embryonic and postembryonic development and a short summary of *C. elegans* genomics

C. elegans development and genomics				
Embryogenesis (20–22 °C)		**Postembryogenesis (20 °C)**		
Time (min)	Event	Time (h)	Stage	Length (μm)
0/−65	Sperm entry	0	Egg laid outside	50 × 30
65/0	First mitosis (2-cell stage)	11	Egg hatches (L1 larvae)	250
80/15	4-cell stage (Fig. 2.1a)	26	L1/L2 molt	360–380
100/35	E cell born (7-cell stage, Fig. 2.1b)	34.5	L2/L3 molt	490–510
125/60	E2 intestine (26-cell stage, gastrulation starts, egg laid outside; Fig. 2.1c)	43.5	L3/L4 molt	620–650
175/110	E4 intestine (Fig. 2.1d)	56	L4/young adult molt	900–940
225/160	E8 intestine (Fig. 2.1e)	65	Adult (959 cells; egg laying begins)	1,110–1,150
325/260	E16 intestine (Fig. 2.1f)	96	Adult, egg laying maximal	1,110–1,150
355/290	E16 intestine (end of gastrulation)	128	Adult, egg laying ends	1,110–1,150
385/320	"Lima bean" (E16 intestine, 558 cells, Fig. 2.2d)	*Based on* Byerly et al. 1976		
445/380	"Comma" (E16 intestine, Fig. 2.2e)	Genomics		
475/410	"Comma" (E20 intestine)	*Base pairs*		100,267,633 bp
485/420	"Tadpole" (1.5 fold stage, Fig. 2.1g)	*Coding sequences*		27,431 (37,474,032 bp; 100 %)
505/440	"Plum" (twofold stage, Fig. 2.3f)	*Confirmed* (*mRNA/EST*)		13,147 (47.9 %)
515/450	"Loop" (threefold stage, Fig. 2.2h)	*Partially confirmed*		12,195 (44.5 %)
585/520	"Pretzel" (fourfold stage, Fig. 2.2a)	*Predicted*		2,089 (7.6 %)
865/800	Hatching (558 cells)	*Protein-coding genes*		20.405
Based on Sulston et al. (1983), McCarter et al. (1999), Leung et al. (1999), www.wormatlas.org		*Based on* ftp://ftp.wormbase.org/pub/wormbase/releases/WS246/letter.WS246		

The life cycle of *C. elegans* includes the embryonic stages (left, see also Fig. 2.1a–g), four larval stages (L1–L4), and adulthood (right, see also Fig. 2.1j). Postembryonic development is triggered by feeding *E. coli* bacteria after hatching. However, if the embryo hatches in the absence of food, such L1 larvae can survive up to 6–10 days without feeding. After food becomes available, these arrested L1-stage larvae progress through normal molting and development. If the environmental

(continued)

Table 2.1 (continued)

conditions are not favorable for further growth, the animal may enter an arrested state, called the L2-*dauer* larva (not shown). The *dauer* state ends when the animal experiences favorable conditions and molts to the L4 stage (Altun and Hall 2009e). The E cells are collectively referred to as the intestine (E lineage, the midgut, or endoderm) and indicate specific stages of the intestine according to the number of E cells present: E2, E4, E8, E16, E18, or E20 (see also Fig. 2.1a–g). At the "lima bean" stage, cell proliferation ceases and the embryo starts morphogenesis and elongation. The shape of the embryo within the eggshell (Wharton 1980; Mansfield et al. 1992; Rappleye et al. 1999; Bembenek et al. 2007; Benenati et al. 2009; Olson et al. 2012) resembles a lima bean (Fig. 2.3b, e, h). The next stage is called the "comma" stage (Fig. 2.2e, f), in which the embryo is slightly folded. A "tadpole"-looking embryo (Figs. 2.1g and 2.2g) consists of an enlarged anterior end with a narrower bit of tail lying just behind it. In the "plum" (Fig. 2.3c, f, i) and "loop" (Fig. 2.2h, i) stages, the elongating embryo adopts a wormlike shape. In the "pretzel" stage (Fig. 2.2a, c), prior to hatching, the animal is now folded into four lengths within the eggshell. The *C. elegans* genome sequencing project (right) was essentially completed and published in Science in 1998 (The_C_elegans_Sequencing_Consortium 1998)

Leung et al. 1999). Thus, the basic anatomy of the E20 intestine can be represented as an a/p sequence of nine rings of intestinal cells (Fig. 2.2a) (the so-called ints: int1, four cells, and ints 2–9, each two cells; Sulston et al. 1983).

Each int forms part of the intestinal lumen at its apical pole and contains a basal lamina at its basal pole, whose constituents are either made by the intestine itself (laminin α and β, nidogen/entactin) or by the muscle and somatic gonad (type IV collagen) (Kramer 2005; Page and Johnstone 2007). The conserved extracellular matrix protein hemicentin stably affixes the anterior- and posteriormost ints to the body wall, hence facilitating passive movement or gliding of the remaining ints during feeding and locomotion and allowing the lumen to fill and empty freely while remaining attached to the body wall (Vogel and Hedgecock 2001; Vogel et al. 2006).

Many microvilli extend into the lumen from the apical surface (Fig. 2.1h, i), forming a brush border. The microvilli are anchored into a cytoskeletal network of cytoplasmic intermediate filaments (IFs) and actin filaments (AFs) at their base (Fig. 2.3d–i), called the terminal web (Hüsken et al. 2008; MacQueen et al. 2005; Bossinger et al. 2004; Carberry et al. 2009; Troemel et al. 2008). The core of each microvillus has a bundle of AFs that connects to this web (MacQueen et al. 2005). Each intestinal cell is sealed laterally to its neighbors by large CeAJs (Figs. 2.1i and 2.2c) (Labouesse 2006; Cox and Hardin 2004; Knust and Bossinger 2002; Pásti and Labouesse 2014) and connects to the neighboring intestinal cells via gap junctions on the lateral sides (Bossinger and Schierenberg 1992a; Altun et al. 2009; Guo et al. 2008).

Transmission electron microscopy of epithelia identifies three electron-dense junctions in *Drosophila* and vertebrates, whereas the *C. elegans* embryo only possesses a single electron-dense junction (Knust and Bossinger 2002), commonly referred to as the *C. elegans* apical junction (Fig. 2.1i) (CeAJ; McMahon et al. 2001). Nevertheless, genetic and cellular analyses have demonstrated that

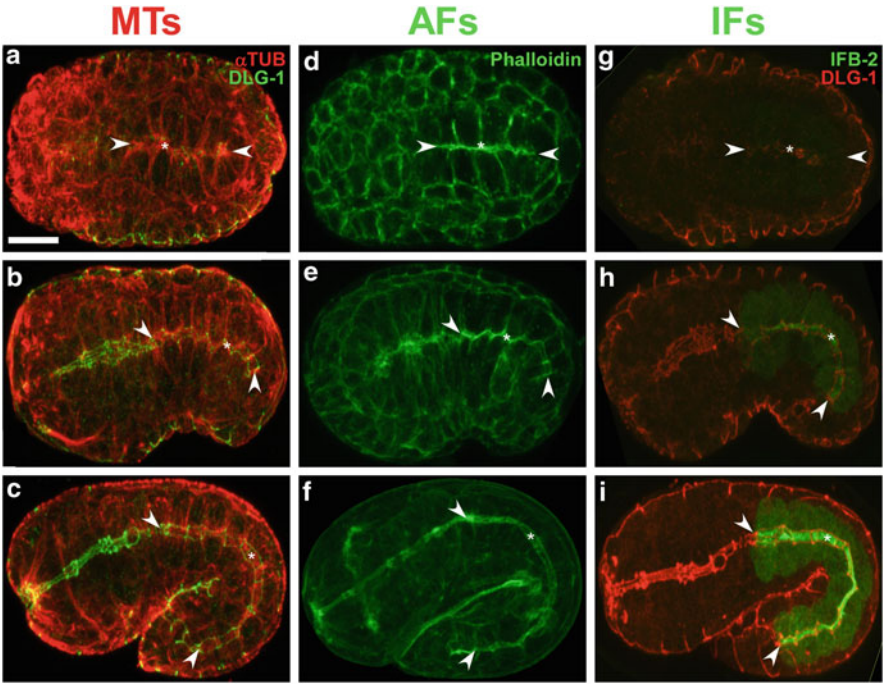

Fig. 2.3 Distribution of the three major cytoskeletal networks during development of the *C. elegans* intestine. (**a**–**c**) Tubulin-based microtubules (MTs, *red*, anti-α-tubulin fluorescence, mab4A1; Piperno and Fuller (1985)). (**d**–**f**) Actin-based microfilaments (AFs, *green*, phalloidin staining). (**g**–**i**) Intermediate filament (IF) protein-based IFs (*green*, anti-IFB2 fluorescence). Anti-DLG-1/Discs large fluorescence (**a**–**c**, *green*; **g**–**i**, *red*) specifies the CeAJ. (**a**–**f**) Immunofluorescence images showing confocal projections of the developing alimentary tract in *C. elegans* embryos (**a**, **d**, and **g,** "lima bean" stage; **b**, **e**, and **h,** "comma" stage; **c**, **f**, and **i,** "plum" stage; for timing see Table 2.1) after MeOH/acetone (**a**–**c**, **g**–**i**) or paraformaldehyde fixation (**d**–**f**). *White arrowheads* and *white asterisks* (**c**–**l**) indicate the anterior and posterior borders of the intestinal primordium and the developing lumen, respectively. The pharynx and hindgut (**h**, **c**, **f**, **i**) are to the left of the anterior and posterior intestinal borders, respectively. Orientation: anterior (*left*), dorsal (*top*). Bar: 10 μm

epithelial cells in *C. elegans* do contain proteins of molecularly and functionally distinct junctional complexes that appear in tight junctions (e.g., CLC-1 to CLC-4/claudins) (Asano et al. 2003) and adherens junctions (e.g., the CCC) (Kwiatkowski et al. 2010; Maiden et al. 2013; Cox-Paulson et al. 2012), desmosomes (e.g., IFB-2/intermediate filament protein) (Bossinger et al. 2004), and septate junctions (e.g., DLG-1/Discs large) (Lockwood et al. 2008b) in other systems (Müller and Bossinger 2003; Cox and Hardin 2004; Armenti and Nance 2012; Labouesse 2006; Pásti and Labouesse 2014).

In the *C. elegans* intestine, the acquisition of apicobasal polarity, the formation of the CeAJ, and the generation of a central lumen are closely connected to each other. The CeAJ aligns in and between each successive a/p pair of intestinal cells

(Fig. 2.2a) and together with the terminal web borders the lumen, making both ideal candidates to limit the width of the lumen that is remarkably uniform throughout the entire length of the intestine. The intestine can change in shape and function dramatically during the *C. elegans*' life cycle. For instance, in the nonfeeding *dauer* larvae, the lumen becomes shrunken and the size and number of microvilli are greatly reduced. When the animal emerges from the *dauer* state, these changes are reversed in the new L4 larva (Albert and Riddle 1988; Popham and Webster 1979). Age-related changes in the intestine include the loss of E cell nuclei; the degradation of intestinal microvilli and changes in size, shape, and cytoplasmic contents of intestinal cells; and the increase of autofluorescent granules (McGee et al. 2011). A reassessment of blue autofluorescence in the *C. elegans* intestine led to the discovery of the phenomenon of death fluorescence, a burst of anthranilate fluorescence that indicates organismal death in *C. elegans* (Coburn et al. 2013; Coburn and Gems 2013).

2.1.2 Proliferation of Intestinal Cells

C. elegans intestinal cells can alter their cell cycle from mitotic cell divisions during embryogenesis to karyokinesis and then endoreplication, which are necessary to promote growth during larval and adult development (Table 2.1, Fig. 2.1j) (Ouellet and Roy 2007). In the L1 larval stage (Byerly et al. 1976), most intestinal nuclei (Fig. 2.2a) undergo karyokinesis (binucleation), resulting in an intestine still composed of 20 E cells but with a total of 30–34 nuclei that have increased their ploidy to 32*n*. Cells of int1 (see above) and int2 (usually) never binucleate, whereas cells of int3 to int7 always binucleate and cells of int8 and int9 may or may not binucleate (Hedgecock and White 1985; Sulston and Horvitz 1977). Postembryonic karyokinesis and endoreplication are not under control of the general cell cycle regulators in *C. elegans* (van den Heuvel 2005; van den Heuvel and Kipreos 2012), like the p21/p27-like cyclin-dependent kinase inhibitor CKI-1 or the positive S-phase regulator CDC-25.1, which are critical to control intestinal cell divisions during embryogenesis (Hong et al. 1998; Kostic and Roy 2002).

CDC25 phosphatase promotes progression through the eukaryotic cell cycle by dephosphorylation of cyclin-dependent kinase (Johnson and Kornbluth 2012). In *C. elegans*, *cdc-25.1* is one of four homologues (Ashcroft et al. 1998). Clucas et al. (2002) and Kostic and Roy (2002) identified the mutant gain-of-function (*gf*) alleles of the *cdc-25.1* gene. Despite the abnormal persistence of the *gf* mutant CDC-25.1 protein in all embryonic cells (Hebeisen and Roy 2008), hyperplasia is only inducible in the intestine at a specific time after the E8 stage (Fig. 2.1e), whereas other aspects of intestinal differentiation are retained. In *cdc-25.1 gf* mutants, between 30 and 45 intestinal cells are produced during embryogenesis. Because the *C. elegans* intestine consists of 20 cells, all cells cannot arise from the E cell by an identical pattern of cell divisions. In the E16 stage (Table. 2.1, Fig. 2.1f), at ~300 min of embryogenesis, only four E cells undergo further cell

divisions. Hence, there is an asymmetry within the E cell lineage that must involve the differential regulation of the cell cycle in the intestine.

One regulator of *cdc-25.1* (*gf*)-induced intestinal hyperplasia is LIN-23 (Segref et al. 2010; Hebeisen and Roy 2008), the *C. elegans* orthologue of the β-transducin repeat-containing protein (β-TrCP), a component of the Skp1/Cul1/F-box (SCF) ubiquitin ligase that, in cultured mammalian cells, has been shown to control cell cycle fluctuations and DNA damage response through the abundance of CDC25A and CDC25B via DSG and DDG motifs, respectively (Busino et al. 2003; Jin et al. 2003; Donzelli et al. 2002). Another regulator which suppresses the *cdc-25.1*(*gf*) embryonic phenotype in the intestine is a subset of splicing factors comprising U2- and U5-specific snRNPs (Hebeisen et al. 2008). Since knockdown of maternal *cdc-25.1* or cyclin E (*cye-1*) can suppress the *cdc-25.1*(*gf*)-induced hyperplasia (Kostic and Roy 2002), it appears plausible that the suppression by a subset of splicing factors depends on the reduction of these two important cell cycle regulators.

The *cdc-25.1*(*gf*) mutations are causing an amino acid substitution (S46F or G47D) within a putative DSG phosphorylation site of CDC-25.1 (Clucas et al. 2002; Kostic and Roy 2002) that is also a consensus glycogen synthase kinase (GSK)3β phosphorylation site. A multiprotein complex containing axin, *adenomatous polyposis coli* tumor suppressor protein (APC), and GSK3β promotes phosphorylation of the DSG motif of mouse β-catenin to target its β-TrCP-dependent degradation (Kitagawa et al. 1999; Kikuchi et al. 2006). Mutations in APC or the β-catenin DSG motif are associated with colorectal cancer in humans (Karim and Huso 2013). In *C. elegans*, RNAi (Fire et al. 1998; Timmons and Fire 1998; Grishok 2013) against the APC orthologue APR (Hoier et al. 2000; Rocheleau et al. 1997) induces hyperproliferation of E cells in the majority of wild-type embryos (Fig. 2.2b) (our unpublished data; Segref et al. 2010; Putzke and Rothman 2010). To test whether *apr-1* has a function mediated through CDC-25.1 controlling the intestinal cell cycle, Segref et al. (2010) repeated RNAi in a *cdc-25.1*(*gf*) background. They observed a significantly increased number of intestinal cells, indicating that *apr-1* is synergistic with *cdc-25.1*(*gf*) and hence does not function through the same pathway as the *gf* mutant CDC-25.1 protein.

The role of APR-1 is puzzling because at the 4-cell stage of early embryogenesis, the protein is also involved in the correct specification of E cell fate by the Wnt/β-catenin asymmetry pathway. Wnt and Src signaling act together to regulate the asymmetry of the EMS blastomere (Fig. 2.1a) that produces the anterior MS and posterior E daughters (Fig. 2.1b), which generate mesoderm and endoderm, respectively (Mizumoto and Sawa 2007; McGhee 2013; Han 1997; Bei et al. 2002; Kim et al. 2013; Sugioka et al. 2011). How can depletion of APR-1 by RNAi cause a complete lack of E cells in ~23 % of embryos (Rocheleau et al. 1997; Segref et al. 2010; Bei et al. 2002), when the majority of embryos show intestinal hyperplasia (see above)?

The first observation can be easily interpreted by the redundancy of the Wnt and Src pathways because only interfering with both signals completely abolishes intestinal differentiation in *C. elegans* embryos (Bei et al. 2002). The second

observation is more complex and to interpret it one has to keep in mind the dual nature of Wnt signaling. For example, hyperactivation of the Wnt pathway, caused by inactivating mutations in APC or activating mutations in β-catenin, is associated with various forms of cancer (Bienz and Clevers 2000; Polakis 2000), and decreased Wnt signaling can lead to increased invasiveness of tumor cells. In case of Wnt signaling in the *C. elegans* embryo, APR-1 acts either negatively on intestine induction early or positively on intestinal cell proliferation late. How can this contradictory observation be explained? The recent work by Putzke and Rothman (2010) suggests that removal of APR-1 (or Fer-type nonreceptor tyrosine kinase FRK-1) results in re-localization of cortical/junctional HMP-2/β-catenin to the nucleus and allows it to substitute for WRM-1, the nuclear β-catenin that normally transduces the Wnt signal during early endoderm induction. In *C. elegans*, HMP-2/β-catenin generally functions in cell adhesion (Costa et al. 1998; Grana et al. 2010; Segbert et al. 2004) and binds to HMP-1/α-catenin and HMR-1/cadherin (Kwiatkowski et al. 2010; Korswagen et al. 2000). So far HMP-2 has not been shown to activate Wnt reporters in tissue culture cells (Korswagen et al. 2000), and intestinal hyperproliferation resulting from excess nuclear HMP-2 appears to occur in the absence of POP-1(TCF/LEF) (Putzke and Rothman 2010), the central transcription factor in the separation of EMS into E and MS cell fates (Fig. 2.1a, b) (Lin et al. 1998; Lin et al. 1995; Yang et al. 2011). However, POP-1 asymmetry in sister cells at each a/p division of the E lineage is intriguing (Lin et al. 1998; Hermann et al. 2000; Schroeder and McGhee 1998) and together with the LIN-12/Notch signaling pathway is necessary for cells in the anterior intestine to undergo reproducible movements that lead to an invariant twist in the embryonic and larval intestine, probably allowing the adult intestine (Fig. 2.1j) to better coil with the developing gonad (Hermann et al. 2000; Neves et al. 2007; Neves and Priess 2005; Priess 2005). In other systems, the Notch signaling pathway is also involved in the development of colorectal tumors (Noah and Shroyer 2013). Notch and WNT signals cooperate to trigger intestinal tumorigenesis (Fre et al. 2009; Kim et al. 2012). In Apc^{Min} mice, the continuous expression of Wnt target genes leads to the development of adenomas. However, inhibition of Notch signaling turned adenoma cells into goblet cells (van Es et al. 2005), whereas activation of Notch signaling in *Apc* mutant mice resulted in an increase in the number of adenoma cells (Fre et al. 2005). Concerning the *apr-1* (*RNAi*)-induced intestinal hyperplasia in *C. elegans*, the role of Notch, if any, still awaits to be investigated.

2.2 Defining the Apicoluminal Membrane Domain of the Intestine

2.2.1 Early Polarization Events

The principal requirement for a biological tube in general is that a lumen must form and the lumen must be sealed (Bryant and Mostov 2008). In the *C. elegans* intestine, the cell surface coating the future lumen of the epithelial tube develops as the ALMD with a prominent microvillar brush border and is sealed by the CeAJ (Fig. 2.1i) to separate from the basolateral membrane domain and to achieve its barrier function.

During polarization of the intestine in the E16 stage (Table 2.1, Fig. 2.1f), the centrally located intestinal nuclei and their centrosomes migrate toward the future apical pole, displacing the cytoplasm to the basal pole as seen by light microscopy (Fig. 2.1f). Although not explicitly described as cytoplasmic polarization, this initial asymmetry in the intestine was already observed by Sulston and coworkers (1983) and further elaborated in great detail by Leung et al. (1999).

Although 12 of the 16 E cells stop dividing, their centrosomes undergo one additional duplication or split to form centrosome pairs each containing two centrioles (Leung et al. 1999; Feldman and Priess 2012). The centrosomes and nuclei then move toward the lateral membrane. During this migration, associated microtubules (MTs) and pericentriolar material (PCM) carrying MT-organizing center (MTOC) activities, such as the MT nucleators γ-tubulin and its interacting protein CeGrip (=GIP-1), are first stripped from the centrosome and then become localized to the lateral membrane near the foci of the polarity proteins PAR-3 and PAR-6. Finally, these proteins move apically, thus defining the ALMD of intestinal cells. E16 cells treated with the MT inhibitor nocodazole show a strong delay in the apical localization of PAR-3 and γ-tubulin (Feldman and Priess 2012). Laser ablation studies and depletion of maternal and zygotic (*m/z*) PAR-3 suggest that both centrosomal and PAR-3 (but not PAR-6) functions are mutually dependent on each other and critical for the progression in MTOC function from centrosomes to the ALMD.

PAR-3, PAR-6, and PKC-3 are present at the ALMD (Fig. 2.2d–f) of the intestine (Bossinger et al. 2001; Köppen et al. 2001; Leung et al. 1999; McMahon et al. 2001; Wu et al. 1998). Deciphering the function of PAR proteins during intestinal polarization involved a sophisticated strategy to rescue their early need in the *C. elegans* zygote by tagging these proteins with the PIE-1 Zn-finger that mediates PIE-1 degradation in the soma (Nance et al. 2003). In *par-3*(*m/z*)-depleted embryos, the ALMD does not become polarized. Many proteins investigated so far (e.g., γ-tubulin, CeGrip, PAR-6, PKC-3, HMR-1, HMP-1, DLG-1, EAT-20, IFB-2) show a significant delay in the arrival at the ALMD and finally localize in aberrant patches (Achilleos et al. 2010; Feldman and Priess 2012; Totong et al. 2007). Hence, PAR-3 is required for the apical clustering and accumulation of polarity and junction proteins. RNAi feeding during *C. elegans* postembryonic development

(Table 2.1) also established that PAR-3 is required to specify the ALMD and to assemble the CeAJ in the spermatheca, another epithelial tube (Aono et al. 2004). PAR-6 does not play a similar role, but instead, as in the epidermis, is essential to consolidate DAC and CCC puncta into a mature apical junctional belt (Totong et al. 2007). PAR-6 and PAR-3 functions appear dispensable to specify the ALMD in the epidermis (Achilleos et al. 2010; Totong et al. 2007). As in other species, the establishment of cell polarity in tubular organs and flat epithelial sheets appears to involve different processes (Nelson 2003; Datta et al. 2011). A role for PKC-3 in *C. elegans* embryonic epithelia (Fig. 2.2f), if any, awaits investigation.

After polarization of the intestine, the MT cytoskeleton appears to emerge in a fountain-like array from the ALMD and extends along the lateral surfaces of intestinal cells (Fig. 2.3a–c) (Leung et al. 1999). Its role during intestinal development is difficult to assess by genetic means. There are nine α-tubulins (TBA-1 to TBA-9) and six β-tubulins (TBB-1 to TBB-6) in the *C. elegans* genome (Table 2.1; wormbase.org). An important issue concerning the function of MTs (Fig. 2.3a–c) in the early intestinal polarization process is the question of additional signals that might participate either by direct release from the MTOC (as postulated for the *C. elegans* 1-cell embryo; Bienkowska and Cowan 2012) or by MT-based transport to the ALMD (as demonstrated during the polarization of the pharynx; Portereiko et al. 2004).

The two other main components of the cytoskeleton, AFs (Fig. 2.3d–f) and cytoplasmic IFs (Fig. 2.3g–i), start to localize at the ALMD around the same time (E16 stage) as the MTOC (Bossinger et al. 2004; Leung et al. 1999; van Fürden et al. 2004). The abundance of genes in both families again makes a genetic analysis difficult. The *C. elegans* genome (Table 2.1; wormbase.org) encodes 5 AFs (ACT-1 to ACT-5) and 11 cytoplasmic IFs (IFA-1 to IFA-4, IFB-1 to IFB-2, IFC-1 to -IFC2, IFD-1 to IFD-2, IFP-1). Nevertheless, the treatment of E16-stage embryos with the AF inhibitor latrunculin A does not affect the apical localization of PAR-3 (Fig. 2.2d) and γ-tubulin (Feldman and Priess 2012). Along the same line, interfering with individual gene functions of several intestine-specific IFs (Fig. 2.3g–i) or the intestinal filament organizer IFO-1 (also referred to as TTM-4) seems not to perturb the establishment of the ALMD in the intestine (Carberry et al. 2012; Hüsken et al. 2008; Karabinos et al. 2001; Bossinger et al. 2004). While AFs and IFs (Fig. 2.3d–i) seem dispensable for the early polarization of the *C. elegans* intestine, both filament systems and their regulators contribute to junction assembly and lumen morphogenesis.

2.2.2 Assembly of the Apical Junctional Belt

During the last decades, various approaches have been used to identify junctional proteins in *C. elegans*. This field was pioneered by Francis and Waterston. After raising monoclonal antibodies against insoluble membrane-associated embryonic extracts (Francis and Waterston 1991; Francis and Waterston 1985), some of these

antibodies (e.g., MH27 or MH33) turned out to recognize proteins of the CeAJ (AJM-1/coiled-coil protein or IFB-2) by immunofluorescence and immunogold staining and provided an excellent platform to investigate the junction assembly and disassembly in *C. elegans* (Bossinger et al. 2004; Köppen et al. 2001; Podbilewicz and White 1994; Priess and Hirsh 1986; Hresko et al. 1994; Williams-Masson et al. 1997; MacQueen et al. 2005). Since then, many genes encoding junctional proteins have been identified by classical forward and reverse genetic means in screens for embryonic elongation defects (e.g., hmp-1, hmp-2, hmr-1, apr-1, vab-9; Costa et al. 1998; Hoier et al. 2000; Simske et al. 2003), enhancer screens (e.g., zoo-1, magi-1, jac-1; Lockwood et al. 2008a; Pettitt et al. 2003; Lynch et al. 2012), chromosomal deficiency screens (Labouesse 1997; e.g. let-413; Chanal and Labouesse 1997; Legouis et al. 2000), promoter trapping screens (e.g., eat-20; Shibata et al. 2000), and screens with stable transgenic strains (e.g., ifo-1; Carberry et al. 2012) or simply by analyzing the functions of homologous proteins after RNAi (e.g., CRB-1, DLG-1, IFC-2, CLC-1 to CLC-4; Bossinger et al. 2001; McMahon et al. 2001; Firestein and Rongo 2001; Asano et al. 2003; Hüsken et al. 2008) or targeted protein degradation (Nance et al. 2003; Totong et al. 2007; e.g., PAR-3, PAR-6; Achilleos et al. 2010). Some components were identified through protein–protein interaction screens (e.g., DLG-1, VANG-1; Köppen et al. 2001; Hoffmann et al. 2010). Tissue-specific RNAi (Bossinger and Cowan 2012; Qadota et al. 2007) and fluorescent protein fusions (Sarov et al. 2012) are now increasingly being used to identify new components of the CeAJ and to study their subcellular localization and kinetics.

In *Drosophila*, the interaction between several protein scaffolds—apically the Bazooka/DmPAR-6/DaPKC and Crumbs/Stardust/Patj complexes and basolaterally the Scribble/Discs large/Lethal giant larvae and Yurt/Coracle complexes—specifies apicobasal polarity and junction assembly (Laprise and Tepass 2011; Knust and Bossinger 2002; Nelson 2003). In *C. elegans*, epithelial polarization may rely on slightly divergent mechanisms and depends upon multiple, probably redundant, cues. For example, loss of HMR-1/cadherin affects neither apicobasal polarity nor cell adhesion as in other systems, and HMR-1/cadherin functions redundantly with SAX-7/L1CAM (Fig. 2.2j, Table 2.2) during *C. elegans* embryogenesis (Grana et al. 2010; Costa et al. 1998).

The polarization of the intestine clearly relies on the function of PAR-3/Bazooka (see above) but not PAR-6 (Fig. 2.2d, e). In *par-6*(*m*/*z*)-deficient embryos, apical junction proteins, PAR-3, and basolateral LET-413/Scribble (Fig. 2.2l) can become positioned asymmetrically, but apical junction proteins and PAR-3 require PAR-6 for their coalescence into belt-like structures, encircling the apex of intestinal cells (Totong et al. 2007). How PAR-6 achieves coalescence is not known. Recent results suggest that DmPAR-6 together with the small GTPase Cdc42 control trafficking events of junctional proteins in *Drosophila* epithelia (Balklava et al. 2007; Harris and Tepass 2010). PAR-6 seems not to function redundantly with the DAC, CCC (Fig. 2.2h, i), or LET-413 (Fig. 2.2k, l) to establish the apicobasal polarity of intestinal epithelial cells (Totong et al. 2007). However, PAR-6 may regulate apicobasal polarity through more redundant interactions,

Table 2.2 Model of cell adhesion during the organogenesis of the *C. elegans* intestine

I	*II*	
HMR-1 (E-cadherin)	SAX-7P (L1CAM)	*Cell adhesion molecules*
HMP-2 (β-catenin)	DLG-1 (Discs large)	*Linker proteins*
HMP-1 (α-catenin)	AJM-1 (coiled coil)	
IFO-1	ERM-1 (FERM)	*Cytoskeletal organizers*
IFB-2, IFC-1/2	ACT-5	*Cytoskeleton*
IFD-1/2, IFP-1 (IFs)	ACT-1/2/3/4 (AFs)	

Genetic data suggest that in the embryonic intestine, at least two redundant cell adhesion systems (*I* and *II*) ensure the integrity of the epithelial tube (see also Fig. 2.4i–l). Both systems act at the level of cell adhesion molecules, linker proteins, and cytoskeletal organizers (of note, only the phosphorylated form of SAX-7 and SAX-7P localizes at the CeAJ) (Chen et al. 2001). At the core of each system, linker proteins and cytoskeletal organizers strongly interfere with the localization of cell adhesion molecules and IFs/AFs, respectively, but in both systems these molecules are not predominantly required for each other's localization

e.g., with the Crumbs/Stardust/Patj complex. Homologues of Crb, Stardust, and Patj exist in the *C. elegans* genome (Table 2.1; wormbase.org). In addition, CRB-1 (Fig. 2.2g), EAT-20, and CRB-3, the three Crumbs homologues, are present at the ALMD of the intestine. However, their absence alone or after double depletions seems not to affect apicobasal polarity (Bossinger et al. 2001; Shibata et al. 2000; our unpublished data). A potential contributory role in apicobasal polarity is revealed by the simultaneous knockdown of CRB-1, HMP-1/α-catenin, and LET-413 (Segbert et al. 2004).

In *Drosophila*, the basolaterally expressed Scribble and Discs large proteins oppose the activity of the apical polarity complexes, thus defining the basolateral position of adherens and septate junctions during epithelial polarization (Elsum et al. 2012). The *C. elegans* homologues LET-413 and DLG-1 have related functions but are not crucial to establish the initial apicobasal polarity (McMahon et al. 2001; Legouis et al. 2000; Bossinger et al. 2001; Firestein and Rongo 2001). Instead, both proteins—like PAR-6—promote compaction of the CeAJ (Fig. 2.4a, e–g) (McMahon et al. 2001; Köppen et al. 2001; Totong et al. 2007; Bossinger et al. 2001). An important future issue will be to determine whether LET-413, DLG-1, and PAR-6 act in the same or parallel pathways. The divergence of compaction defects and its enhancement in LET-413- and DLG-1-depleted embryos (Köppen et al. 2001) argue for the latter possibility.

In addition, in LET-413- and DLG-1-deficient embryos, junctional proteins reach their subapical position less efficiently (Bossinger et al. 2001; Köppen et al. 2001; McMahon et al. 2001). Moreover, after depletion of LET-413, the ALMD progressively spreads into the lateral and basal membrane domains of intestinal cells (Fig. 2.4h), suggesting that LET-413 function is a prerequisite to maintain the apicobasal polarity during *C. elegans* embryogenesis (Bossinger et al. 2004; McMahon et al. 2001). How LET-413 acts at the molecular level is unknown. With regard to the process of junction compaction, an unexpected cue recently emerged from the observation that loss of the inositol-triphosphate

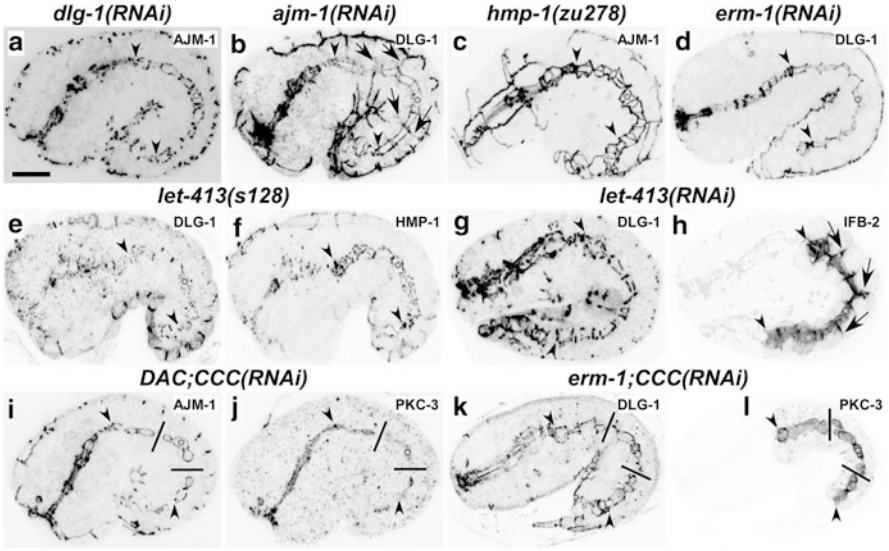

Fig. 2.4 Distribution of junctional and apicoluminal markers in *C. elegans* RNAi and mutant embryos. (**a**, **b**) RNAi against the DAC (DLG-1/Discs large–AJM-1 complex). Note that AJM-1 puncta do not consolidate into a mature apical junctional belt (**a**, compare to Fig. 2.2c), whereas DLG-1 spreads to the lateral membrane domain (LMD, *black arrows*) of intestinal cells (**b**). (**c**) Distribution of AJM-1 in a HMP-1/α-catenin mutant embryo. (**d**) Depletion of ezrin–radixin–moesin/ERM-1 yields a narrowing of the developing lumen (*asterisk*), as indicated by junctional constrictions (compare to Fig. 2.2c). (**e**, **f**) Double staining against the DAC (**e**) and the CCC (HMP-1/α-catenin–HMP-2/β-catenin–HMR-1/E-cadherin complex, **f**) in a *let-413* mutant embryo. (**g**, **h**) Double staining against the DAC (**g**) and the intermediate filament protein IFB-2 (**h**) after depletion of LET-413/Scribble. Note the spreading of IFB-2 to the LMD (*black arrows*). (**i**, **j**) Double depletion of the DAC (*dlg-1*(*RNAi*)) and the CCC (*hmp-1*(*RNAi*)) induces rupture of the ALMD (*straight black lines*) as indicated by the double immunofluorescence of AJM-1 and PKC-3. (**k**, **l**) Double depletion of ERM-1 (*erm-1*(*RNAi*)) and the CCC (*hmp-1*(*RNAi*)) induces rupture of the ALMD (*straight black lines*) as indicated by double immunofluorescence of DLG-1 and PKC-3. (**a**–**l**) Inverted immunofluorescence images (antibody staining indicated in the *top right corner*) showing confocal projections of the developing alimentary tract in *C. elegans* embryos ("tadpole" and "plum" stages, for timing see Table 2.1) after MeOH/acetone fixation. *Black arrowheads* and *white asterisks* (**c**–**l**) indicate the anterior and posterior borders of the intestinal primordium and the developing lumen, respectively. The pharynx and hindgut are to the left of the anterior and posterior intestinal borders, respectively. *Black arrows* (**b**, **h**) indicate spreading of intestinal markers to the LMD. *Straight black lines* (**i**–**l**) indicate rupture of the ALMD. Orientation: anterior (*left*), dorsal (*top*). Bar: 10 μm

receptor ITR-1 or loss of the inositol polyphosphate 5-phosphatase IPP-5 can partially compensate the knockdown of LET-413 by RNAi, suggesting that it might be Ca^{2+} sensitive (Pilipiuk et al. 2009). Intriguingly, ITR-1 interacts with myosin II (Walker et al. 2002), raising the possibility that myosin II is involved in junction compaction in *C. elegans*. Indeed, recent studies demonstrated the need for myosin II in the development of adherens junctions in cell culture (Yonemura et al. 2010).

2.2.3 *Formation of the Lumen*

Lumen formation in general enables essential functions such as nutrient uptake, gas exchange, and circulation. The reduced function of the ALMD or perturbation of the finely balanced control of lumen diameter is often fatal. Investigating the molecular mechanisms controlling the formation and maintenance of the lumina is key to better understand common human diseases (Datta et al. 2011). For instance, hyperdilated tubules associated with renal dysfunction occur in polycystic kidney diseases (Wilson 2011; Nagao et al. 2012), and reduction of lumen size is associated with vascular diseases such as hypertension (Iruela-Arispe and Davis 2009). Furthermore, early stages of many epithelial cancers display luminal filling, such as in ductal carcinomas in situ (Hebner et al. 2008).

The ALMD of the *C. elegans* intestine is bordered, in general, by only two cells. However, in the E16 stage, the nascent ALMD becomes established mostly between pairs of four radially symmetrical cells, two from the dorsal and two from the ventral layer of E cells (Leung et al. 1999). Remarkably, the intercalation of ventral intestinal cells into the dorsal layer does (see above) not define a new ALMD, and its rather *ventral* position matches with the location of the future lumen (Leung et al. 1999). Hence, intestinal cells must undergo a complex cytoskeletal rearrangement to take on a crescent shape and to form the lumen centrally.

The *C. elegans* intestinal lumen seems to form by *cord hollowing* (Lubarsky and Krasnow 2003). In *cord hollowing*, intracellular vesicles are thought to contain fluid that is taken up by endocytosis, *trans*-Golgi-derived material, and apical proteins. Their movement and delivery to the cell surface at a coordinated point between closely opposed cells creates a luminal space de novo (Bryant and Mostov 2008). In the E16 intestine, vesicles appear continuously and remain concentrated near the ALMD. If these apical vesicles are exocytosed, they might contribute to both apical membrane biogenesis and initial lumen formation (Leung et al. 1999).

In the developing zebrafish and mouse intestines, fusion of multiple rudimentary lumina into a single lumen occurs in a PKC- and ezrin-dependent manner respectively (Horne-Badovinac et al. 2001; Saotome et al. 2004). In *C. elegans*, multiple microlumina appear at the E16 to E20 stage (Leung et al. 1999), and loss of the *C. elegans* ezrin–radixin–moesin homologue ERM-1 yields luminal obstructions (van Fürden et al. 2004), suggesting that fusion is critical to form a central lumen in the intestine.

ERM-1 and SMA-1/β_H-spectrin act as scaffolding proteins to connect AFs (Fig. 2.3d–f) to the luminal membranes of intestinal cells. Both proteins are involved in lumen formation and the organization of the brush border (Brown and McKnight 2010; Praitis et al. 2005; McKeown et al. 1998; van Fürden et al. 2004; Göbel et al. 2004; Saotome et al. 2004). ERM-1 is required along with the branched actin nucleator Arp2/3 and one of its activators (WAVE/SCAR, GEX-2/Sra1/p140/PIR121 and GEX-3/NAP1/HEM2/Kette, but not WASP) for apical F-actin enrichment in the embryonic intestine. Intestines developing with reduced ERM-1, Arp2/3, or WAVE/SCAR accumulate less apical F-actin and show altered lumen

morphogenesis (Bernadskaya et al. 2011; van Fürden et al. 2004; Patel et al. 2008). Along the same line, depletion of formins, which promote linear actin formation, or *C. elegans* members of the TOCA family (TOCA-1, TOCA-2), which control actin dynamics through their interactions with actin remodeling factors (WAVE/SCAR, WASP), also leads to lower levels of phalloidin at the ALMD (Giuliani et al. 2009). Phalloidin staining also becomes reduced in *ifo-1*, which encodes a novel, histidine-rich, polyproline tract-containing nematode protein and interferes with the localization of intestine-specific IFs (Fig. 2.3g–i) (Carberry et al. 2012). Finally, apical enrichments of F-actin (Fig. 2.3d–f) and DLG-1 (Fig. 2.3a–c) (but not HMR-1/E-cadherin) are mutually dependent on each other (Bernadskaya et al. 2011).

The reduction of apical F-actin in the embryonic intestine has opposite effects on the width of the lumen. While the absence of TOCA and Arp2/3 complex proteins causes the lumen to become wider, the loss of ERM-1 yields extreme narrowing of the lumen and the reduction of IFO-1 and IFs seems to generate a rather wild-type-like lumen (Carberry et al. 2012; Bossinger et al. 2004; Hüsken et al. 2008). TOCA and Arp2/3 complex proteins seem to maintain lumen morphogenesis in controlling early endocytosis and the morphology of early endosomes (Patel and Soto 2013; Giuliani et al. 2009). Of note, endocytosis mutants, including *chc-1*/clathrin heavy chain, *dyn-1*/dynamin GTPase, and *rab-5*/Rab5 GTPase, show similar intestinal lumen expansion as observed after depletion of GEX-3 (Patel and Soto 2013). In mature epithelial cells of rat small intestine, immunogold localization of ezrin shows that most gold particles are associated with the microvilli. However, a low level of staining is also seen in the terminal web region, whereas no staining is seen in the region of adherens junctions (Berryman et al. 1993). Ezrin was initially believed to laterally tether the microvilli core bundle to the membrane (Takeuchi et al. 1994; Berryman et al. 1995; Crepaldi et al. 1997; Bonilha et al. 1999). However, this hypothesis was questioned in a recent work by Brown and McKnight (2010). Instead, as demonstrated by its knockout in mice, ezrin is believed to be important in maintaining a connection between the terminal web and the ALMD (Saotome et al. 2004). Ezrin is not absolutely required for the formation of brush border microvilli in mice and *C. elegans* (Saotome et al. 2004; Göbel et al. 2004).

Arp2/3–ERM-1 and IFO-1–IFs affect each other's protein levels. Depletion of GEX-3 or IFO-1 leads to an increased junctional accumulation of ERM-1 or IFs respectively (Bernadskaya et al. 2011; Carberry et al. 2012). This supports a role for Apr2/3 and IFO-1 in maintaining the levels of ERM-1 and IFs in the terminal web and downregulating their levels at the CeAJ. F-actin, either nucleated by Arp2/3 and formins or enriched by ERM-1 and IFO-1, could provide stiffness to the lumen. The junctional enrichment of ERM-1 and IFs as seen in *gex-3* and *ifo-1* mutants may indicate that WAVE/SCAR proteins and IFO-1 prevent excessive flexibility of the lumen by upregulating ERM-1 and IFs in the terminal web.

erm-1 interacts genetically with *ifo-1*. An enhanced phenotype is observed for apical F-actin and anti-IFB-2 signals in the intestine, which are significantly more reduced in *erm-1–ifo-1* mutant embryos (Carberry et al. 2012). Remarkably, a novel luminal defect becomes obvious. In contrast to the respective single mutants, the DLG-1-positive CeAJ and the junctional IFB-2 meshwork are discontinuous,

indicative of luminal rupture in these embryos. In addition, *erm-1* (Fig. 2.4k, l) and *ifo-1* also genetically interact with the components of the CCC and DAC, respectively. During morphogenesis of the *C. elegans* intestine, only double knockdowns of ERM-1 and HMR-1/E-cadherin or IFO-1 and DLG-1/Discs large but not IFO-1 and HMR-1 or ERM-1 and DLG-1 (Carberry et al. 2012; van Fürden et al. 2004) generate a similar phenotype as that observed after depletion of ERM-1 and IFO-1. These genetic data suggest two parallel pathways (Table 2.2), ERM-1 + DAC and IFO-1 + CCC, which are both necessary to ensure luminal and junctional integrity, presumably by promoting cell adhesion (Fig. 2.4i, j). In the case of the ERM-1/DAC pathway, the L1CAM SAX-7 (Fig. 2.2j), a single-pass transmembrane cell adhesion receptor belonging to the immunoglobulin superfamily, has the potential to interact with ERM-1 and DLG-1 (Chen and Zhou 2010; Zhou et al. 2008; Chen et al. 2001). Although the loss of SAX-7 seems not to interfere with the junctional localization of the DAC (Bernadskaya et al. 2011), depletion of the DAC disturbs junctional localization of phosphorylated SAX-7 in the embryonic intestine (our unpublished data). Very recently, it has been demonstrated that SAX-7/L1CAM and HMR-1/E-cadherin also function redundantly in blastomere compaction and non-muscle myosin accumulation during *C. elegans* gastrulation (Fig. 2.1c) (Grana et al. 2010). Of note, during morphogenesis of the *C. elegans* epidermis, SAX-7 interacts with MAGI-1/MAGUK and its adapter protein AFD-1/afadin to maintain a stable, spatially ordered CeAJ (Lynch et al. 2012).

2.2.4 Formation of the Brush Border

The surface of most animal cells lining the intestinal lumen is characterized by a brush border. It consists of regularly spaced and evenly shaped microvilli that are anchored to the cytoskeleton-rich, organelle-free cytoplasmic terminal web and its associated apical junctions. Microvilli increase the absorptive and resorptive surface areas of the intestine and are characterized by a core of membrane-attached longitudinal F-actin filament bundles whose rootlets extend into the subapical terminal web region. The terminal web has been investigated at the ultrastructural level (Hirokawa et al. 1982; Bement and Mooseker 1996), and the principal components are known to be AFs (Fig. 2.3d–f), IFs (Fig. 2.3g–i), myosin, spectrin, and an assortment of actin-binding proteins (Fath and Burgess 1995; Ku et al. 1999; Mooseker 1985; Thomas 2001; Drenckhahn and Dermietzel 1988).

The intestinal terminal web in many nematodes contains a discrete and prominent substructure termed the *endotube* (Munn and Greenwood 1984). In *C. elegans*, the reactivity of actin proteins and the IF protein IFB-2, as detected by immunoelectron microscopy, decorates the *endotube* and continues into the region where the *endotube* joins the electron-dense structure of the intestinal CeAJ (MacQueen et al. 2005; Bossinger et al. 2004). Electron microscopy reveals a discontinuous endotube with large intermittent gaps in worms whose intestinal cells were infected with microsporidia (Troemel et al. 2008). A complete loss of

the *endotube* and disordered but still intact microvilli are observed in *ifo-1* animals (Carberry et al. 2012).

Within the *C. elegans* intestinal brush border (Fig. 2.1h, i), AFs, probably built by association of ACT-5 monomers, form long bundles. These bundles are capped at their barbed end by EPS-8A, the long isoform of the *C. elegans* homologue of the epidermal growth factor receptor substrate Eps8, which is localized at the tips of the brush border intestinal microvilli (MacQueen et al. 2005; Croce et al. 2004). *act-5* seems not to encode the only actin in the embryonic intestine because in *act-5* loss-of-function mutants, E cells are able to divide and terminally differentiate into polarized epithelial cells. Nevertheless, sequence differences between ACT-5 and ACT-1 to ACT-4 most likely render ACT-5 functionally distinct and specialized for microvilli formation. Ultrastructural analysis of animals grown on *act-5*(*RNAi*) reveals a complete loss of intestinal microvilli. The lumen is frequently round instead of ellipsoid and associated with an abnormally thick terminal web structure (MacQueen et al. 2005). In *eps-8A*(*RNAi*) L4 larvae, microvilli form an irregular layer, with an overall lower microvillar density and total absence of microvilli in some areas. Many microvilli are longer than in wild-type animals, indicating a lack of termination of microvilli elongation. In addition, the terminal web seems to detach from the microvillar layer (Croce et al. 2004).

How establishment of apicobasal polarity in the *C. elegans* intestine (see above) leads to the subsequent formation of the brush border (Fig. 2.1i) and how the distribution/density of microvilli in the ALMD is regulated are not understood. In human intestinal epithelial cell lines LKB1, the homologue of the PAR-4 polarity protein can induce complete apicobasal polarity in a cell-autonomous fashion in single isolated colon cells after activation by its specific adapter protein STRAD. Furthermore, upon LKB1 activation, single cells rapidly remodel their AFs to form an apical brush border and junctional proteins reallocate in a belt peripheral to the brush border (Baas et al. 2004). In this system, apicobasal polarity is translated directly into the acquisition of a brush border through a small G protein (Rap2A) signaling module whose action is positioned by a cortical lipid cue and finally executed by activated ezrin (Gloerich et al. 2012). During intestinal brush border formation, this signaling pathway from Rap2A to ezrin seems to be evolutionarily conserved. In *C. elegans*, immunostaining of wild-type L3 larvae for ERM-1 phosphorylated at its activating threonine (Thr 544) revealed its strong enrichment at the ALMD of the intestine. After depletion of the *C. elegans* Rap2 homologue, the level of anti-phospho-ERM-1(Thr544) staining becomes substantially decreased (Gloerich et al. 2012). Of course, the molecular details concerning microvilli morphogenesis in *C. elegans* (Fig. 2.1i) still await investigation.

2.3 Expansion and Maintenance of Intestinal Membrane Domains During the *C. elegans* Life Cycle

From late embryogenesis through larval and adult development (Table 2.1, Fig. 2.1g, j), the intestine, comprising roughly one third of the total somatic mass of *C. elegans* (McGhee 2007), expands by growth alone without further cell divisions. For instance, the volume of intestinal cells roughly doubles during embryogenesis, presumably by the internalization of yolk proteins, which are secreted from most blastomeres (Bossinger and Schierenberg 1992b; Yu et al. 2006; Bossinger et al. 1996).

The *expanding C. elegans* intestine has become an attractive in vivo model for the analysis of polarized membrane biogenesis. Because the conversion of polarized membrane domains and the formation of ectopic intestinal lumen can be easily followed during the *C. elegans* life cycle, a recent work has revealed that Lats kinase, glycosphingolipids (GSLs), clathrin heavy chain (CHC) and its AP-1 adapter, and RAB-11 recycling endosomes (REs) are important for sorting to the apical membrane and the maintenance of epithelial cell polarity (Zhang et al. 2012; Zhang et al. 2011; Shafaq-Zadah et al. 2012; Kang et al. 2009; Winter et al. 2012).

The *warts* (*wts*) gene, encoding a Lats kinase homologue in *Drosophila*, was first identified in genetic studies (Justice et al. 1995; Xu et al. 1995). In *Drosophila* and mammals, *wts* acts in the conserved Hippo pathway that promotes inhibition of apoptosis and drives cell proliferation (Enderle and McNeill 2013; Hergovich 2013). Surprisingly, *wts-1* function in *C. elegans* primarily maintains the integrity of the intestinal ALMD but is not involved in the establishment of apicobasal polarity (Kang et al. 2009). In *wts-1* homozygous L1 larvae, ACT-5::GFP, the CCC, and the DAC (Fig. 2.2i, h) gradually spread to the lateral membrane domain, and finally lumen-like structures, sealed by the CeAJ and containing a brush border, develop. Dependent on the function of the exocyst complex, which is known to be important for targeting proteins to the basolateral membrane (Grindstaff et al. 1998), only newly synthesized ACT-5::GFP becomes ectopically enriched (Kang et al. 2009). The exocyst is an evolutionarily conserved multisubunit protein complex implicated in tethering secretory vesicles to the plasma membrane. It localizes to restricted regions of the plasma membrane, where it mediates the delivery of proteins and lipids necessary for polarized membrane expansion (Heider and Munson 2012). From the phenotype caused by the *wts-1* mutation in *C. elegans*, it seems plausible that WTS-1 function normally ensures that AFs (Fig. 2.3d–f) and CeAJ protein are properly transported and maintained near the ALMD to preserve normal expansion of the ALMD (Kang et al. 2009).

Several genes encoding enzymes of the GSL biosynthetic pathway, as well as CHC-1/AP-1, act as mediators of polarized transport to the ALMD in *C. elegans* late embryonic and larval intestines. Surprisingly, depletion of these genes does not affect the initial establishment of apicobasal polarity in the intestine (see above), but induces the mislocalization of apical molecules to lateral membrane domains, and thus promoting the formation of additional ectopic lumens exclusively during

late embryonic (Zhang et al. 2012; Shafaq-Zadah et al. 2012) or larval development (Zhang et al. 2011) of intestinal cells (Table 2.1). Because the reduction-of-function phenotypes of GSLs and CHC-1/AP-1 produce strong synergistic effects, Zhang et al. (2013) proposed that both pathways contribute to the same or a parallel apical sorting function during biogenesis of the intestinal ALMD.

In epithelial cells, the apical and basolateral plasma membranes are generally enriched in GSLs/sphingomyelin and phosphatidylcholine, respectively, to form the so-called membrane/lipid rafts that are required in vivo for trafficking pathways and can act as hubs for many molecular scaffolds (Simons and Ikonen 1997; Head et al. 2014). In the *C. elegans* intestine, GSLs are the common apical polarity-affecting lipid species, and exogenous lipids supplied by food, including GSL, can partially rescue germline mutations in fatty acid biosynthetic enzymes. For instance, in *let-767* larvae ectopic lateral lumina become closed, the central lumen is rebuilt, and the growth arrest and lethality are rescued (Zhang et al. 2011).

The functions of clathrin and AP1B in mammalian epithelial cell culture so far are both implicated in basolateral sorting, and neither clathrin nor AP1B seem to be required for the overall epithelial polarity maintenance (Weisz and Rodriguez-Boulan 2009; Gonzalez and Rodriguez-Boulan 2009; Fölsch et al. 1999). In contrast, in the *C. elegans* intestine, AP-1 is required to apically enrich RHO GTPase CDC-42 and RAB-11 recycling endosomes (REs), suggesting that AP-1 might function at the level of this compartment (Zhang et al. 2012; Shafaq-Zadah et al. 2012). Interestingly, another study in *C. elegans* found that PAR-5/14-3-3 protein and RAB-11–REs play a central role in maintaining the apicobasal polarity of the adult intestine. After depletion of PAR-5, RAB-11–REs become mispositioned basally along with patches of AFs in a process that depends on the kinesin-1 orthologue UNC-116 and AF modulators, such as ADF/cofilin and profilin (Winter et al. 2012).

In summary, during postembryonic development (Table 2.1) of the *C. elegans* intestine, GSL raft-dependent trafficking, clathrin/AP-1-dependent pathways, and the PAR-5 regulatory hub seem to intersect on the RAB-11–REs to control the expansion of the ALMD and to preserve the identity of the basolateral membrane domain (BMD). Whether the exocyst complex is a requirement for the mislocalization of apicoluminal membrane components to the BMD, as demonstrated in the case of *C. elegans* Lats kinase mutations (see above, Kang et al. 2009), remains to be investigated.

2.4 Future Perspectives

Despite the considerable progress in uncovering the basic mechanisms that are involved in the maintenance of cell polarity through trafficking during late embryonic, larval, and adult development of the *C. elegans* intestine, future progress should address the issue of how the vesicle trafficking machinery participates in the establishment of the apicoluminal membrane domain (including microvilli and

lumen formation) and how a cross talk with the MT and F-actin networks is regulated. In addition, the molecular mechanism of LET-413/Scribble function is still a challenge in the early polarization events. We have probably reached a plateau in terms of describing the function of key molecules of the *C. elegans* apical junction in the embryonic intestine. Future progress should now approach the still mysterious issue of how the epithelial junctional belt and cytoskeletal filaments are organized and regulated during larval and adult development to support the intestine's major roles in the response of *C. elegans* to environmental (e.g., toxins or infections) and mechanical stresses.

Acknowledgments We thank R. Durbin for kindly providing electron micrographs (Fig. 2.1h, i); L. Chen, M. Fuller, J. McGhee, K. Kemphues, and J. Priess for providing antibodies and strains; and R. Leube, A. Dineen, B. Lancaster, and members of the Bossinger lab for the critical reading of the manuscript. T. W. is supported by the Canadian Institutes of Health Research operating grant to J. D. McGhee. Work in the laboratory of O. B. is funded by the DFG and Bayer Crop Science. Some nematode strains used in this work were provided by the *Caenorhabditis* Genetics Center, which is funded by the National Institutes of Health National Center for Research Resources (NCRR). The monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biology, Iowa City, IA 52242, USA.

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Chapter 3
Apicobasal Polarity and Lumen Formation During Development

Adam Navis and Michel Bagnat

Abstract Networks of interconnected tubes form the basic structural element of many organs. Tubes are composed of polarized epithelia that enclose a lumen. During organogenesis, lumens form by several distinct mechanisms, ranging from wrapping of an epithelial sheet to generation of a lumen de novo within a rod of cells. Nevertheless, all tube formation processes share some basic common principles that result in the generation of a single, continuous lumen. Interactions with the surrounding environment direct epithelial cell polarization that governs physiological regulators of lumen formation including the actin cytoskeleton, adhesion, vesicular transport, and fluid secretion. Polarized physiological processes mediate the mechanical interactions between epithelial cells and their environment. Polarity within actin cytoskeleton generates contractile forces generating morphogenetic movements. Secretion of fluid or matrix into the lumen generates outward forces driving lumen opening and expansion. Thus, cell polarity is crucial for vectorial transport processes and structural asymmetries during lumen formation. Here we focus on recent discoveries illuminating the relationship between lumen formation and cell polarity in vivo.

Keywords Polarity • Lumen formation • Organogenesis

3.1 Introduction

Biological tubes are a fundamental unit of construction in nearly all organs, and proper tube formation is integral to their function. These tubes are composed of epithelial cells that enclose a central lumen. To properly form a lumen, cells in the epithelium must generate and maintain cell polarity, which typically includes an apical membrane contacting the lumen and a basolateral membrane that contacts surrounding cells and a basement membrane composed of extracellular matrix (ECM). Cell polarity is required for diverse processes during organogenesis

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K. Ebnet (ed.), *Cell Polarity 2*, DOI 10.1007/978-3-319-14466-5_3

including actin contractility, adhesive forces, migration, and secretion. Lumen morphogenesis depends on the precise coordination of these polarized processes.

Cell polarity of the apical and basolateral membranes is established and maintained through many intracellular processes. The apical membrane is associated with a variety of polarity regulators including Crumbs, Cdc42, and the Par complex. These proteins regulate numerous downstream processes including apical polarization of the actin cytoskeleton, vectorial trafficking, and localization of ion transporters (Tepass 2012). The basolateral membrane is specified by the Scribble complex composed of Scribble, Disks large, and Lethal giant larvae and is populated by several types of adhesion proteins including members of the claudin, cadherin, and integrin families (Schlüter and Margolis 2012).

Lumen formation proceeds through several phases. Cell polarization is initiated through interaction with the ECM, which directs the orientation of the apicobasal axis (Rasmussen et al. 2012; Yu et al. 2005). The mechanisms by which ECM interactions result in cell polarization are not well understood, but likely involve integrin, lipid, and cytoskeletal signaling. Polarity in the cytoskeleton is necessary for morphogenetic events such as apical constriction and cell motility that mediate the cellular rearrangements leading up to lumen formation (Sawyer et al. 2010). Sorting directs proteins to the appropriate pathway where they can be delivered to specific membrane domains by intracellular trafficking. Opening and expansion of the lumen involve polarized secretion of fluid or matrix (Cartwright et al. 2009; Luschnig and Uv 2013). The apical and basolateral membranes are studded with complementary sets of ion transporters that coordinate to produce osmotic gradients that drive the transport of water into the lumen (Cartwright et al. 2009). This activity depends on asymmetric localization of channels in the apical and basolateral membranes, regulated by trafficking and sorting events. Secretion at the apical membrane leads to luminal membrane expansion and may also produce an accumulation of matrix. Either fluid or matrix accumulation within the lumen drives lumen expansion (Luschnig et al. 2006; Navis et al. 2013; Tiklová et al. 2013; Wang et al. 2006).

The mechanisms regulating cell polarity govern the generation of polarized physiological processes that drive mechanical forces and directly mediate lumen formation. Many of these processes converge on the basolaterally localized sodium potassium ATPase (Na^+/K^+-ATPase, covered in detail in Part 2, Chap. 4) and the establishment of tight junctions. Utilizing polarity provided by the apicobasal axis, the Na^+/K^+-ATPase is central to the establishment of a physiological axis controlling fluid secretion, cell adhesion, and secretory functions. Together, these processes coordinate to generate a functional lumen during organogenesis.

During development, epithelial cells form lumens within complex organs and interact with a number of different cell types. To understand the mechanisms that drive lumen formation in vivo, organogenesis has increasingly been studied in animal models, which incorporate the intercellular interactions occurring throughout development. In this review, we will focus on studies of in vivo lumen formation and how they have advanced our knowledge of luminogenesis. Particularly, we focus on how cell polarization machinery synergizes with biophysical

forces that mediate lumen formation. Investigating the mechanisms of lumen formation is important for understanding the progression of human diseases associated with organ development and maintenance including atherosclerosis and polycystic kidney disease. The studies highlighted here have led to important advances in our understanding of the diversity of processes required for lumen formation and their commonalities across the animal kingdom.

3.2 Tubulogenesis: Cellular Mechanisms of Lumen Formation

During organogenesis, cells form lumens through several distinct mechanisms. A lumen may form from a polarized sheet of cells, within a rod of cells, or even through a hollowed cell (Lubarsky and Krasnow 2003).

A sheet of polarized cells can form a tube through a process of epithelial wrapping. In these tissues, a layer of polarized cells undergoes apical constriction, which contracts the apical surface. This generates a force that causes the cell to change shape from columnar to pyramidal, bending and eventually wrapping the epithelium, which encloses a new lumen. In mammals, chicks, and frogs, epithelial wrapping gives rise to the gut and neural tube during development (Sawyer et al. 2010).

Tubes may also branch in new directions by budding. Typically budding is initiated by apical constriction at the new bud, which generates an invagination that extends to generate the lumen. Budding occurs during the development of the mammalian lung, vasculature, *Drosophila* tracheal system, and salivary gland (Hogan and Kolodziej 2002; Uv et al. 2003).

The vasculature undergoes several types of lumen formation during development. Recently new modes of lumen formation have been described. Ensheathment is characterized by migration of endothelial cells to surround a lumen. The zebrafish common cardinal vein initially develops as an open-ended tube, and then endothelial cells are specified and migrate to incorporate into an extending vascular wall that grows to enclose the common cardinal vein lumen (Helker et al. 2013). During development of the zebrafish caudal vein, endothelial cells sprout from the dorsal aorta and generate a separate lumen in a process reminiscent of budding. However, rather than branching a continuous lumen, the cells migrate and form a distinct blood vessel (Herbert et al. 2009). It is likely that these types of lumen formation identified by live imaging in zebrafish are also present in other animals.

Within a rod of cells, lumens form by cavitation or cord hollowing. Cavitation occurs through apoptosis of the cells within the lumen, sparing the epithelial cells at the periphery. In the mammalian mammary and salivary glands, cells contacting the ECM are polarized and receive survival cues, while the cells not recruited to the epithelium undergo apoptosis. The apoptotic cells are then cleared, opening the new lumen (Mailleux et al. 2007; Melnick and Jaskoll 2000; Tucker 2007). Although

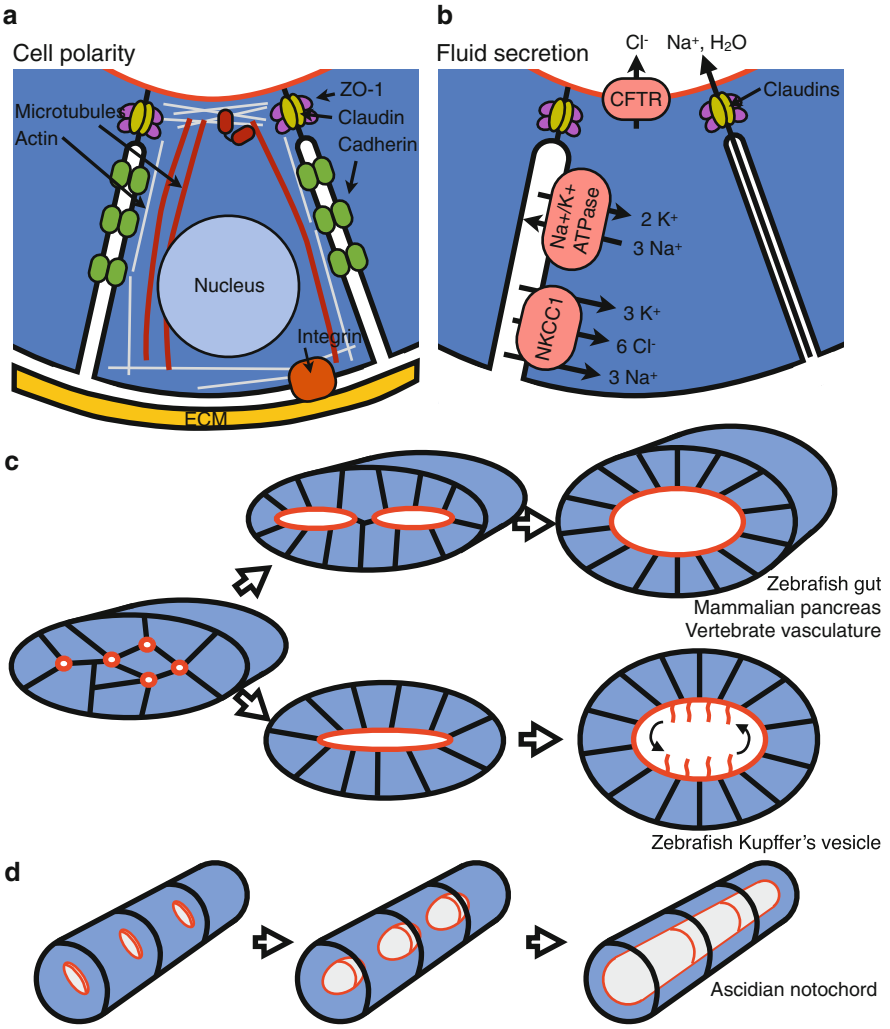

Fig. 3.1 Cell polarity during lumen morphogenesis. (**a**) Schematic of a polarized epithelial cell to illustrate the organization of the adhesion proteins, cytoskeleton, nucleus, ECM, and membrane domains. The apical, luminal membrane is at the top, and the basal membrane facing the ECM at the bottom. (**b**) Schematic representation of a cell driving fluid secretion. The channels are marked by *ovals*, and *arrows* indicate the movement of ions during fluid secretion. (**c**) Illustration of single lumen formation by cord hollowing in various organs. (**d**) Diagram of lumen formation by cell hollowing in the Ascidian notochord

apoptosis has been observed during development of the mammalian salivary and mammary glands, a definitive role for apoptosis directly driving lumen formation has not been established. A rod of cells may also form a central lumen in the absence of apoptosis by rearranging the epithelial cells in a process termed cord hollowing (Fig. 3.1c). This process generates several lumens throughout an organ,

which coalesce as the lumen expands (Bagnat et al. 2007). Tube formation in the mammalian kidney, pancreas, large blood vessels, and zebrafish gut forms through cord hollowing (Herwig et al. 2011; Horne-Badovinac et al. 2001; Kesavan et al. 2009; Yang et al. 2013).

Cell hollowing is a specialized type of lumen formation where a lumen forms intracellularly within rod of cells (Fig. 3.1d) and may be accompanied by anastomosis as seen in the mammalian vasculature (Herwig et al. 2011). This is generally characterized by the formation of an apical compartment that grows and extends through the length of the cell and merges with compartments from neighboring cells to generate a lumen extending through the middle of hollowed cells. The *C. elegans* excretory system, terminal and fusion branches of the *Drosophila* trachea, mammalian vasculature, and ascidian notochord form lumens by cell hollowing (Buechner 2002; Dong et al. 2009; Kamei et al. 2006; Levi et al. 2006).

Organs may form a lumen through a variety of mechanisms, but the underlying intracellular processes remain constant. Several unifying principles link cell polarity and lumen formation across a variety of organs and systems. To form a lumen, cells require contact with the ECM, polarized signaling, cytoskeletal assembly, vectorial trafficking, and lumen expansion through fluid accumulation or matrix secretion. These polarized processes have been well studied in vitro, but their precise function in vivo, especially during lumen formation, has only recently begun to emerge.

3.3 Breaking Symmetry: Specifying Membrane Polarity

To establish and maintain a functional lumen, the epithelium must correctly partition a wide variety of molecules. The apical and basolateral membranes are maintained by opposing complexes, the Par complex at the apical membrane and the Scribble complex at the basolateral membrane. These complexes are required at the earliest stages of development where they mediate cellularization in the fly and asymmetric distribution of the cytosol during the stereotypic divisions of the *C. elegans* embryo (Suzuki and Ohno 2006). Functions for these complexes have been detailed by many studies of epithelia in vivo as well as in vitro, including three-dimensional cultures, but their precise role during lumen formation in vivo remains unclear.

Properly oriented cell polarity is essential for lumen formation. A central polarity determinant, the Rho GTPase, Cdc42, plays an early role in the specification of the apicobasal axis. In the mammalian pancreas, coordination of cell polarity is necessary to complete cord hollowing and generate a single lumen. Loss of Cdc42 in the pancreas disrupts cell polarity and leads to disorganization of the lumen (Kesavan et al. 2009).

A key regulator of cell polarity is the Par complex of proteins, composed of Par3, Par6, and atypical protein kinase C (aPKC). Mutants in the Par complex typically display severe defects in lumen formation due to disorganized cell polarity. The

zebrafish neural tube, which forms by cord hollowing, has been a focal point of genetic screens for mutants affecting cell polarity. A zebrafish mutant for *pard6γb*, an orthologue of Par6, displays defective lumen formation in the neural tube due to disorganized cell polarity (Munson et al. 2008). The zebrafish *heart and soul* (*has*) mutant encodes a defective PKCλ, an aPKC. These mutants display defects in cell polarity and spindle orientation, leading to improperly partitioned and positioned cells throughout the gut and neural tube (Horne-Badovinac et al. 2001). These studies highlight the importance of the Par complex during single lumen formation; disrupted polarity often leads to the formation of multiple lumens in organs that form by cord hollowing.

Cell polarity is also regulated by an extensively investigated apical determinant, Crumbs, which functions in concert with the Par complex (Tepass et al. 1990). Crumbs regulates cell polarity during the development of the *Drosophila* tracheal and salivary gland lumens. In the salivary gland, Crumbs is necessary for delivery of new apical membrane as the lumen expands (Myat and Andrew 2002). Overexpression of Crumbs leads to increased secretion and expansion of the tracheal lumen, while loss of Crumbs function leads to a smaller lumen (Letizia et al. 2011). The role of Crumbs in the specification of apical secretion is balanced by the septate junction-associated proteins Yurt and Scribble. Loss of these proteins allows Crumbs to drive apical secretion unchecked, causing expansion of the tracheal lumen (Laprise et al. 2010). Crumbs is also important for the specification of epithelial polarity in zebrafish. The mutant *oko meduzy* (*ome*) encodes a mutant Crumbs orthologue and develops defects in several polarized tissues leading to kidney cysts and retinal disorganization (Malicki and Driever 1999; Omori and Malicki 2006). Thus, Crumbs is a central regulator of cell polarity, which governs lumen formation across the animal kingdom.

Genetic screens in the zebrafish neural tube have identified other regulators of cell polarity. The membrane-associated guanylate kinase (MAGUK) family protein, *nagie oko* (*nok*), is necessary for lumen formation in the neural tube. Loss of *nok* results in junctional disorganization and disrupts single lumen formation in the developing brain ventricle, leading to the development of discontinuous lumens in the neural tube (Lowery and Sive 2005).

Physiologically, cell polarity is important to direct the localization of proteins that mediate lumen morphogenesis including adhesions and ion channels. Orientation of cell polarity in the mouse kidney is regulated by a Ras/Rap effector protein, Afadin. During the initial formation of a lumen, Afadin delivers nectin family adhesion proteins to junctional complexes, which mediate cadherin localization and lumen formation. Loss of Afadin function disrupts formation of the Par complex, leading to defects in epithelial polarity and formation of multiple discontinuous lumens throughout the kidney (Yang et al. 2013). Luminal fluid secretion requires tightly regulated ion channel localization. This requirement can be seen in the zebrafish Kupffer's vesicle (KV) and neural tube. In KV, the chloride channel Cftr is directed specifically to the apical membrane early in lumen morphogenesis to expand the developing lumen (Navis et al. 2013). The electrochemical gradients that drive apical chloride transport are driven by basolateral localization of the Na^+/

K^+-ATPase. In zebrafish, the Na^+/K^+-ATPase is required to inflate the brain ventricles during hindbrain development and for single lumen formation in the zebrafish gut (Bagnat et al. 2007; Lowery and Sive 2005). Generation of cell polarity directs the localization of physiologically important channels and adhesion proteins that mediate the forces that drive lumen formation.

3.4 This Way Up: Polarity Cues from the Extracellular Matrix

Epithelial cells enclosing a lumen are typically surrounded by several layers of ECM and mesenchyme. The ECM plays several important roles during lumen morphogenesis: it can act as a cue for orientation of apicobasal polarity, a substrate for mechanical tension, or a signal for survival. The ECM is secreted by mesenchymal cells that surround the luminal epithelium, and the basement membrane is layered on the ECM through basolateral secretion by epithelial cells (Fig. 3.1a) (Frantz et al. 2010). Mechanically, the ECM serves as a structural support for the epithelium and provides a substrate for mechanical tension during cell migration and rearrangement. It also signals the orientation of epithelial polarity early during lumen formation and provides survival cues to epithelia. In vivo studies have been instrumental in investigating the interactions between the epithelia and the surrounding ECM and mesenchyme, which is challenging to model in simplified cell culture systems.

An early step in the orientation of cell polarity occurs through interactions between the epithelium and the ECM. Signals from the basement membrane are required to correctly orient the distribution of key polarity determinants, Par3, Par6, and aPKC, which regulate the polarity of many cellular processes (Yeaman et al. 1999). For example, in the *C. elegans* pharynx, loss of laminin leads to mislocalization of Par3 and disrupts cell polarity. Loss of properly oriented cell polarity leads to defects in the formation of the pharynx lumen and causes the formation of several disconnected lumens rather than a single, continuous lumen (Rasmussen et al. 2012).

The actin cytoskeleton mediates signaling from the basement membrane to pathways within the cell. The ECM is bound by integrins, which use adaptor proteins to link the actin cytoskeleton and activate polarized signaling. This process is highlighted by lumen formation in the *Drosophila* trachea, where the adaptor *talin* connects the actin cytoskeleton to integrins (Levi et al. 2006). Loss of *talin* function disrupts the connection to the actin cytoskeleton and prevents integrins from delivering the polarized cues. *Talin* mutants have disrupted cell polarity, develop multiple discontinuous lumens, and display a general disorganization of the tracheal system (Levi et al. 2006). Lacking properly oriented cell polarity, disorganized epithelial cells are unable to form single lumen.

In addition to signaling, physical contact with the basement membrane also acts as a substrate for migration as cells rearrange to form a lumen. In *Drosophila*, mutant for laminin β lumen formation fails in several organs, including the gut, trachea, and nervous system due to defects in cell migration and rearrangement (Urbano et al. 2009). Interaction between integrins and the ECM is also necessary for migration during branching morphogenesis. In the vasculature, cells must migrate in the direction of the new bud to extend the lumen. Loss of β1-integrin function results in the disruption of the connection to the ECM and causes a failure of migration and increased apoptosis which ultimately prevents budding (Carlson et al. 2008; Lei et al. 2008). The structural support provided by the ECM is integral to the morphogenetic events driving lumen formation.

The ECM also provides survival cues during the formation of the mouse salivary gland. In the mouse submandibular gland (SMG), lumen formation was thought to occur through cavitation (Jaskoll and Melnick 1999). However, more recent evidence suggests that the salivary gland lumen forms instead via cord hollowing in the absence of apoptosis (Nedvetsky et al. 2014). Surrounding the lumen is a layer of cells in contact with the ECM that receive survival and polarization cues to become the luminal epithelium (Jaskoll and Melnick 1999).

3.5 Bent Out of Shape: Polarized Cytoskeletal Tension Drives Cell Shape Change

The cytoskeleton plays vital roles throughout all stages of lumen formation. It provides mechanical support for the cells, powers cell shape changes, and acts as a substrate for vesicular traffic during lumen morphogenesis. In many organs, actin is polarized toward the apical surface of epithelial cells, which allow it to generate differential forces along the apicobasal membranes to mediate morphogenetic events such as apical constriction (Fig. 3.1a). Additionally, the apically concentrated actin network, or terminal web, can provide a substrate for vesicular traffic near the apical surface. The contractility of the actin network is carefully balanced to control lumen morphogenesis.

Polarization of the actin cytoskeleton is regulated by a large number of proteins. In the zebrafish vasculature, cell-cell contacts through vascular endothelial cadherin (VE-cadherin) help establish apicobasal polarity in the actin cytoskeleton. VE-cadherin prevents the apical localization of Moesin, which links the actin network to the membrane (Wang et al. 2010). The expansion of the apical membrane and organization of the cytoskeleton are similarly regulated in the *Drosophila* trachea. A transcription factor, Ribbon, activates expression of Crumbs and downregulates Moesin (Kerman et al. 2008). Crumbs mediates expansion of the apical membrane, while limiting apical localization of Moesin allows the actin to accumulate apically, establishing polarity in the actin network (Wang et al. 2010).

Actin filaments are assembled into a network by nucleation and polymerization of actin monomers. Formins catalyze polymerization and extension of actin filaments. Fly mutant for a formin family protein, Diaphanous (Dia), has defective lumen formation in several organs. The defects in *dia* mutants are due to disruption of the apical actin network, which also disrupts vesicular transport powered by myosin V. Diaphanous protein and RNA are both localized apically, suggesting its localization is tightly controlled (Massarwa et al. 2009). The highly specific localization of Dia protein is regulated by apical localization of Rho1 and production of PI(4,5)P2 by a PIP5 kinase, Skittles (Rousso et al. 2013); however, the mechanisms underlying apical localization of *dia* RNA remain unknown.

Regulation of actin polymerization is also important for lumen formation in the *Drosophila* trachea. An apically localized tyrosine kinase, Src42, directs tracheal extension while limiting diameter of the tube (Förster and Luschnig 2012; Nelson et al. 2012). Src42 regulates another formin, dDaam (Nelson et al. 2012), and promotes recycling of E-cadherin (Förster and Luschnig 2012) suggesting that the extension of the *Drosophila* tracheal lumen depends on changes in the actin cytoskeleton and epithelial remodeling. Curiously, Src42 functions independently of the planar cell polarity pathway, which typically regulates morphogenetic changes in the plane of an epithelium.

One of the most common cell shape changes during lumen formation is apical constriction. Increased actin contractility at the apical membrane leads to constriction at the apical surface and initiates a cell shape change from columnar to pyramidal. During the formation of the *Xenopus* neural tube, the apically localized actin binding protein Shroom recruits increased actin and is required for apical constriction (Haigo et al. 2003). The polarity determinant, Crumbs, also regulates the initiation of apical constriction. In the *Drosophila* trachea, Crumbs accumulation at the apical surface facilitates assembly of the apical actin cytoskeleton and regulates construction of the contractile bundles (Letizia et al. 2011; Röper 2012).

Apical constriction is also required during the formation of the *Drosophila* salivary gland, where the primordium undergoes a characteristic invagination preceding lumen formation. The site of invagination is initiated by cells expressing a transcription factor, huckebein, and a cell adhesion molecule, faint sausage, which together initiate apical constriction and invagination of the future salivary gland lumen (Myat and Andrew 2000). The regulation of actin contractility also specifies lumen size in the salivary gland. A GTPase, Rho1, induces apical actin polymerization and prevents Moesin from localizing apically by limiting its phosphorylation (Xu et al. 2011b). Mutants for Rho1 disrupt Moesin localization and lead to defects in the size of the salivary gland lumen.

The lung is a highly branched organ produced by numerous budding events. In the chick lung, the buds are initiated by apical constriction of the epithelial cells, and the bud is extended by processes including proliferation and properly oriented cell divisions (Kim et al. 2013). Apical constriction is also required for the formation of the *C. elegans* vulva (Farooqui et al. 2012). Signaling through Ras and Notch balances actin contractility in the vulva, which regulates lumen formation. Notch signaling leads to an increase in actin contractility, while Ras signaling decreases actin contractility (Farooqui et al. 2012). The interplay between contraction and relaxation specifies the size of the vulval lumen.

In mouse endothelial cells, Ras signaling is a key regulator of actin contractility and epithelial polarity. As mouse blood vessels form, actin contractility drives cell shape changes that form a lumen. Rasip1, a Ras-interacting protein, and Arhgap29, a Rho GTPase binding partner, regulate Ras signaling. Defects in Ras signaling drive increased actin contractility, disrupt cell polarity, and lead to a disorganized and substantially smaller lumen. Concordantly, overexpression of Rasip1 and Arhgap29 led to activation of Cdc42 and Rac1, key components of epithelial polarity and also important players in lumen formation (Xu et al. 2011a).

The apical actin network is extended by branching from existing actin filaments. Arp2/3, an actin branching protein, is important for the formation of dense actin networks, especially during the formation of the apical actin belt (Bernadskaya et al. 2011). In the *C. elegans* intestine, Arp2/3 is required to generate the dense actin belt and for the formation of apical junctional complexes. Loss of Arp2/3 results in mislocalization of ERM-1 and DLG-1 and causes expansion of the intestinal lumen (Bernadskaya et al. 2011). ERM-1 recruits F-actin to the apical membrane and links the cytoskeleton to the plasma membrane, while DLG-1 is a scaffold protein that mediates junction formation in the worm. ERM-1 and DLG-1 functions are required for single lumen formation in the worm; loss of these proteins results in the development of multiple lumens along the length of the intestine (Van Fürden et al. 2004).

Relaxation of the actin network is required during the development of the zebrafish hindbrain ventricle (Gutzman and Sive 2010). The brain ventricle lumen forms in two distinct phases: first the lumen is formed, and then it must expand. During lumen expansion, fluid is secreted into the neural tube, which generates a luminal force (Lowery and Sive 2005); however, the lumen cannot expand without relaxation of the actin network engaged during the initial stages of lumen formation. In zebrafish, loss of a myosin phosphatase regulator, *mypt1*, prevents expansion of the hindbrain by failing to relax actin contractility leading to a substantially smaller ventricle lumen (Gutzman and Sive 2010).

The cytoskeleton is coupled to adhesions through a variety of linker proteins. In the *Drosophila* trachea, loss of *talin* leads to highly disorganized tracheal terminal branch lumens (Levi et al. 2006). In these mutant flies, early tracheal morphogenesis occurs normally, but the fine branching during late tracheal morphogenesis is severely disrupted, indicating that maintenance of polarity in the tracheal system depends on the linkage between the ECM and the actin network.

During the formation of villi in the mammalian intestine, maintenance of cell polarity preserves epithelial integrity. Apically localized ezrin, which links the actin cytoskeleton to the plasma membrane, maintains the cell polarity and the actin terminal web. Loss of ezrin in the intestine leads to defects in cell polarity and disrupts the integrity of the epithelium causing fusions between villar epithelia. The epithelial fusion across villi leads to a grossly disorganized epithelium and formation of cysts within the intestinal wall (Saotome et al. 2004).

The microtubule network mediates polarized vesicular traffic within the cell and also participates in cell shape changes. Trafficking of vesicles along the microtubule cytoskeleton is mediated by a complement of motor proteins (Caviston and

Holzbaur 2006). During formation of the zebrafish neural tube, microtubules mediate trafficking to the apical membrane in preparation for lumen opening. Disruption of vesicular transport along the microtubules disrupts localization of Rab11a and Par3, which prevents the neural tube lumen from opening (Buckley et al. 2013). Additionally, the microtubule cytoskeleton has been implicated in cell elongation during lumen formation. During apical constriction in the *Xenopus* neural tube, the epithelial cells elongate along their apicobasal axis in addition to constricting at the apical membrane (Lee et al. 2007). Loss of microtubule-dependent cellular elongation leads to defects in the wrapping morphogenesis and lumen formation in the neural tube.

Similarly, cell elongation is mediated by the actin and microtubule networks in the *Ciona* notochord. The ascidian notochord provides an interesting example of lumen formation. The notochord is a feature of all chordates and functions as an early hydrostatic support structure (Adams et al. 1990). The notochord develops as a single rod of cells and, in ascidians, generates a central lumen through a process of cell hollowing. The cells of the notochord form opposing apical membranes that are drawn through the cell and fuse with neighboring lumens to generate a central lumen (Dong et al. 2011). The actin and microtubule cytoskeleton mediate cell elongation and basal constriction, which promote fusion of the developing lumens (Dong et al. 2011).

3.6 The Ties that Bind: Cell Adhesions During Lumen Formation

Cell adhesions perform several crucial functions during lumen formation. They properly orient cell polarity, provide mechanical linkage to other cells, and facilitate remodeling of the epithelium (Fig. 3.1a). Adhesions are typically regulated by endosomal recycling, which removes them from the membrane and allows the cell to remodel its junctions. Cell adhesion can be inhibited by secreting repulsive molecules to the luminal membrane. Coordinated regulation of cell adhesion and repulsion is necessary for lumen morphogenesis.

The tight junctions in vertebrates and the septate junctions in invertebrates serve several important roles. They provide a molecular fence between the apical and basolateral membranes, mediate the formation of an intercellular barrier to restrict diffusion of luminal solutes, and also help regulate cell polarity (Schneeberger and Lynch 2004). The *Drosophila* septate junctions are integral to tracheal lumen formation. Disruption of the septate junctions leads to defects in matrix secretion into the lumen (Behr et al. 2003; Nelson et al. 2010; Wu et al. 2004). Classically, the Na^+/K^+-ATPase hydrolyzes ATP to generate an extracellular sodium gradient that powers fluid secretion, glucose import, cell volume, and membrane potential (Rajasekaran and Rajasekaran 2009). Additionally, the Na^+/K^+-ATPase has more recently been found to participate in cell adhesion (Rajasekaran et al. 2001). The

Na^+/K^+-ATPase participates in the assembly of septate junctions independent of its channel function. Loss of the Na^+/K^+-ATPase leads to defects in tracheal expansion (Paul et al. 2007).

Adhesive bonds between cells are typically mediated by the cadherin family of proteins. Cadherin contacts are relatively stable, so they must be remodeled to facilitate cellular rearrangements (Desclozeaux et al. 2008). During formation of zebrafish blood vessels, junctional contacts are rapidly remodeled (Herwig et al. 2011). The rate of cadherin turnover is important for epithelial rearrangement and lumen formation (Pirraglia et al. 2010). Lack of remodeling prevents cells from rearranging and disrupts lumen formation. In the zebrafish gut, cell rearrangements preceding single lumen formation are mediated by the recycling of cadherins through Rab11 (Alvers et al. 2014). Disruption of adhesion recycling prevents cell rearrangements and fusion of adjacent lumens. To break cell adhesions, cadherins must be internalized via endocytosis, where they can be recycled to the membrane to generate a new contact or be targeted for degradation (Xiao 2003). Conversely, overactive recycling can also disrupt lumen formation, as highlighted by increasing the activity of a regulator of recycling, Pak1 (Pirraglia et al. 2010).

Just as regulated adhesion is important for lumen formation, so is luminal repulsion, which limits adhesion and promotes opening of a lumen. To open a lumen, opposing membranes must be separated by releasing adhesions at the presumptive luminal membrane. Adhesions can be released by removing them from the membrane or secreting negatively charged proteoglycans to the apical membrane to generate a repulsive force. In the mammalian vasculature, cord hollowing generates a lumen within a rod of cells. As the cells are organized and prepare to form the nascent lumen, they secrete negatively charged proteoglycans such as podocalyxin to the apical surface which generate repulsive forces between apical membranes (Strilić et al. 2010). In these systems, injection of positively charged molecules disrupts lumen formation, highlighting the importance of electrostatic repulsion during the initial stages of lumen opening. In a similar process, inhibition of cell adhesion during lumen formation occurs during the development of the heart tube. Syndecan regulates Slit and Roundabout (Robo), which are known regulators of repulsion between cell membranes (Santiago-Martínez et al. 2008). Loss of Syndecan function results in the mislocalization of Slit and Robo away from the luminal surface. This mislocalization prevents the luminal membrane from undergoing repulsion necessary for lumen formation in the *Drosophila* heart tube (Knox et al. 2011). Of note, the *Drosophila* heart tube luminal membrane shares many characteristics with basolateral membranes, a unique feature of invertebrate vascular development (Knox et al. 2011; Kučera et al. 2009).

Overall, cell adhesion performs many roles throughout lumen morphogenesis. Regulated adhesion controls epithelial rearrangements as organs initiate lumen morphogenesis. The separation of luminal membranes is facilitated by secretion of negatively charged molecules. The polarization of intracellular processes may also depend on adhesive interactions, especially in the *Drosophila* trachea where septate junctions regulate polarized secretions that determine lumen size. Together,

these studies highlight the range of functions that adhesions perform throughout lumen morphogenesis.

3.7 Special Delivery: Trafficking and Sorting During Lumen Formation

The localization of polarized proteins depends on vesicular transport to specific locations within the cell. Protein sorting directs proteins to the appropriate transport pathway where they may be trafficked to the appropriate membrane or secreted from the cell. Sorting and trafficking mediate the delivery of physiologically important molecules like ion channels and adhesion proteins to the apical and basolateral membranes, where they mediate processes like fluid secretion and cellular rearrangements.

The development of the *Drosophila* trachea has been used as a model to investigate the role of trafficking during lumen formation. *Drosophila* tracheal cells secrete a chitinous matrix, which inflates the lumen and expands the trachea. Once lumen formation is completed, the matrix is cleared by endocytosis and is followed by removal of the fluid allowing the trachea to fill with gases (Tsarouhas et al. 2007). Genetic screens for regulators of tracheal morphogenesis have identified many mutants that disrupt the secretory pathway (Ghabrial et al. 2011). Flies mutant for γCOP, a member of the COPI complex, develop an abnormally narrow tracheal lumen. COPI, which mediates anterograde secretory transport out of the Golgi apparatus, is necessary for secretion of many proteins, including the luminal matrix (Grieder et al. 2008; Jayaram et al. 2008). Similarly COPI is also required in the *Drosophila* salivary gland, where γCOP regulates secretion of matrix and expansion of the salivary gland lumen (Abrams and Andrew 2005; Fox et al. 2010; Grieder et al. 2008).

Function of the COPII complex is also important for apical secretion and regulation of lumen size. *Drosophila* mutant for *stenosis*, a cargo-binding subunit of the COPII complex, displays defects in the expansion of the tracheal lumen (Förster et al. 2010). The COPII complex facilitates cargo transport from the ER to the Golgi apparatus (Aridor et al. 1995). Loss of stenosis drastically reduces tracheal lumen size due to the failure of protein secretion into the lumen. These mutants also display defects in the cell shape changes required during tracheal lumen formation and develop disrupted organization of the ER (Förster et al. 2010). Loss of secretory function in the trachea disrupts transport to the apical membrane limiting membrane expansion, disrupting cell rearrangements, and inhibiting the delivery of luminal components and apical membrane proteins.

To form a lumen, cells must often rearrange themselves within an organ primordium. To rearrange, cells must break their adhesive contacts to generate new contacts by internalizing and recycling adhesive components. In the *Drosophila* trachea, cells adhere to one another through E-cadherin interactions. To remodel

these contacts, cadherins must be recycled from the membrane through Rab11-positive compartments to release the adhesion between two cells (Shaye et al. 2008). In flies mutant for a transcription factor, Sal3, that controls expression of trafficking genes, defects in recycling prevent remodeling of the cadherin contacts and disrupt lumen formation (Shaye et al. 2008). Rab11 also mediates transport of proteins and new membrane to the apical surface during apical membrane expansion in the *Drosophila* trachea. Endosomal recycling through Rab11 is regulated by the transcription factor, Ribbon. Flies mutant for *ribbon* lose apical localization of Rab11-positive endosomes and develop defects in tracheal lumen expansion (Kerman et al. 2008). Similarly, in the zebrafish gut, Rab11-dependent recycling regulated by *smoothened* is required for apical membrane expansion and basolateral contact remodeling during single lumen formation (Alvers et al. 2014). Thus, recycling of cell-cell contacts and apical membranes is a key component of epithelial morphogenesis and lumen formation.

The establishment and maintenance of epithelial polarity depend on protein sorting to specific compartments. A screen for defects in *C. elegans* intestinal lumen morphogenesis identified sphingolipids as key regulators of epithelial polarity (Zhang et al. 2011). In the worm intestine, loss of the enzymes that regulate production of sphingolipids disrupts cell polarity and leads to the formation of accessory lumens throughout the intestine. Sphingolipids are thought to function by clustering with other lipids in the membrane to mediate sorting of membrane proteins (Schuck and Simons 2004). While still poorly explored during lumen formation, sphingolipids may represent a central class of polarity regulators that may facilitate apical membrane formation through sphingolipid-dependent clustering. Intriguingly, loss of either AP-1 or clathrin in the *C. elegans* intestine also leads to mislocalization of apical proteins including Par6 and disrupts the polarization of the epithelium. Worms lacking AP-1 or clathrin develop ectopic lumens throughout the intestine (Shafaq-Zadah et al. 2012; Zhang et al. 2012). This phenotype may reflect a role for basolateral sorting machinery in apical membrane biogenesis or that basolateral sorting, particularly of ion channels and adhesion molecules, plays a key role in lumen formation. In any event, these studies highlight the importance of protein sorting during the establishment of polarity and lumen formation in *C. elegans*.

Sorting of proteins to the apical membrane is also regulated by N- and O-glycosylation (Schuck and Simons 2004). Specification and expansion of the apical membrane is important for lumen formation, but whether sorting of glycans is important for lumen formation in vivo is unknown. In contrast to the role of lipids during apical sorting in lumen formation, genetic evidence linking lumen formation to apical sorting of glycosylated proteins in vivo is lacking.

Sorting of the Na^+/K^+-ATPase to the basolateral membrane is integral for several physiological processes required during lumen formation. The regulation of Na^+/K^+-ATPase localization by association with ankyrin has been well studied in vitro (Devarajan et al. 1997), but the mechanisms that determine localization of this fundamental transporter during lumen formation in vivo are largely unknown.

Understanding the functions of membrane polarity in vivo will provide a deeper understanding of lumen formation.

3.8 Opening and Expanding a Lumen from Within

A hallmark of many tubular organs is the presence of a fluid-filled lumen. Luminal secretion provides an internal force on the epithelium that drives lumen expansion and promotes lumen coalescence during single lumen formation (Bagnat et al. 2007; Navis et al. 2013). Lumens are expanded using different strategies in vertebrates and invertebrates. While vertebrates utilize fluid pressure to expand a lumen, many invertebrate organs are expanded by secretion of luminal matrix. Though the details are distinct, fluid and matrix accumulation represent common mechanisms for lumen opening.

Luminal fluid secretion depends on polarized trafficking of specific ion transporters to the apical and basolateral membranes, where their coordinated function establishes electrochemical and osmotic gradients that drive fluid secretion. Misregulated fluid secretion leads to defects in organogenesis and organ function. Diseases such as cystic fibrosis are characterized by loss of fluid secretion in many organs, while secretory diarrheas and polycystic kidney disease occur due to excessive fluid secretion.

Fluid secretion is necessary for lumen formation in many tubular organs. To secrete fluid, apical and basolateral ion channels coordinate to regulate electrochemical and osmotic gradients that ultimately drive fluid secretion. A classic example of fluid secretion occurs in the mammalian intestine where ion gradients are powered by the basolaterally sorted Na^+/K^+-ATPase, which uses energy from ATP to import potassium ions and secretes sodium ions, generating an extracellular sodium gradient (Fig. 3.1b). The concentration of sodium ions outside the cell is used to import chloride ions into the cell. The chloride ions are then secreted through apical anion channels, which generates a luminal electrochemical gradient that draws sodium into the lumen through intracellular or paracellular cation channels. These movements generate an osmotic gradient that transports fluid into a lumen (Barrett and Keely 2000).

In the zebrafish gut, fluid secretion drives single lumen formation (Fig. 3.1c). During cord hollowing in the zebrafish gut, multiple lumens coalesce into a single, central lumen (Bagnat et al. 2007). Luminal fluid pressure participates in the resolution of these lumens during single lumen formation. When the Na^+/K^+-ATPase is inhibited during gut morphogenesis, lumen coalescence fails and multiple lumens are observed throughout the gut (Bagnat et al. 2007).

Hydrostatic fluid pressure is also required for lumen expansion during organogenesis. A clear example of lumen expansion due to fluid secretion comes from the zebrafish Kupffer's vesicle (KV), a fluid-filled organ necessary for the specification of left-right asymmetry (Fig. 3.1c). An important regulator of vertebrate fluid secretion is the chloride channel, Cftr (Anderson et al. 1991). In KV, loss of Cftr

function specifically disrupts fluid accumulation and prevents expansion of the lumen (Navis et al. 2013). In *cftr* mutants, the epithelium in KV is and polarized properly specified, including the formation of cilia necessary for KV function, but the lumen is uninflated. The lack of luminal fluid prevents the cilia from beating and disrupts specification of left-right asymmetry (Navis et al. 2013).

A similar requirement for fluid-driven lumen expansion is observed during ventricle formation in the zebrafish brain. The zebrafish brain forms a central lumen through cord hollowing, followed by filling of the ventricles with fluid (Lowery and Sive 2005). Fish lacking Na^+/K^+-ATPase activity lose expansion of the ventricular lumen but have otherwise normal neuroepithelial cells and an uninflated but continuous single lumen. Additionally, in the ascidian notochord, fluid secretion is important for expansion of the lumen and to provide a hydrostatic support structure for the larval body (Fig. 3.1d). Fluid secretion in the *Ciona* notochord is driven in part by an Slc26 family chloride/bicarbonate exchanger, which likely regulates osmotic gradients and luminal pH (Deng et al. 2013). A coordinated network of ion transporters governs the osmotic gradients that drive fluid secretion and lumen expansion in diverse organs.

Regulated apical fluid secretion is also necessary for proper lumen formation and organ function. In the zebrafish *baobab* mutant, which encodes Cse1l, fluid secretion in the gut becomes misregulated leading to dramatic expansion of the lumen (Bagnat et al. 2010). Loss of Cse1l function leads to increased Cftr activity. Unregulated chloride secretion drives excessive fluid accumulation, leading to massive expansion of the gut lumen and cell stretching similar to secretory diarrheas.

Work in a number of diverse epithelia indicates that the establishment of a tight barrier is crucial to regulate the movement of water and ions that direct the accumulation of fluid in a wide variety of organs. Claudins are instrumental regulators of barrier function and direct the permeability of the barrier (Furuse et al. 2002; Van Itallie et al. 2001). Without claudin function to regulate paracellular ion flow, the epithelial cells are unable to properly regulate lumen expansion.

The electrochemical gradients that drive fluid secretion rely on the formation of an epithelial barrier to regulate the paracellular flow of water and ions. Barrier function is modulated by insertion of tight or leaky adhesion molecules that either restrict or allow the flow of molecules between cells. Claudin family adhesion molecules regulate barrier function and permeability (Furuse et al. 2002). In the mammalian kidney, Claudin 4 and 8 interact to allow the movement of chloride ions across the barrier (Hou et al. 2010). Loss of Claudin 4 leads to excessive accumulation of water due to a failure to reabsorb chloride ions (Fujita et al. 2012). Similarly, Claudin 2 is required for absorption of sodium ions, which also helps promote absorption of water within the renal system (Muto et al. 2010). Loss of Claudin 2 also causes kidney cyst formation in the mouse since loss of ion absorption leads to excessive fluid accumulation within the kidney.

In the zebrafish gut, Claudin 15 is required for fluid secretion during single lumen formation. Loss of Claudin 15-dependent fluid secretion leads to multiple

lumens in the gut (Bagnat et al. 2007). Claudin function is also known to be required in the developing zebrafish brain. Loss of *claudin 5a* causes lumen expansion to fail due to an inability to form a tight paracellular barrier and properly regulate the movement of ions necessary for expansion of the ventricular lumen (Zhang et al. 2010).

Claudins are also responsible for the expansion of the earliest example of a mammalian lumen. Very early in mammalian development, the embryo forms a blastocyst with a fluid-filled lumen prior to implantation. The fluid in this lumen is regulated by Claudin 4 and Claudin 6, which are necessary for barrier function and regulation of fluid accumulation (Moriwaki et al. 2007). Without a proper barrier, the blastocyst lumen fails to inflate and the embryo is incapable of implantation.

The movement of water is driven by osmotic gradients, but transepithelial water transport is also facilitated by aquaporins. In the *C. elegans* excretory canal, aquaporin 8 (AQP-8) is required for fluid secretion to elongate the canal lumen (Khan et al. 2013; Kolotuev et al. 2013). The channel is recruited to the luminal membrane by the apically polarized ERM-1 where it mediates the transport of water into the lumen. Lacking AQP-8 activity, due to either loss of AQP-8 expression or treatment with mercury, an aquaporin inhibitor, the lumen fails to expand as water is not properly secreted into the lumen (Khan et al. 2013). Fluid secretion also regulates the formation of the *C. elegans* excretory canal. In the excretory canal, pros-1, an orthologue of Prox-1, regulates osmotic gradients that control fluid secretion (Kolotuev et al. 2013). Worms mutant for pros-1 fail to sufficiently inflate the excretory canal lumen. These studies point to conserved roles for fluid secretion during lumen morphogenesis throughout the animal kingdom.

3.9 Matrix Secretion Driving Lumen Formation and Expansion

In invertebrates, the expansion of luminal spaces is commonly mediated by apical secretion of matrix proteins, which generate an outward force on the epithelium. Polarized matrix secretion has been instrumental in identifying regulators of the secretory pathway and septate junction assembly. Lumen expansion through apically polarized secretion of matrix proteins has been best studied in the *Drosophila* trachea, hindgut, and salivary gland; however, it is unclear whether matrix secretion is a conserved mechanism for lumen expansion in vertebrates or an invertebrate-specific strategy for tube expansion. While vertebrates do not secrete chitin, many lumens secrete charged proteoglycans, such as podocalyxin, during the initial stages of lumen opening. Nonetheless, secretion of luminal matrix in invertebrates provides fundamental lessons underlying vertebrate lumen morphogenesis and cell polarity. A more in-depth discussion focused on invertebrate luminal secretion can be found in a recent review of luminal matrix (Luschnig and Uv 2013).

Expansion of the *Drosophila* trachea is regulated by secretion of chitin matrix into the lumen. Chitin proteins are regulated by apical secretion of two chitin deacetylases, Serp and Verm, that modify the chitin to generate a more rigid structure. This change to the chitin matrix limits the diameter of the tracheal lumen and promotes its extension (Luschnig et al. 2006; Wang et al. 2006). Recently, the activity of Serp and Verm was shown to function in balance with another class of chitin-binding proteins, Gasp and Obst-A (Tiklová et al. 2013), which also regulate lumen diameter and length. These proteins are necessary for the dilation of the tracheal lumen and for the assembly of the chitin matrix in the lumen.

In the *Drosophila* trachea, several claudin family proteins are required for the apical secretion of luminal components. The claudins Megatrachea, Sinuous, and Kune-kune are each required for the formation of septate junctions and secretion of the chitin deacetylases that regulate the expansion of the luminal matrix (Behr et al. 2003; Nelson et al. 2010). Loss of these claudins inhibits secretion of *serp* and *verm*, leading to the characteristic expansion of the *Drosophila* trachea associated with these mutants. Similarly, the Na^+/K^+-ATPase regulates septate junction assembly independently of its well-characterized ion transport activity (Paul et al. 2003, 2007). Mutants for the Na^+/K^+-ATPase disrupt secretion of *serp* and *verm* into the tracheal lumen leading to its expansion.

Luminal matrix can also drive the expansion of lumens in the fly gut and eye. The O-glycosylated protein Tenectin (Tnc) is secreted by the *Drosophila* hindgut epithelium to expand the lumen (Syed et al. 2012). Spatially regulated Tnc secretion mediates local changes in the diameter of the hindgut. Similarly, during the formation of the *Drosophila* eye, the retina forms many ommatidia, which will become the photoreceptive unit of the eye. As the retina develops, the *Drosophila* ommatidium forms a small lumen termed the intrarhabdomeral space (IRS). To open this lumen, the ommatidial cells secrete a unique glycoprotein, Eyes shut, into the IRS (Husain et al. 2006). Mutants for *eyes shut* fail to expand their IRS, which is necessary for *Drosophila* vision.

3.10 Divide and Expand: Asymmetric Cell Division During Lumen Formation

The formation and maintenance of a single lumen also depend on properly oriented cell divisions. During mitosis, cells must properly orient their division to segregate their contents and preserve barrier function of the epithelium. The direction of division is specified by the orientation of the mitotic spindle poles and often proceeds in two different axes. Symmetric division occurs in the plane of the epithelium equally distributing cellular contents of the parent cells into the daughter cells, while an asymmetric division is often perpendicular to the plane of the epithelium and results in an uneven segregation of intracellular contents. Asymmetric divisions are often involved in the differentiation of one cell type into

another, whereas symmetric divisions typically promote the maintenance of cell fate.

Polarized signaling also regulates spindle orientation, which is necessary for epithelial maintenance. In the mammalian kidney, a receptor tyrosine kinase, ErbB4, regulates epithelial polarity, spindle orientation, and proliferation. In ErbB4 mutants, the spindle orientation and proliferation defects combine to generate a smaller lumen, while overexpression of ErbB4 leads to expansion of the kidney lumen and development of accessory cysts, likely due to misoriented cell divisions (Veikkolainen et al. 2012).

Cell polarity and spindle orientation are important during the development of the mammalian lung. Properly oriented cell divisions, regulated by Cdc42, are necessary for branching morphogenesis. Loss of Cdc42 leads to disorganized cell division and disrupts lung patterning (Wan et al. 2013). Cell divisions can also be regulated along the longitudinal and transverse axes within a tubular epithelium to promote extension of a branch. In the mouse lung, Ras signaling helps to properly orient cell division. Loss of function of the extracellular regulated kinases, ERK1 and ERK2, leads to defects in the extension of lung branches (Tang et al. 2011). In mutant lungs, spindle orientation was perturbed leading to misoriented cell divisions and a thickened epithelium.

Lumen resolution within the zebrafish neural tube depends on a specialized type of cell polarity. The cells of the neural tube initially span the width of the tube and then generate apical membrane containing the polarization marker, Par3, at the midline of the cell and neural tube (Tawk et al. 2007). The cell then executes a mirror-symmetric division generating two cells with opposing apical membranes. To properly establish the plate of apical membrane at the midline, the cells utilize Rab11a-dependent recycling and a specialized microtubule network to direct the localization of apical determinants (Buckley et al. 2013). Another regulator of cell polarity, *pard6γb*, regulates oriented cell divisions in the zebrafish neural tube (Munson et al. 2008). In fish mutant for *pard6γb*, aberrant cell divisions cause defects in the neural tube leading to the formation of multiple lumens within the epithelium. Similarly, the zebrafish aPKC mutant, *heart and soul* (*has*), causes defects in spindle orientation in a variety of epithelial tissues and leads to the formation of multiple lumens within the gut (Horne-Badovinac et al. 2001).

Studies in mammals and zebrafish highlight that properly oriented cell divisions are crucial for the development and maintenance of polarized epithelia surrounding a lumen. The central polarity determinants regulate the orientation of cell divisions and influence the differentiation and organization of cells within the epithelium. Disrupting the plane of cell division causes the formation of multiple lumens or leads to changes in the thickness of the epithelium.

3.11 Closing Thoughts and Perspectives

Although the cellular processes generating a lumen are often distinct between organs, common physiological processes coordinate their morphogenesis. Central to the establishment of a functional lumen is the generation and maintenance of epithelial polarity. The complexes that regulate the apical and basolateral membranes are highly conserved and regulate cell polarity in many epithelia. These complexes govern downstream physiological processes including sorting, trafficking, adhesion, and the cytoskeleton. The precise coordination of these fundamental regulators of cell physiology determines the morphogenetic events leading to lumen formation.

To better understand lumen formation in vivo, it will be important to address several outstanding questions. Interaction with the ECM is integral for the orientation of cell polarity, but the mechanisms translating signals from the ECM to the establishment of cell polarity in vivo are poorly understood. Investigating the mechanisms that regulate cell polarity during organogenesis will be important to better understand lumen formation and disease in tubular organs. Apical secretion is an important process during many types of lumen formation, but the mechanisms that direct its targeting in vivo are also largely unknown. Investigating the roles of apical secretion in vivo will provide a better understanding of the processes that regulate lumen formation.

Several basolateral proteins perform integral functions during lumen formation, such as the Na^+/K^+-ATPase and cadherins. The processes that regulate sorting and trafficking of these proteins to the basolateral membrane have been extensively investigated in vitro for various cargoes (e.g., VSVG, transferrin receptor, LDL receptor), but it remains unclear whether the same processes and machinery also function in vivo during lumen formation, particularly for the Na^+/K^+-ATPase and cadherins. Similarly, N- and O-glycosylation are well known to regulate sorting of apical proteins (Schuck and Simons 2004), but evidence demonstrating a requirement for apical sorting of glycans during lumen formation in vivo is still lacking. Translating discoveries of processes that regulate membrane polarity and lumen formation from in vitro to in vivo models will be necessary to better understand organ development and disease.

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Part II
Asymmetric Cell Division and Stem Cells

Chapter 4
Asymmetric Cell Division and Development of the Central Nervous System in *Drosophila*

Manu D. Tiwari and Andreas Wodarz

Abstract Asymmetric cell division is the fundamental mechanism through which stem cells generate distinct types of cells. The central nervous system of *Drosophila* develops by asymmetric divisions of neural stem cells called neuroblasts. In this chapter, we delineate the chief molecular factors that enact the asymmetric division of neuroblasts. We discuss the events leading to establishment of cortical polarity and its interdependence with spindle asymmetry. We highlight cases where aberrations in these processes can derail the normal developmental program and cause tumors, and also examine future prospects for the field.

Keywords Asymmetric cell division • Cell polarity • Drosophila neurogenesis • Neuroblast • Spindle alignment

The mammalian central nervous system (CNS) is the most complex organ of the body and is typified by the existence of diverse, and distinct, neuronal and glial subtypes. Its development rests on a finite set of ectodermally derived neural precursors, the neural stem cells (NSCs), which divide asymmetrically to self-renew and produce terminally differentiated progeny. The fundamental tenet in generation of these differently fated daughter cells is the selective apportioning of molecular information during cytokinesis (Fig. 4.1) (Horvitz and Herskowitz 1992). Although seemingly unrelated, this mode of NSC division and CNS development exhibits remarkable parallels between vertebrates and insects, specifically mice and flies (Wodarz and Huttner 2003). For instance, asymmetric divisions in both are driven by an almost identical set of molecular factors that are regulated by similar mechanisms. Furthermore, the availability of various genetic engineering methods and the ability to induce transplantable malignant neoplasms translate *Drosophila* into an attractive

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K. Ebnet (ed.), *Cell Polarity 2*, DOI 10.1007/978-3-319-14466-5_4

system for studying neural tumors. In this chapter, we focus on the development of the *Drosophila* CNS with special emphasis on asymmetric cell division.

4.1 An Overview of *Drosophila* CNS Development

4.1.1 Embryonic NBs Generate the Larval CNS

The ganglionic CNS of *Drosophila* is a segmented organ that comprises of the brain and the ventral nerve cord. Its biphasic development begins in the embryo through spatiotemporally phased asymmetric divisions of NSCs, called neuroblasts (NBs), which delaminate from grid-like domains of neuroectodermal cells with distinct gene expression termed neural equivalence groups (Bossing et al. 1996; Schmidt et al. 1997). NB specification within a neural equivalence group is driven by high proneural gene (*achaete* (*ac*), *scute* (*sc*), and *lethal of scute* (*l'sc*)) activity, coupled with low neurogenic (Notch) signaling (Villares and Cabrera 1987; Vässin et al. 1987; Johansen et al. 1989; Cabrera 1990; Skeath and Carroll 1992). Following delamination, the NB divides in a typical asymmetric mode to self-renew and generate a progenitor cell, the ganglion mother cell (GMC), which divides once to produce two terminally differentiated progeny, neuron(s) and/or glia (Hartenstein and Wodarz 2013). Altogether, NB asymmetric divisions produce the larval CNS neuropil comprising of distinct interneurons (~90 %), motoneurons (~5 %), and glia (~5 %) by embryonic stage 14. This signals the end of embryonic neurogenesis and is marked by segment-specific cessation of NB activity.

NB fate post-cessation involves a choice between cell death or dormancy and depends on its location. NB apoptosis is driven by the expression of RGH locus genes, particularly *reaper* (*rpr*) and *grim* (*grm*) (White et al. 1994; Peterson et al. 2002; Tan et al. 2011). Surviving NBs in each segment enter a mitotically dormant phase termed quiescence, and this appears to depend upon the frequency of NB division and reduction in the nucleocytoplasmic ratio below a critical level (Edgar et al. 1986; Hartenstein et al. 1987). These, in turn, hinge on cooperative interactions between the Hox genes, the Nab transcription cofactor, and cell-intrinsic mechanisms enacted by the sequential expression of a series of transcription factors in the consecutive order: *hunchback (hb)* → *seven up (Svp)* → *krüppel (kr)* → *POU homeodomain protein 1* (*Pdm1*, *nubbin)* → *castor (cas)* (Brody and Odenwald 2000; Isshiki et al. 2001; Kanai et al. 2005; Tsuji et al. 2008). Although not all NBs express the complete complement of these factors, it seems plausible that they might leave the cell cycle and transit into quiescence at specific stages of the temporal series.

4.1.2 Quiescent NBs Reproliferate During the Larval Stage

The link between embryonic and larval phases of neurogenesis is provided by quiescent NBs that enlarge and resume proliferation as postembryonic NBs

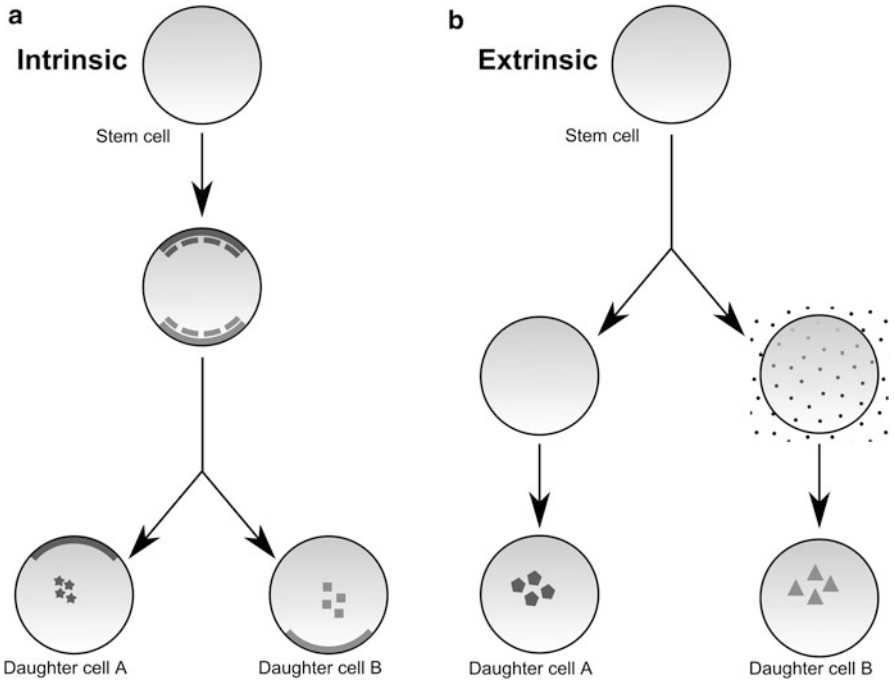

Fig. 4.1 Mechanisms of asymmetric stem cell division. In intrinsic mechanism (**a**), molecular factors called cell fate determinants induce polarity in the parent stem cell, which divides to generate differently fated daughter cells; this mechanism governs the division of *Drosophila* neuroblasts. In extrinsic mechanism (**b**), division of the stem cell produces two equivalent daughter cells at birth that acquire distinct fates as a consequence of different environmental cues; the germline stem cells in *Drosophila* divide via this mechanism

(pNBs) in response to several extrinsic inputs during the first larval instar (Prokop and Technau 1991; Britton and Edgar 1998). Under amenable nutrient conditions, a fat body-derived systemic mitogen induces surface glia to produce insulin-like peptides that bind insulin-like receptors on quiescent NBs; this causes activation of the insulin, phosphatidylinositol 3-kinase (PI3K), and target of rapamycin (TOR) signaling pathways (Chell and Brand 2010; Sousa-Nunes et al. 2011). Secondly, the steroid hormone ecdysone (20-hydroxyecdysone) stimulates reactivation of older NBs in non-starved larvae and increases the rate of NB cell cycle, but not the frequency, in CNS explants (Truman et al. 1994; Datta 1999). Additionally, NB reactivation also appears to be modulated by the fragile X mental retardation protein (FMRP) that is expressed first in the NBs, followed by glia, and acts by regulating the insulin signaling pathway (Callan et al. 2012).

The final signal that coaxes quiescent NBs into active mode is terribly reduced optic lobes (trol; perlecan in mammals) that binds several growth factors and liberates NBs from anachronism (Ana, secreted by the surface glia)-induced cell cycle arrest (Ebens et al. 1993; Voigt et al. 2002; Lindner et al. 2007). Glia-derived jelly belly (Jeb) acts as a ligand for anaplastic lymphoma kinase and can potentiate

NB proliferation even under nutrient-deprived conditions (Cheng et al. 2011). Given their restrictive as well as permissive influences on NB reactivation and proliferation, surface glia have been surmised to represent the larval pNB niche, although not a conventional one since cultured pNBs also express the temporal series, divide asymmetrically, and generate differentiated progeny (Grosskortenhaus et al. 2005; Ceron et al. 2006; Chai et al. 2012)

4.1.3 Asymmetric Division of pNBs Generates the Adult CNS

Reactivated pNBs propagate in different ways to sculpt the larval CNS into the adult CNS. Based on their location, proliferative capacity, division mode, and marker expression, several types of pNBs have been identified. The most populous, and closest in relation to embryonic NBs, are type I pNBs that divide, albeit without shrinking, in a typical asymmetric fashion to generate the majority of neuronal progeny (Fig. 4.2). Type I pNBs are Deadpan $(Dpn)^{+}$ Asense $(Ase)^{+}$ Prospero $(Pros)^{cytoplasmic+}$ and are located throughout the larval CNS but exclusively in the dorsoanterior lateral region of the central brain (Boone and Doe 2008).

Interspersed within the dorsoposterior and medioposterior regions of a central brain hemisphere are eight type II Dpn^{+} Ase^{-} $Pros^{-}$ pNBs which produce arborizing neurons of the central complex and also act as neuroglioblasts to generate glia (Fig. 4.2) (Bello et al. 2008; Boone and Doe 2008). In each such division, a type II pNB generates a self-renewing Dpn^{+} Ase^{+} $Pros^{cytoplasmic+}$ intermediate neural progenitor (INP) that again divides asymmetrically multiple times to produce

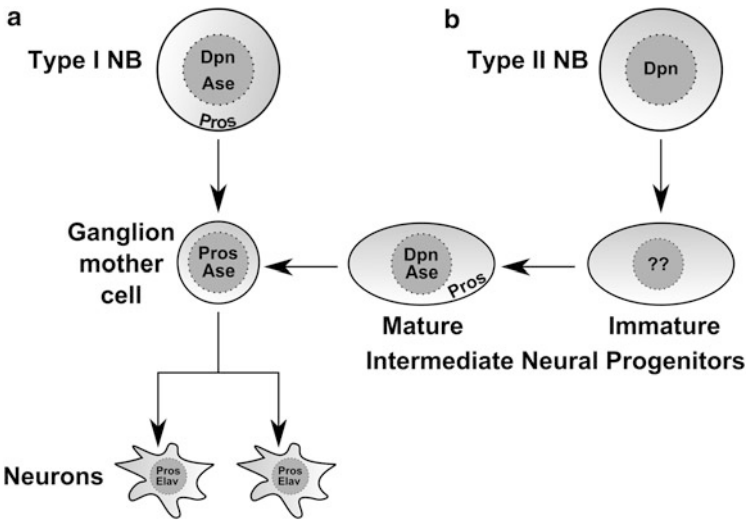

Fig. 4.2 Progeny and molecular markers of type I (**a**) and type II (**b**) neuroblasts. *Ase* Asense, *Dpn* Deadpan, *Elav* embryonic lethal abnormal vision, *Pros* Prospero

GMCs. Correspondingly, the extent of progeny clones generated by type II pNBs is far more than those by type I pNBs. In addition to type I and type II NBs, the larval CNS also harbors mushroom body pNBs that divide asymmetrically to produce collections of intrinsic neurons called Kenyon cells and optic lobe pNBs that generate the medulla and lamina neurons (Ito et al. 1997; Egger et al. 2007; Kunz et al. 2012).

Most pNBs populate the CNS with distinct neuronal progeny under directions from a putative postembryonic temporal series. Thoracic Cas^+ NBs retain their temporal memory upon reactivation as pNBs and follow it up with an asynchronous wave of Svp expression. Both these transcription factors result in deep-layer neurons that express the BTB zinc finger protein, Chinmo, and smaller, superficially located neurons that express Broad Complex (Br-C) (Maurange et al. 2008). Interestingly, a majority of INPs also express three transcription factors in the sequential order *dichaete* (*d*) → *grainyhead* (*grh*) → *eyeless (ey)* to generate distinct neuronal progeny over respective expression windows (Bayraktar and Doe 2013). D and Grh are necessary, although not exclusively, for generating Brain-specific homeobox$^+$ (Bsh) neurons. Ey, on the other hand, is required for specifying later-born neurons and glia that become part of the central complex.

A few obligatory and gratuitous molecular factors that contribute to NB character have been delineated. The Snail family transcription factor Worniu (Wor) promotes NB cell cycle progression, cortical polarity, and survival; *wor* mutant NBs undergo precocious maturation due to heightened activity of the neuronal marker, embryonic lethal abnormal vision (ELAV) (Lai et al. 2012). Additionally, the zinc-finger transcription factor klumpfuss (klu; Wilms tumor 1 in mammals) also aids in maintaining NB identity since its loss leads to premature maturation, overexpression causes transplantable tumors, and misexpression in INPs reverts them into type II NBs (Xiao et al. 2012; Berger et al. 2012). Type II NB identity is contingent upon Dpn that appears to function independently of Notch; *dpn* mutant type II NBs morph into Ase^+ $Pros^+$ type I NBs before undergoing Pros-induced premature terminal divisions (Zhu et al. 2012). Also, PntP1, a constitutively active isoform of the E26 transformation-specific (ETS) family transcription factor Pointed (Pnt), suppresses Ase in type II NBs and is necessary as well as sufficient for INP formation (Zhu et al. 2011).

4.1.4 pNBs Cease Divisions After Formation of the Adult CNS

The final event in the life of a NB involves termination of its activity after requisite progeny clones have been generated. Transient expression of Cas in the late embryo results in repression of D and upregulation of Grh that ingrains a temporal series-responsive D^-Grh^+ state in pNBs (Almeida and Bray 2005). Type I $D^-Grh^+Cas^-$ pNBs terminate proliferation by expressing the nuclear cell fate determinant Pros, which induces cell cycle exit (Maurange et al. 2008). Type II $D^-Grh^+Cas^-$ pNBs,

on the other hand, undergo RHG-dependent apoptosis, which occurs earlier in the abdominal segments due to a burst of AbdA (Cenci and Gould 2005). Altogether, fueled by the asymmetric divisions of NBs, neurogenesis in the wild-type fly completes before eclosion, and the adult does not harbor any known NBs; even the transplantation of larval brain tissue into adult flies does not elicit any neurogenic response. Intriguingly, however, abrogating the activity of Foxo, together with RHG family genes, spares NBs from apoptosis, and neurogenesis can be detected in the adult mushroom body (Siegrist et al. 2010). Furthermore, the medulla cortex of optic lobes contains slowly dividing Dpn^+ progenitors, presumably adult NBs, which can be stimulated by injury-induced dMyc (Fernández-Hernández et al. 2013).

4.2 Asymmetric Division of Drosophila NBs

4.2.1 Drosophila NBs Divide Asymmetrically via an Intrinsic Mechanism

The asymmetric division of *Drosophila* NBs is largely driven by an intrinsic mechanism as they can self-renew and reproducibly generate progeny clones in culture (Ceron et al. 2006; Lüer and Technau 2009). Except for the lack of any defined orientation of the pNBs with respect to an external or organismal axis, most *Drosophila* NBs divide asymmetrically based on an underlying theme. It involves a sequence of four well-orchestrated steps: establishment of a polarity axis in the parent NB, proper positioning of the mitotic spindle with reference to the polarity axis to ensure asymmetric cleavage, localization of molecular factors called fate determinants to specific locations within the parent NB, and cytokinesis to produce differently fated daughter cells.

4.2.2 Several Proteins Partition to Distinct Regions of the NB to Induce Polarity

Drosophila NBs acquire polarity as a consequence of the differential localization of distinct molecular factors at their apical and basal cortical domains. The symmetry-breaking events that generate NB polarity are dynamic in nature since these cortical domains disappear following cytokinesis and reappear prior to the onset of next mitosis. Apicobasal polarity in embryonic NBs is established during delamination when they inherit the partitioning defective (Par) complex through a transient apical stalk from the neuroectoderm. pNBs, on the other hand, remain unpolarized during most of the interphase, and Par complex localization to the apical cortex becomes evident only during late G2/early prophase.

4.2.3 *Two Distinct, But Linked, Molecular Complexes Adorn the Apical Cortex of NBs*

The evolutionarily conserved Par complex comprises of the *Drosophila* homologue of atypical protein kinase C (aPKC), the PDZ domain scaffolding protein Bazooka (Baz; Par3 in mammals), and the semi-CRIB and PDZ domain protein Par6 (Fig. 4.3) (Kuchinke et al 1998; Wodarz et al. 1999, 2000; Kaltschmidt et al. 2000; Petronczki and Knoblich 2001). Apical assembly of the Par complex is maintained, but not initiated, by Cdc2/B-type cyclin complexes since diminishing Cdc2 activity causes defects in asymmetric division (Tio et al. 2001). Another important cell cycle factor implicated in Par complex assembly is Cdc42 which drives aPKC and Par6 localization to the apical cortex in a Baz-dependent manner (Atwood et al. 2007). Baz appears to be the cornerstone of apical polarity as its localization is unaffected in *cdc42*, *par6*, or *aPKC* mutants, while Par6 and aPKC are cytoplasmic in *baz* mutants (Rolls et al., 2003).

The second evolutionarily conserved molecular complex that localizes apically in NBs exhibits receptor-independent heterotrimeric G-protein activity. The active entity in this tripartite complex is Gαi-GDP that is generated from Gαi-GTP by the action of non-receptor guanine nucleotide exchange factor for Gαi, Ric-8 (Hampoelz et al. 2005; Wang et al. 2005). Gαi-GDP activates and recruits the

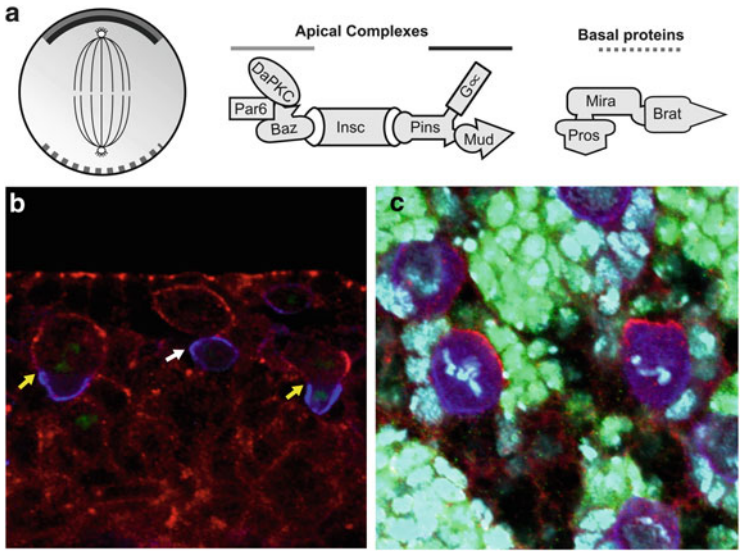

Fig. 4.3 Apical and basal protein complexes in *Drosophila* neuroblasts. Several proteins adorn the apical and basal cortical domains of *Drosophila* neuroblasts as shown in the cartoon in (**a**). Immunofluorescence staining of embryonic (**b**) and larval (**c**) neuroblasts shows localization of Baz (*red*) and Mira (*blue*) to the apical and basal cortices, respectively. *Yellow arrows* in (**b**) indicate dividing neuroblasts, and white arrow shows a ganglion mother cell after neuroblast division; neurons are indicated by Elav (*green*) and DNA by DAPI (*cyan*) in (**c**)

tetratricopeptide (TPR, 34-amino acid repeats that contain Leu-Gly-Asn)-GoLoco protein Partner of Inscuteable (Pins; LGN-AGS3 in mammals), which enables it to bind the coiled-coil microtubule-associated dynein-binding protein, mushroom body defective (Mud; NuMA in mammals) (Schaefer et al. 2000; Siegrist and Doe 2005; Bowman et al. 2006; Izumi et al. 2006; Siller et al. 2006; Nipper et al. 2007). Both the apical complexes appear to act downstream of the other subunit of heterotrimeric G-protein signaling, Gβ13F, as abrogation of its activity results in a phenotype similar to when both the apical complexes have been disrupted (Fuse et al. 2003). Gβ13F also restricts Gα to the apical domain which then aids in priming the NB for asymmetric division (Wang et al. 2005)

The two apical complexes are linked by the cytoskeletal adaptor protein Inscuteable (Insc), that interacts simultaneously with Baz and Pins through its ankyrin-like repeats and also localizes apically in presumptive NBs during late interphase (Schober et al. 1999; Wodarz et al. 1999; Schaefer et al. 2000). *insc* mutant NBs display defects in cell polarity as well as spindle orientation highlighting its importance in both these processes (Kraut et al. 1996; Siegrist and Doe 2005). Its misexpression in epithelial cells results in asymmetric division due to realignment of the mitotic spindle along the apicobasal axis. Insc recruitment to the apical cortex is contingent upon: (a) Baz - embryos without maternal and zygotic Baz exhibit a uniform cytoplasmic distribution of Insc and misoriented mitotic spindles (Kraut and Campos-Ortega 1996; Schober et al. 1999; Wodarz et al. 1999), and (b) microfilaments along with microtubules - their chemical inhibition also renders it cytoplasmic (Broadus and Doe 1997). Facilitated by Insc, the two apical complexes direct localization of cell fate determinants to the basal cortex of NBs.

4.2.4 Cell Fate Determinants Localize at the Basal Cortex of NBs

During late prometaphase, NBs start exhibiting basal accumulation of three proteins, collectively termed cell fate determinants, which partition into the GMC upon completion of division (Fig. 4.3). The phosphotyrosine-binding domain protein Numb coaxes GMCs into maturation by abrogating Notch signaling via alpha-adaptin-mediated endocytosis of the Notch receptor (Berdnik et al. 2002). First identified in sensory organ precursors, it localizes asymmetrically to the basal cortex through its N-terminus in an actin-dependent manner (Uemura et al. 1989; Knoblich et al. 1995, 1997). The Polo kinase-phosphorylated form of the adaptor protein Partner of Numb (Pon) also aids in this process, although its requirement is dispensable (Lu et al. 1998).

The second protein that acts as a cell fate determinant and partitions basally in NBs is the homeodomain transcription factor Pros (Prox1 in mammals) (Hirata et al. 1995; Knoblich et al. 1995). Within the NB, cyclin E contributes to tether

cytoplasmic Pros to the cortex and to inhibit its function (Berger et al. 2010). Upon completion of NB division, Pros segregates into the GMC where it acts as a binary switch that shuts off NB fate genes (*ac*, *aPKC*, *asense*, *baz*, *dpn*, *grh*, *hb*, *insc*, *kr*, *pdm*, *sc*, *snail*) and cell cycle genes (*cyclin E*, *string*, and *E2F*), and simultaneously upregulates maturation-promoting genes (*bangles and beads*, *even skipped*, *fushi tarazu*, *gilgamesh*, *glial cells missing*) (Li and Vaessin 2000; Choksi et al. 2006). Within the differentiating GMCs, Pros provides a transient signal for governing their cell cycle exit by upregulating expression of the cyclin-dependent kinase inhibitor Dacapo (Colonques et al. 2011).

Correspondingly, *pros* mutant daughter cells continue exhibiting NB markers and do not differentiate. Interestingly, *pros* mutant type II NBs correctly specify INPs but exhibit GMC tumors, as compared to *numb* mutants that exhibit NB tumors (Bowman et al. 2008). Forced misexpression of *pros* in type II NBs blocks their proliferation but does not transform them into type I NBs (Bayraktar et al. 2010). In INPs, the evolutionarily conserved transcription factor dFezf/Earmuff (Erm; Fezf in mammals) activates Pros and antagonizes Notch to coax them into maturation (Weng et al. 2010); *erm* mutant INPs exhibit normal cortical polarity but dedifferentiate into type II NBs.

The third cell fate determinant that accumulates basally is the NHL domain translation repressor brain tumor (Brat; TRIM32 in mammals), which inhibits the oncogenic dMyc posttranscriptionally (Sonoda and Wharton 2001; Betschinger et al. 2006). Brat suppresses NB self-renewal and promotes maturation: type I *brat* mutant NBs exhibit defective Pros partitioning into the GMC, leading to supernumerary NBs; similarly, type II *brat* mutant NBs fail to correctly specify INPs and exhibit tumor-like overgrowth (Lee et al. 2006c; Bello et al. 2006; Bowman et al. 2008). Along with Numb, Brat segregates into and promotes the maturation of type II NBs into INPs. Furthermore, inhibition of Klu activity in *brat* mutants, or in flies with constitutively active N signaling, blocks the reversion of INPs to type II NBs (Xiao et al. 2012).

4.2.5 Miranda Is a Vehicle for Pros and Brat

Basal segregation of Pros and Brat in type I NBs and INPs is essentially contingent upon the coiled-coil adaptor protein Miranda (Mira; no known ortholog in mammals). Mira is a scaffold protein that possesses an N-terminal domain which tethers it to the cell cortex, a central cargo-binding domain which positions it apically in interphase NBs and confers it with the ability to bind the double-stranded RNA-binding protein Staufen (Stau), Pros, *pros* mRNA, and Brat, and a C-terminal domain which aids in releasing its cargo when in the GMC (Matsuzaki et al. 1998; Schuldt et al. 1998; Shen et al. 1998; Fuerstenberg et al. 1998; Lee et al. 2006c). Although Mira also interacts with Numb in vitro, it does not appear to be involved in its asymmetric segregation. *mira* mutants exhibit uniform

cytoplasmic distribution and, consequently, equal partitioning of Pros and Brat among daughter cells.

Mira exhibits a dynamic apical-to-basal translocation within the NB. Its apical positioning is aided through binding to Insc; this interaction, however, is not obligatory since *insc* mutants still exhibit asymmetric Mira localization (Shen et al. 1998). Besides actomyosin, its basal translocation depends on the mitotic regulator, anaphase-promoting complex/cyclosome (APC/C), although very low levels of Mira still localize basally in *Apc*/*c* mutants (Slack et al. 2007). APC/C is an E3 ubiquitin ligase that mono-ubiquitinylates Mira at its C-terminal, which might serve as a cue for its basal transport or cortex retention. Following cytokinesis, Mira partitions into the GMC along with its cargo proteins, persists for some time, and is undetectable when Pros enters the nucleus (Ikeshima-Kataoka et al. 1997; Shen et al. 1998).

4.3 Mechanisms of Cortical Enrichment and Spindle Alignment

4.3.1 Phosphorylation and Dephosphorylation Events Effectuate Asymmetric Protein Localization

Photobleaching experiments with Numb-GFP and Pon-GFP indicate that they maintain a dynamic equilibrium with the cortex, and this cortical exchange governs their translocation from the cytoplasm to the basal cortex (Mayer et al. 2005). Tethering of Numb to the membrane appears to depend upon aPKC that inactivates it by phosphorylating at its N-terminus (Smith et al. 2007; Wirtz-Peitz et al. 2008). Additionally, Polo kinase phosphorylates Pon, and also possibly Numb, to sequester Numb at the basal cortex (Wang et al. 2007, 2009). Phosphorylated Numb regulates homeostasis in type II NB lineages and is antagonized in a non-apoptotic, non-catalytic mode by Dronc caspase (Nedd2-like caspase in mammals) (Ouyang et al. 2011).

Cell fate determinants are prevented from occupying apical locations through an exclusion mechanism enacted by several phosphorylation events. Entry of the NB into mitosis sets off a phosphorylation cascade wherein the mitotic kinase Aurora-A (Aur-A) phosphorylates Par6, thus activating aPKC that then phosphorylates and inactivates the cytoskeletal protein Lethal (2) giant larvae (Lgl), paving the way for Baz to associate with Par6 (Fig. 4.4) (Betschinger et al. 2003, 2005; Lee et al. 2006a; Wang et al. 2006b; Wirtz-Peitz et al. 2008). Aur-A acts as a tumor suppressor since its mutants exhibit cortical polarity as well as spindle alignment defects, which leads to ectopic self-renewal of NBs. *lgl* mutants fail to segregate Numb indicating that Lgl serves to modulate aPKC activity (Haenfler et al. 2012). Reducing aPKC activity in *lgl pins* double mutants reduces NB number, while localized expression of a membrane-targeted form of aPKC in NBs induces their

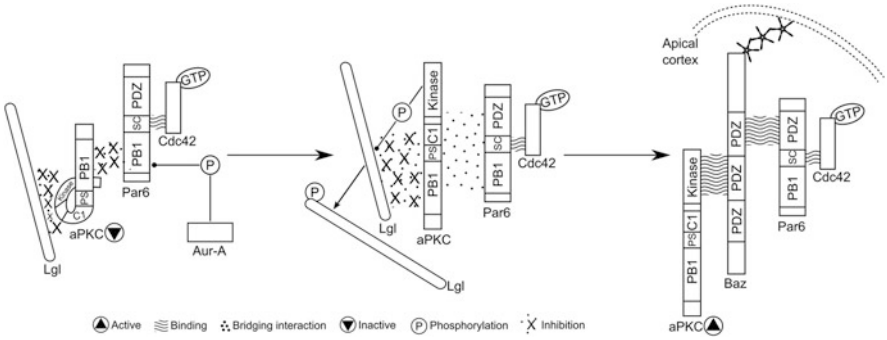

Fig. 4.4 Apical localization of the Par complex. Initially, aPKC is inactive due to auto-inhibition by its pseudosubstrate (PS) domain and inhibitory interactions with Lgl and PB1 domain of Par6. Phosphorylation of the Par6 PB1 domain by Aur-A induces a conformational change in aPKC that allows its kinase domain to phosphorylate Lgl, which then disengages from the complex. Activated aPKC and Par6 then complex with Baz, which is bound to phosphoinositides at the apical cortex through its C-terminal, thus establishing the Par complex apically. Par6 also interacts with Cdc42-GTP through its semi-CRIB (SC) domain. The modular structures of proteins show relevant interacting domains

ectopic self-renewal highlighting its necessity in correct localization of cell fate determinants and, consequently, NB self-renewal (Lee et al. 2006b).

aPKC also phosphorylates Mira whose basal localization appears to be contingent upon protein phosphatase 4 (PP4)-mediated dephosphorylation and reattachment to the cortex (Sousa-Nunes et al. 2009; Atwood and Prehoda 2009). During mitosis, membrane association of the regulatory subunit of PP4, PP4R3/Falafel (Flfl), serves to localize Mira and its cargo to the basal cortex, while during interphase/prophase, nuclear Flfl blocks Mira and Pros entry into the nucleus. Another phosphatase implicated in asymmetric protein localization is protein phosphatase 2A (PP2A) that reverses Aur-A phosphorylation via its catalytic subunit microtubule star (mts) (Chabu and Doe 2009). PP2A mutants exhibit NB hyperproliferation at the expense of differentiating neurons (Wang et al. 2009). PP2A can also dephosphorylate the Par1-phosphorylated Baz, causing an "upside-down" polarity phenotype in NBs (Krahn et al. 2009; Sousa-Nunes and Somers 2010).

The central role of aPKC in asymmetric protein localization is highlighted by the observation that its activity in daughter cells of mutants with altered spindle orientation is sufficient to induce distinct fates (Cabernard and Doe 2009). Consequently, it has been suggested that the relative ratio of apical proteins (specifically, aPKC) to the basal cell fate determinants governs cellular fate (Cabernard and Doe 2009; Knoblich 2010). aPKC, itself, is transcriptionally repressed by the zinc-finger transcription factor Zif; in NBs, however, a reciprocally repressive loop exists in which aPKC phosphorylates Zif to exclude it from the nucleus and render it inactive (Chang et al. 2010). At the protein level, aPKC appears to be activated by the apically enriched dynamin-associated protein 160 (Dap160) (Chabu and Doe 2008).

4.3.2 Phosphoinositide Signaling and TOR Pathway Are Involved in Maintaining NB Asymmetry

Phosphatidylinositol lipids mediate intracellular signaling through metabolites such as phosphatidylinositol-4,5-bis-phosphate (PIP_2) and phosphatidylinositol-3,4,5-tris-phosphate (PIP_3). Phosphorylation of PIP_2 to PIP_3 is catalyzed by the oncogenic PI3K, while the reverse reaction requires the phosphatase and tensin homolog (PTEN). Baz appears to bind to phosphoinositide membrane lipids through a conserved region in its C-terminus (Krahn et al. 2010). PTEN also colocalizes apically with Baz to establish a putative link between the Par complex and PI3K signaling (von Stein et al. 2005). Intriguingly, PI3K is involved in a cross talk with TOR signaling in NBs since diet-restricted *pins* mutant larvae, as well as rapamycin (a TOR inhibitor)-exposed *pins* mutant, or *pi3k pins* double mutant larvae, exhibit excessive growth of $Mira^+$ cells (Rossi and Gonzalez 2012).

4.3.3 Actomyosin Might Influence the Cortical Transport of Cell Fate Determinants

Studies using chemical inhibitors and genetic mutants point toward the involvement of actomyosin, and not microtubules or vesicles, in asymmetric segregation of NB proteins (Broadus and Doe 1997; Knoblich et al. 1997; Siegrist and Doe 2005; Halbsgut et al. 2011). However, a direct cause-effect relationship appears dubious since the Rho-associated protein kinase inhibitor used to inhibit non-muscle myosin II phosphorylation also impedes aPKC activity (Barros et al. 2003; Atwood and Prehoda 2009). Nevertheless, actomyosin appears to influence the intriguing case of Mira localization. Newly formed Mira localizes apically at interphase but distributes ubiquitously at prophase in a myosin II-dependent manner (Erben et al. 2008). It then passively diffuses toward the basal cortex where it entrenches through the activity of unconventional myosin VI (Jaguar, Jar) (Petritsch et al. 2003). Interestingly, the basal segregation of Pon seems to depend only upon myosin II indicating that both these adaptor proteins utilize subtly distinct actomyosin mechanisms for their asymmetric localization (Lu et al. 1999; Erben et al. 2008).

4.3.4 Accessory Pathways Coordinate Cortical Polarity with Spindle Alignment

Apart from the "classical" Par-complex-directed basal segregation of cell fate determinants that serves to link cortical polarity with CNS stratification, additional pathways exist to ensure correct polarization of the NB. The first such pathway acts during metaphase, is colcemid sensitive, and effectuates basal segregation of Numb

and Mira even in absence of the Par complex (Ohshiro et al. 2000; Peng et al. 2000). This microtubule-to-cortex pathway involves the plus-end-directed motor protein kinesin heavy chain 73 (Khc73) and the PDZ domain guanylate kinase disks large 1 (dlg1), along with Insc and Pins. Its activity seems to be directed by Insc which ensures spindle coupling to the cortex through Pins-Dlg before activation of spindle-pulling forces through Mud (inset in Fig. 4.5) (Mauser and Prehoda 2012). Embryonic *insc* NBs do not possess a functional Par complex at interphase but exhibit cortical Dlg-Pins-Gαi crescents over one of the spindle poles at metaphase (Siegrist and Doe 2005). Apparently, astral microtubules transport Khc73 at their plus ends to recruit Dlg, which then binds Pins; the complex then coordinates with Mud to ensure proper spindle alignment (Siegrist and Doe 2007).

lgl, *dlg1*, and *scribble (scr)* mutants exhibit symmetric or inverted NB divisions due to smaller apical cortices and spindles; this results in abnormally small NBs and large GMCs indicating that cortical polarity, and not cell size, plays a primal role in determining cell fate (Albertson and Doe 2003). Another pathway that operates in parallel to the microtubule-to-cortex pathway involves the evolutionarily conserved protein suppressor-of-G2-allele-of-skp1 (Sgt1), which interacts with heat shock protein 90 (Hsp90). *sgt1* mutants fail to establish apical cortical polarity at prophase

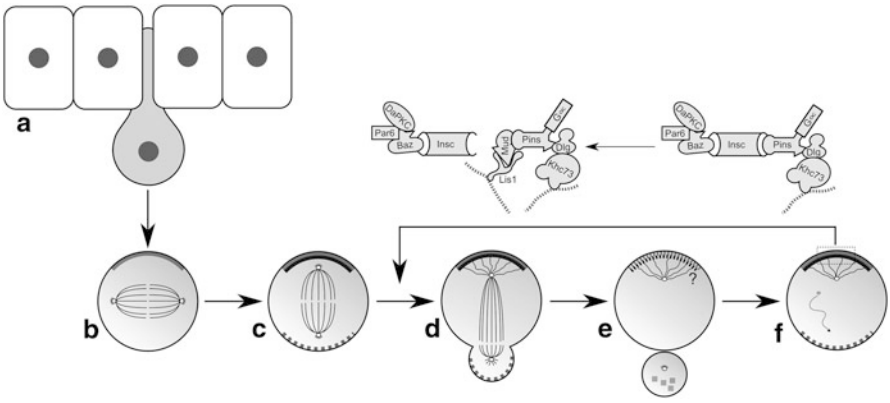

Fig. 4.5 Spindle orientation in *Drosophila* neuroblasts. The delaminating neuroblast inherits the Par complex from the neuroectoderm ((**a**); see Fig. 4.3 for apical and basal complexes). Its duplicated centrosomes organize a spindle that rotates by 90° at metaphase to align the spindle and polarity axes ((**b**) and (**c**)). During anaphase, the spindle becomes asymmetric due to differential microtubule-organizing activity of the two centrosomes (**d**); this leads to unequally sized daughter cells upon cytokinesis (**e**). The renewed neuroblast loses apical polarity cues and retains the daughter centrosome which anchors apically through as yet unknown microtubule-cortex interactions (? in (**e**)). During most of the interphase, the mother centriole of this centrosome wanders randomly (**f**) but establishes itself basally by early prophase. In subsequent divisions of embryonic neuroblasts and in all divisions of postembryonic neuroblasts, the cycle repeats to generate asymmetric progeny ((**d**) to (**f**)). *Inset* in (**f**) shows the major molecular events: Insc prevents the binding of Mud to Pins to ensure spindle attachment to the cortex through Dlg-Khc73; once this occurs, Insc disengages from the TPR domains of Pins, allowing Mud to bind to Pins. Mud then acts through Lis1 to exert pulling forces on the spindle leading to asymmetry

in larval NBs, while *sgt1* and *pins* double mutants exhibit NB tumors (Andersen et al. 2012).

Finally, a third pathway operates during anaphase/telophase to rectify mislocalized cell fate determinants. It involves the snail family of zinc-finger transcription factors—*snail (sna)*, *escargot (esg)*, *and wor*—that coordinate asymmetry through two modes. In the first mode, they regulate *insc* expression and, consequently, Baz and Pins localization, throughout mitosis. In the second, they enable partial segregation of Numb and Pros into the GMCs during telophase, independently of Insc (Cai et al. 2001). Although not completely efficient, as some *insc* NBs still exhibit loosely pieced basal crescents, this "telophase rescue" of basal cell determinants serves to amend localization errors in the absence of Insc. Additionally, telophase rescue of Pros and Mira also requires the *Drosophila* homologue of the mammalian tumor necrosis factor (TNF) receptor-associated factor (DTRAF1) and Eiger (Egr; TNF in mammals). In *insc* NBs, Egr and Baz sequester DTRAF1 apically during metaphase, which then salvages mislocalized Mira complex during telophase (Wang et al. 2006a).

Orientation of NB cortical polarity relative to the overlying neural epithelium is important for the correct stratification of the developing CNS and appears to depend on the sanctity of contacts between the NBs and the epithelium. Disruption of these contacts, as in isolated NBs, results in arbitrary "budding-off" of GMCs over consecutive cell divisions indicating that cortical polarity exists but is randomized (Siegrist and Doe 2006). The NB-epithelium interaction is regulated by the rhodopsin family orphan G-protein-coupled receptor trapped in endoderm (Tre1) (Yoshiura et al. 2012). Tre1 functions in NBs to activate the Goα subunit that recruits Pins and thus link apical polarity to spindle orientation. Surprisingly, *tre1* mutants display normal coupling and localization of the Pins-Gαi complex to Insc, reaffirming the non-cell-autonomous nature of this signal.

4.3.5 NB Spindle Reorients Along the Cortical Axis of Polarity

Establishment of NB cortical polarity paves the way for realignment of the mitotic spindle along the polarity axis such that the GMC always buds off from the same cortical section. This is achieved through an obligatory 90° rotation of the spindle apparatus in the first cell cycle that follows delamination and occurs in a two-step process that is driven by precise centrosomal positioning and spindle-cortex interactions (Fig. 4.5) (Kaltschmidt et al. 2000; Cabernard and Doe 2009; Rebollo et al. 2009). Subsequent divisions are then directed by this pre-oriented spindle setup, obviating the need for spindle rotation in each division. Chemical depolymerization of microtubules during interphase disengages the apical centrosome from the cortex and abolishes the memory of orientation of the previous spindle alignment (Siller and Doe 2009).

4.3.6 NB Mitotic Spindle Is Asymmetric

The apical centrosome serves as a reference point for polarity between successive mitotic divisions of the NB (Januschke and Gonzalez 2010). It is retained by the parent NB after division, possesses more pericentrosomal centrosomin (Cnn), and is the predominant microtubule-organizing center of the NB (Fig. 4.5). The other centrosome is smaller, devoid of pericentrosomal material, and traverses randomly throughout the cytoplasm to finally settle at the basal cortex during prophase, which results in its translocation into the GMC upon NB division (Rebollo et al. 2007; Rusan and Peifer 2007). A key factor in this process is Centrobin (Cnb), which depends on Pins and Polo kinase-mediated phosphorylation to be active, and is necessary and sufficient for a centriole to retain the pericentrosomal material (Januschke et al. 2013). Coupled with myosin II-driven cortical extension, this centrosomal asymmetry transforms into spindle asymmetry during anaphase due to displacement of the mitotic cleavage plane toward the basal side of the cell (Connell et al. 2011). Ultimately, it culminates into unequally sized daughter cells with the basal daughter (GMC) being the smaller one.

Spindle asymmetry can be effectuated by either of the apical complexes (aPKC/Par or Pins/Gαi) since it is only upon abrogation of both of them that the cell starts dividing symmetrically (Cai et al. 2003). Disruptions of individual apical complexes highlight their main roles in asymmetric division: abolishing Mud activity causes moderately increased numbers of NBs with normal cortical polarity but misaligned mitotic spindle (Siller and Doe 2008; Cabernard and Doe 2009); here, symmetric divisions result if the misalignment places the spindle orthogonally to the polarity axis. On the other hand, *aPKC* and *Par6* mutants exhibit properly aligned mitotic spindles but disorganized cortical polarity (Rolls et al. 2003).

Spindle asymmetry does not appear to be vital for spindle orientation as mutants for most centrosomal proteins such as Cnn, Asterless (Asl), or Spindle defective 2 (Spd2) exhibit normal asymmetric division (Giansanti et al. 2001; Cabernard et al. 2010; Conduit and Raff 2010). However, mutations in spindle assembly abnormal 4 (DSas4) block centrosome duplication and lead to NBs without centrosomes; these NBs exhibit reduced telophase rescue indicating dependence of this process on centrosomes (Basto et al. 2006). Furthermore, the Pins-dependent formation of a basal furrow of spindle-associated proteins such as the kinesin-like protein Pavarotti (Pav), the actin-binding protein Scraps (Scra), and the heavy chain of non-muscle myosin II Zipper (Zip) can occur even in the complete absence of a mitotic spindle (Cabernard et al. 2010; Connell et al. 2011).

Correspondingly, the common factors involved in the establishment of cortical polarity and spindle alignment do not appear to be exclusively dependent on each other. Gαi-Pins-Mud directs NB spindle orientation at metaphase possibly by engaging the dynein-dynactin complex which pulls and stabilizes one of the centrosomes beneath the apical cortex. Mud interacts with the dynein-dynactin protein Lissencephaly-1 (Lis1), which seeds dynamic rocking movements along both the spindle poles (Siller and Doe 2008). Disruption of Lis1/dynactin or astral

microtubules results in abnormal spindle alignment which is rescued by telophase suggesting that Lis1/dynactin acts early in the process to align the mitotic spindle.

Another player implicated in the correct orientation of the mitotic spindle is the PDZ protein Canoe (Cno) that colocalizes apically with Baz and interacts functionally with Pins and Mud (Speicher et al. 2008). Cno also channels signals from the Ral guanine nucleotide exchange factor Rgl, which acts via the Ras-like small guanosine triphosphatase Rap1 to orient the mitotic spindle (Carmena et al. 2011). Positioning of Mud at the apical complex and centrosome is also regulated independently of Gαi-Pins by the centriolar protein Anastral spindle 2 (Ana 2) that docks up with the dynein light-chain protein Cut up (Ctp) to recruit Mud to the centrosomes (Wang et al. 2011).

4.4 Conclusions and Perspectives

The development of *Drosophila* CNS is driven by asymmetric cell divisions of NBs. Technical breakthroughs in *Drosophila* genetic engineering and microscopy have resulted in several key advances pertaining to mechanisms of asymmetric cell division over the last couple of decades. We now know that two conserved protein complexes establish NB cortical polarity which, directly and indirectly, influences spindle alignment. Precise spindle asymmetry and alignment is required for daughter cell asymmetry and depends upon contractile and centrosomal proteins. Deficiencies in any of these processes can lead to aberrant division and, in many cases, tumor formation (Knoblich 2010; Chang et al. 2012). Nevertheless, several crucial pieces of the puzzle still remain to be deciphered. These include, but are not limited to, timing of cell cycle entry of quiescent NBs, origin of pNBs, establishment of polarity in reactivated NBs, self-renewal factors and their interactions in NBs, mutual correspondence between Notch signaling and cell fate determinants, coordination of centrosomal dynamics with polarity cues, correspondence between NB transcriptional states and identity of the neuronal progeny, and the extent of functional overlap between fly and mammalian proteins and mechanisms.

It is expected that the advent of techniques for precision genomic engineering, coupled with advances in next-generation sequencing, shall aid in better elucidation of the mechanisms of asymmetric cell division (Bassett et al. 2013; Koboldt et al. 2013). At the translational level, it shall be tempting to apply these fundamental discoveries in cancer research (Wodarz and Näthke 2007). Altogether, the study of asymmetric cell division in *Drosophila* CNS shall continue to provide enticing opportunities for basic as well as clinical research.

Acknowledgments We thank the members of the Wodarz lab for keen insights and fruitful discussions and apologize to colleagues whose work we could not cite due to space restrictions. Work in the Wodarz lab is supported by a grant from the DFG in the Cluster of Excellence "Nanoscale Microscopy and Molecular Physiology of the Brain (CNMPB)".

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Chapter 5
Polarity Control of Spindle Positioning in the *C. elegans* Embryo

Lars-Eric Fielmich and Sander van den Heuvel

Abstract Cell and tissue polarity guides a large variety of developmental processes, including the choice between symmetric and asymmetric cell division. Asymmetric divisions create cell diversity and are needed for the maintenance of tissue-specific stem cells. Symmetric divisions, on the other hand, promote exponential cell proliferation. Polarized cells often divide symmetrically by cleaving along the axis of polarity. Alternatively, cell cleavage in a plane perpendicular to the polarity axis results in asymmetric division. To control this decision, developmental cues position the mitotic spindle, which instructs the plane of cell cleavage. In animal cells, the positioning of the spindle depends on evolutionarily conserved interactions between a heterotrimeric G-protein alpha subunit, TPR–GoLoco domain protein, and NuMA-related coiled-coil protein. This trimeric complex recruits the dynein microtubule motor and captures astral microtubules at the cortex. The interplay between dynein movement and depolymerizing microtubules generates cortical pulling forces that promote aster movement and spindle positioning. Through mechanisms that are poorly understood, cell polarity and other developmental signals control the microtubule-pulling forces to instruct the orientation and plane of cell division. In this chapter, we review the current understanding of the connection between cell polarity and spindle positioning, with a focus on studies of the early *C. elegans* embryo. The nematode *C. elegans* develops through a highly reproducible division pattern and has proven to be a powerful model for studying the regulation and execution of asymmetric cell division.

Keywords Asymmetric cell division • *C. elegans* • Polarity • Pulling forces • Spindle positioning

Abbreviations

AB	Anterior blastomere
EMS	Endomesodermal blastomere

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K. Ebnet (ed.), *Cell Polarity 2*, DOI 10.1007/978-3-319-14466-5_5

GAP GTPase-activating protein
GEF Guanine nucleotide exchange factor
LIN Lineage abnormal
NB Neuroblast
P1 Posterior blastomere 1
PAR Partitioning defective
PCP Planar cell polarity
SOP Sensory organ precursor

5.1 Spindle Positioning and Asymmetric Cell Division

When cells divide, chromosome segregation is followed by cleavage of the cytoplasm. The microtubule spindle apparatus instructs the cytokinetic furrow to form perpendicular to, and usually midway through, the central spindle. By positioning the spindle with respect to the polarity axis of the cell or tissue, daughter cells are formed at the proper place, with the right size and developmental fate. Hence, accurate spindle positioning is critical for tissue integrity, morphogenesis, and the balance between symmetric and asymmetric division of stem cells and tissue-specific progenitor cells. How cell polarity information is translated into proper spindle positioning has been a subject of intense study over the past 15 years. Information from a variety of systems has resulted in a general model for spindle positioning in animal cells (for reviews: Galli and van den Heuvel 2008; Knoblich 2010; Morin and Bellaïche 2011). While some aspects are understood in considerable detail, cell type-dependent variations are still emerging, and many questions remain unanswered even for the best-studied systems.

The distinction is often made between intrinsic and extrinsic asymmetric division (Horvitz and Herskowitz 1992) (Fig. 5.1). In intrinsic asymmetric division, anterior–posterior, apical–basal, or planar polarity guides the asymmetric distribution of cell fate determinants in mitosis. By also aligning the mitotic spindle with this polarity axis, cytoplasmic cleavage segregates the localized components into a single daughter cell. Thus, intrinsic asymmetric division generates different daughter cells during the cell division process. As an alternative mode of asymmetric division, external signals may instruct a different fate in daughter cells that are initially identical after division. As an example, tissue-specific stem cells may depend on contact with a niche for the maintenance of the uncommitted state. If the spindle orients perpendicular to the niche during cell division, a single daughter cell will remain associated with the niche as an uncommitted stem cell, while the other daughter cell loses this interaction and initiates a differentiation program (Fig. 5.1). Thus, spindle positioning is crucial for the unequal partitioning of determinants during intrinsic asymmetric division and for properly positioning daughter cells during extrinsic asymmetric division.

Spindle positioning has been best studied in the context of asymmetric cell division in invertebrate models. While this review focuses on the nematode

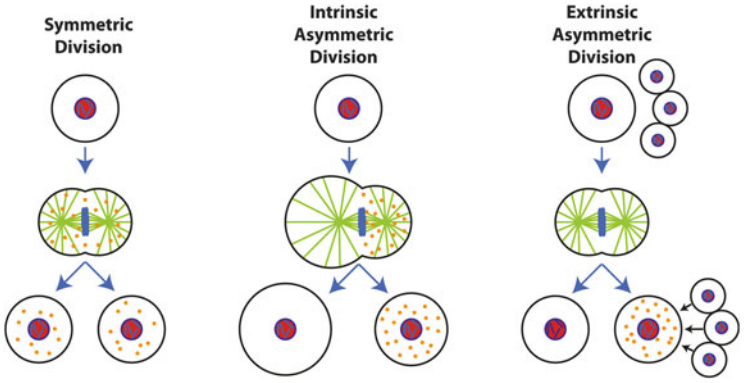

Fig. 5.1 Symmetric and asymmetric cell division. The *left panel* illustrates a symmetric cell division in which cell fate determinants (*orange*) are distributed equally over the two daughter cells. In the *middle panel*, an intrinsic asymmetric cell division is depicted. The plane of the cell cleavage and asymmetric localization of fate determinants in the mitotic mother cell ensure that cell division creates daughter cells with different cytoplasmic determinants and cell fates. Off-center positioning of the spindle causes the division to be asymmetric in size as well. The *final panel* illustrates an extrinsic asymmetric division. The two daughter cells do not inherit different fate determinants during mitosis, but receive different extrinsic signals that promote their distinct cell fates

C. elegans, other examples should be mentioned to illustrate the importance of this process. Excellent examples are the *Drosophila* central nervous system and larval brain, which are formed through repetitive rounds of intrinsic asymmetric division of neuroblasts (NBs). The embryonic neuroblasts become specified within a polarized epithelium known as ventral neuroectoderm (Knoblich 2008, 2010; Morin and Bellaïche 2011). They delaminate from this epithelium and go through several rounds of asymmetric divisions in which the spindle aligns along the apical–basal polarity axis. Critical in spindle orientation is the expression of the Inscuteable adaptor protein upon NB specification. Inscuteable forms a link between proteins that determine apical cell polarity and proteins that anchor astral microtubules, thereby ensuring apical–basal orientation of the spindle. The apical daughter cells are larger and retain self-renewing capacity, while the smaller basal cells (known as ganglion mother cells) undergo one symmetric division to form two neurons. Remarkably, the size asymmetry does not follow from asymmetric spindle positioning, but from asymmetry in spindle geometry during anaphase (Kaltschmidt et al. 2000). Consequently, the cell cleavage plane is placed toward the basal side. Asymmetric cell cleavage in neuroblasts can also occur independently of the spindle, presumably induced by basally enriched actomyosin (Cabernard et al. 2010).

Another well-studied model for asymmetric division is the *Drosophila* sensory organ precursor (SOP, also called pI) cell. SOP cells generate the mechanosensory organs of the peripheral nervous system of the fly. These organs consist of a sensory hair, connected to a socket cell and neuron, which is surrounded by a glial-like sheath cell (Knoblich 2008; Morin and Bellaïche 2011). To form these four

different cells, SOP cells go through two rounds of intrinsic asymmetric cell division. These divisions are coordinated with the anterior–posterior (A/P) body axis of the fly to align the orientation of sensory bristles. Hereto, Frizzled (Fz)-dependent planar cell polarity (PCP) signaling aligns the spindle along the A/P body axis in mitotic SOP cells. During this division, the Notch-antagonist Numb localizes to the anterior cell cortex and becomes asymmetrically segregated to the anterior pIIb cell. This cell continues to divide to form a neuron and sheath cell, while division of the posterior pIIa cell creates the hair and socket cell of the sensory organ.

Stem cell divisions in the male and female *Drosophila* germ line provide examples of niche-dependent asymmetric divisions. The mechanisms that control these divisions differ from the focus of this chapter and are therefore not discussed. Insight obtained in *C. elegans* studies has substantially contributed to the molecular understanding of asymmetric divisions in mammals, in particular those that occur during mammalian skin and brain development. The development of the mouse skin from a single to multilayered structure (stratification) coincides with the switch from symmetric to mostly asymmetric divisions around day 14 of embryogenesis (E14) (Lechler and Fuchs 2005; Williams et al. 2011). The spindle orients in the plane of the epithelium during symmetric divisions. Asymmetric division involves the rotation of the spindle to align with the apical–basal axis of cell polarity and leads to the formation of a differentiating daughter cell above the basal cell layer. The mammalian brain develops from neuroepithelial progenitor cells (Fietz and Huttner 2011). These cells are connected by adherens junctions close to their apical surface. Cell cycle-dependent apical–basal movements of the cell nuclei create a pseudostratified epithelium. When these cells enter mitosis, the nucleus is always at the apical side. Initially, the mitotic spindle orients within the plane of the epithelium, and symmetric divisions expand the pool of progenitors. Similar to skin development, a switch to asymmetric division leads to neurogenesis, which peaks around day E14–E15 of mouse embryogenesis and coincides with subtle spindle rotations. Because the apical surface is narrow, even a subtle spindle rotation leads to the creation of a daughter cell that lacks apical surface and adherens junction attachment (Fietz and Huttner 2011). This cell initiates neural differentiation either directly or after further division. In all these examples, apical polarity and spindle positioning use molecular mechanisms that have been discovered in substantial part through studies of the early *C. elegans* embryo.

5.2 Spindle Positioning in the Early *C. elegans* Embryo

The nematode *C. elegans* develops through a highly reproducible pattern of asymmetric and symmetric divisions. The division of the *C. elegans* zygote has served as a particularly informative model for the concerted steps that are required for intrinsic asymmetric cell division: establishment of polarity, asymmetric localization of fate determinants, and proper positioning of the spindle to instruct the plane

of cell cleavage. The *C. elegans* oocyte is not polarized before fertilization. Sperm entry initiates a symmetry-breaking event, which defines the posterior end and leads to the formation of the embryonic A/P axis. The establishment of A/P polarity is excellently reviewed in another book chapter (Carrie Cowan, Volume 2). In short, the anterior PAR protein complex consists of the PDZ-domain proteins PAR-3 and PAR-6 in association with atypical protein kinase C (PKC-3). This complex occupies the oocyte cortex at the time of fertilization (Fig. 5.2). Two posterior PAR proteins, the PAR-2 ring finger protein and PAR-1 MARK family Ser/Thr kinase, are present in the cytoplasm at that time, because PKC-3 phosphorylates PAR-2 and prevents its cortical localization. While this distribution is stable, fertilization-dependent processes disturb the equilibrium. A sperm-derived Rho-GAP, CYK-4, and cortical depletion of the Rho-GEF ECT-2 by maturated sperm-derived centrosomes disrupt the actomyosin cytoskeleton and cause it to retract toward the opposite (anterior) pole. Coincident with this actomyosin flow, anterior PAR proteins are removed from the posterior cortex. Moreover, microtubules nucleated at the mature centrosomes bind PAR-2 and protect it from PKC-3 phosphorylation. This allows PAR-2 to occupy the cortex and to recruit PAR-1 near the paternal pronucleus (Fig. 5.2). PAR-1 then phosphorylates PAR-3, which antagonizes posterior localization of the anterior PAR complex. The mutual antagonism between the anterior and posterior PAR proteins results in a new equilibrium

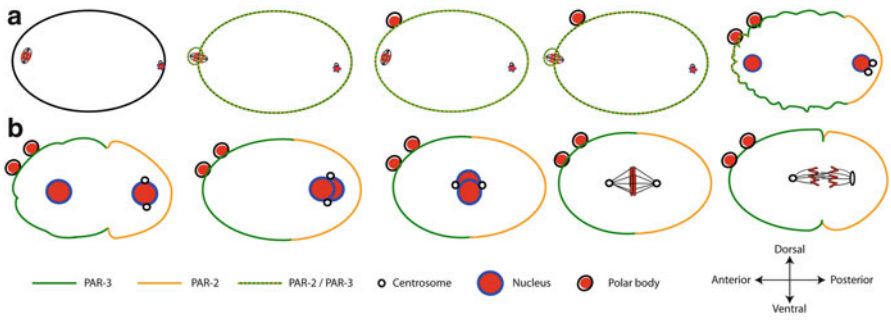

Fig. 5.2 Establishment of polarity in the *C. elegans* zygote. (**a**) At the moment of fertilization by the male sperm, the oocyte is stalled in prophase of meiosis I, and PAR polarity proteins are distributed uniformly. Sperm entry breaks the symmetry and marks the future posterior pole of the embryo. The oocyte then finishes meiosis I and II, resulting in two polar bodies and one maternal pronucleus. Simultaneously, cortical actomyosin starts to retract anteriorly, while the posterior cortex smoothens. Coincident with the actin flow, anterior PAR proteins (*green*) are removed from the posterior cortex, allowing for cortical localization of posterior PAR proteins (*yellow*). (**b**) Polarization is complete when an equilibrium between the opposing PAR domains is reached. After the pronuclei have met in the posterior, the pronuclei–centrosomal complex centrates and rotates while assembling the mitotic spindle along the A/P PAR polarity axis. At the embryonic midplane, the nuclear envelopes break down and chromosomes become aligned at the metaphase plate. Higher posterior pulling forces acting on the spindle cause the spindle to displace posteriorly, positioning the cleavage plane off-center. This sequence of events, initiated by male sperm entry, results in a division that is unequal in size and contents. Figure adapted after Galli and van den Heuvel (2008)

with PAR-3/PAR-6/PKC-3 occupying the anterior half of the cortex, while PAR-2 and PAR-1 occupy the posterior half (Cowan and Hyman 2007; Hoege and Hyman 2013).

Polarity establishment coincides with two consecutive highly asymmetric meiotic divisions. These divisions produce two small polar bodies, as the compact meiotic spindle segregates the chromosomes in close proximity to the cortex (Fig. 5.2). Subsequently, the haploid maternal pronucleus migrates to the posterior to meet the paternal pronucleus, followed by the movement of the adjoined nuclei toward the center (centration). The pronuclei–centrosomal complex rotates during this anterior migration, coincident with spindle assembly along the A/P axis of the zygote (P0). Following nuclear envelope breakdown, the spindle aligns the chromosomes at the metaphase plate in the middle of the zygote. The spindle relocates slightly toward the posterior in metaphase and during elongation in anaphase. During this translocation, the posterior pole shows extensive lateral oscillations, named "rocking," while the anterior pole remains relatively fixed. The off-center placement of the spindle results in an unequal first division that gives rise to a larger anterior blastomere (AB) and smaller posterior daughter (P1) (Figs. 5.2 and 5.3). Coincidently, cytoplasmic determinants become unequally partitioned, creating intrinsic differences between the AB somatic blastomere and P1 germ line precursor cell. All these asymmetries depend on A/P polarity. In embryos that lack PAR protein function, the spindle remains in the center of the zygote, and cell cleavage is symmetric in size and fate. Inactivation of anterior PAR complex function results in the rocking of both spindle poles and exaggerated spindle elongation (Kemphues et al. 1988). In contrast, inactivation of posterior PAR function causes both spindle poles to remain quite stationary, resembling the normal behavior of the anterior pole.

The P1 blastomere reestablishes opposing PAR protein domains. The duplicated centrosomes migrate around the nucleus in AB and P1 to initiate a transverse spindle position (Fig. 5.3). In P1, however, the nucleus and associated centrosomes rotate by 90° to align with the A/P polarity axis in prometaphase and promote asymmetric cell division (Fig. 5.3). This division generates another precursor of the germ line (P2), which continues cell-autonomously controlled asymmetric division, just like its daughter germ line precursor cell P3. In contrast, asymmetric division of EMS, a precursor of endoderm (intestine) and mesoderm, requires signaling from the neighboring P2 blastomere at the four-cell stage. This involves a Wnt/Fz pathway and parallel acting MES-1/SRC-1 tyrosine kinase signaling. These pathways coordinate spindle orientation along the long axis of the embryo with endoderm specification in the daughter cell that contacts P2 (Bei et al. 2002). The division of ABa and ABp, the other two blastomeres of the four-cell embryo, uses a small rotation of the spindle to divide left–right under a slight angle to create reproducible left–right asymmetry of the animal (Bergmann et al. 2003). The right daughter cell of this division, ABar, rotates its spindle again dependent on a Wnt-signal from the neighboring C blastomere. In summary, the position of the

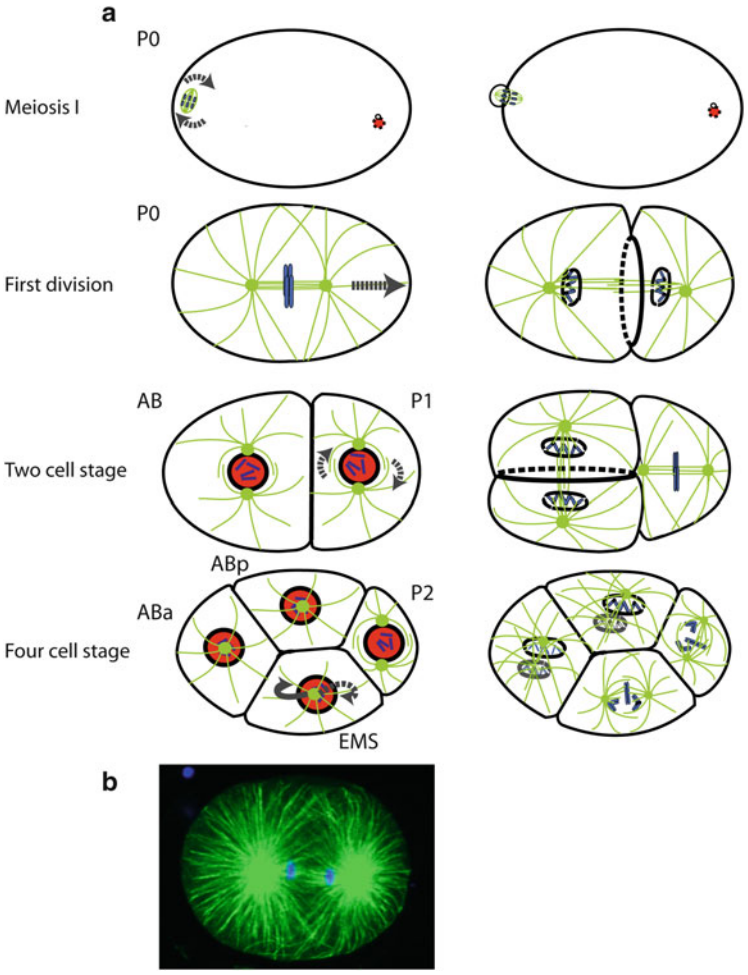

Fig. 5.3 Asymmetric cell divisions in the *C. elegans* early embryo. (**a**) During meiosis, the oocyte divides highly asymmetrically because the meiotic spindle locates close to the cell cortex and rotates such that small polar bodies are formed, and a large zygote remains. During the first mitotic division, pulling forces are asymmetric in P0, resulting in a larger AB and smaller P1 blastomere. In P1, the spindle rotates and aligns with the A/P polarity axis as in P0. In the four-cell embryo, ABa and ABp divide under a slight angle to generate left–right asymmetry. The EMS spindle rotates under the influence of extrinsic signals emanating from the P2 cell. These rounds of asymmetric division are highly reproducible and regulated, making them a strong experimental model for studies of asymmetric cell division. (**b**) Immunofluorescent labeling of tubulin (*green*) and DNA (*blue*) marks the mitotic spindle, which is visibly shifted posteriorly during P0 division. Figure adapted after Galli and van den Heuvel (2008)

spindle and cleavage plane is highly regulated in *C. elegans* and critical in generating the proper cell lineages throughout development. While substantial insight has been obtained in the proteins that contribute to spindle positioning, the coordination with cell polarity is only partly understood.

5.3 The Molecular Components of Cortical Force Generation

In animal cells, pulling forces that act between the cell cortex and astral microtubules position the spindle in mitosis. These forces are generated by depolymerization of the microtubule plus ends, in association with dynein minus-end-directed motor proteins. The dynein motor is recruited to the cortex by a trimeric protein complex that is conserved throughout the animal kingdom. In *C. elegans*, the complex contains a GOA-1 or GPA-16 heterotrimeric G-protein α-subunit, which functions as a cortical anchor. The TPR and "G-protein regulator" motif proteins GPR-1/GPR-2 form a bridge between Gα at the membrane and the LIN-5 coiled-coil protein. LIN-5 interacts directly or indirectly with subunits of the cytoplasmic dynein complex (Fig. 5.4). Genetic studies place PAR polarity proteins upstream of Gα-GPR-1/GPR-2–LIN-5 in the control of spindle positioning. Multiple possible links between polarity regulators and the pulling force complex have been suggested, but a comprehensive picture of spatiotemporal control of spindle positioning has yet to emerge.

5.3.1 Noncanonical G-Protein Signaling

G-protein signaling has long been known as a major route to convey extracellular signals over the plasma membrane. In this pathway, ligand binding induces a

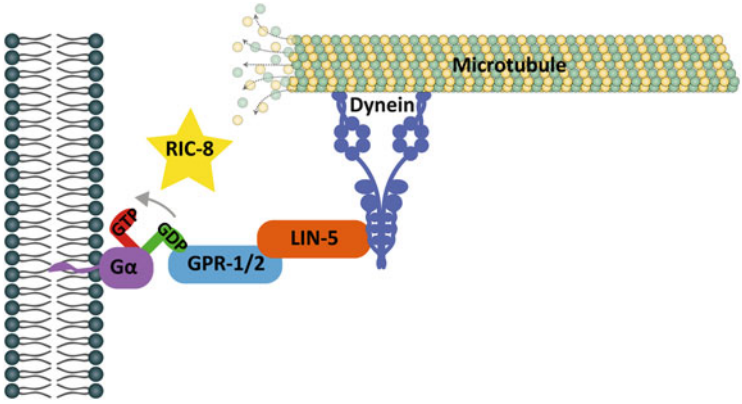

Fig. 5.4 Model illustrating the generation of cortical pulling forces at microtubule plus ends. Dynein is tethered to the cortex by a trimeric complex of Gα·GDP–GPR-1/GPR-2–LIN-5. Cortical pulling forces are generated by microtubule depolymerization and dynein minus-end-directed motor activity. The GEF protein RIC-8 facilitates the GDP/GTP exchange on GOA-1 Gα and promotes GPA-16 Gα plasma membrane localization. Inactivation of any of these components abrogates cortical pulling force generation. See text for further explanation

transmembrane G-protein-coupled receptor (GPCR) to act as a guanine nucleotide exchange factor (GEF). The exchange of GDP to GTP causes dissociation of the receptor-associated Gα·GDP–Gβγ complex, allowing Gα·GTP and Gβγ to activate downstream targets. Surprisingly, spindle positioning turned out to use a receptor-independent noncanonical G-protein pathway. The first support for heterotrimeric G-protein contribution to spindle positioning came from the Plasterk group, who found that inactivation of *gpb-1*, one of two *C. elegans* Gβ genes, randomizes mitotic spindle orientation in early divisions and thus perturbs the tightly regulated process of asymmetric cell division (Zwaal et al. 1996). Subsequently, Miller and Rand found that mutation of *goa-1* Gαo also affects the position and orientation of the mitotic spindle in early *C. elegans* embryos (Miller and Rand 2000). While in *goa-1* mutant embryos defects were observed with low penetrance, combining mutations and/or RNA-mediated interference (RNAi) of *goa-1* and *gpa-16* Gαi/o causes near-complete absence of spindle positioning and results in symmetric division of the one-cell embryo (Gotta and Ahringer 2001). Thus, the GOA-1 and GPA-16 Gα subunits (together referred to as Gα) act redundantly in spindle positioning. Finally, RNAi of one of two *C. elegans* Gγ-subunit genes, *gpc-2* (Gγ-2), was found to cause spindle defects comparable to *gpb-1* RNAi (Gotta and Ahringer 2001). At that time, a complete heterotrimeric G protein, consisting of GOA-1/GPA-16 Gα, GPB-1 Gβ, and GPC-2 Gγ subunits, had been implicated in asymmetric cell division in *C. elegans*. There was no evidence, however, for the contribution of a G-protein-coupled receptor (GPCR), and transmembrane signaling appeared unlikely in a one-cell embryo.

Support for noncanonical G-protein signaling in asymmetric cell division came simultaneously from studies in *Drosophila*. Inscuteable was found to control asymmetric NB divisions in association with the Partner of Inscuteable (Pins) protein and a Gα subunit (Schaefer et al. 2000). In addition, Gαi and Pins, but not Inscuteable, were shown to determine the division orientation of SOP cells in *Drosophila* (Bellaïche et al. 2001; Schaefer et al. 2001). Thus, cell autonomous control of spindle positioning in the *C. elegans* zygote and *Drosophila* NB and division orientation control by planar cell polarity all turned out to use a novel form of G-protein signaling.

5.3.2 TPR–GPR Domain Proteins

Three groups simultaneously identified additional positive regulators of Gα in the control of asymmetric division of the *C. elegans* zygote (Colombo et al. 2003; Gotta et al. 2003; Srinivasan et al. 2003). The G-protein regulator (GPR) genes *gpr-1* and *gpr-2* were first observed to affect spindle positioning in a high-throughput RNAi screen of all chromosome III-encoded genes (F22B7.13, C38C10.4; (Colombo et al. 2003)). Independently, GPR-1 and GPR-2 proteins were co-immunopurified with the spindle-positioning protein LIN-5 (Srinivasan et al. 2003). The *gpr-1* and *gpr-2* coding sequences share 96 % nucleotide identity; hence, RNAi for one

inhibits the other gene simultaneously. The predicted proteins are 97 % identical and are commonly referred to together as GPR-1/GPR-2 or simply GPR. Importantly, GPR-1 and GPR-2 are related to *Drosophila* Pins and part of a protein family that includes the closely related mammalian LGN (Leu–Gly–Asn repeat-enriched protein) and AGS3 (activator of G-protein signaling), as well as *C. elegans* AGS-3. These proteins all contain multiple N-terminal tetratricopeptide (TPR) protein interaction motifs and at least one C-terminal GoLoco/GPR domain (Colombo et al. 2003; Gotta et al. 2003; Srinivasan et al. 2003).

The GoLoco/GPR domain interacts with Gαi/o·GDP, inhibits GDP release, and competes with Gβγ association. Crystal structure studies showed that Gβγ and the GoLoco/GPR motif interact with Gα·GDP through overlapping binding sites (Kimple et al. 2002; Martin-mccaffrey et al. 2005). Hence, spindle positioning was initially thought to depend on Gβγ release (Gotta and Ahringer 2001; Schaefer et al. 2001). In *C. elegans*, RNAi of *gpr-1/gpr-2* closely mimics *goa-1/gpa-16* RNAi, which indicates that GPR-1 and GPR-2 act positively with Gα. In contrast, *gpb-1* Gβ and *gpc-2* Gγ RNAi do not resemble *gpr-1/gpr-2* RNAi or alter the *goa-1/gpa-16* RNAi phenotype. Such observations demonstrated that, rather than Gβγ, the interaction between Gα·GDP and GPR-1/GPR-2 is required for spindle positioning in asymmetric cell division (Colombo et al. 2003; Gotta et al. 2003; Srinivasan et al. 2003). This confirmed alternative use of G-protein signaling in spindle positioning, as Gα·GDP instead of Gα·GTP is the active form. Gα·GDP interacts with a GPR/Pins family member, which in turn uses the TPR motifs, and possibly a linker between the TPR and GoLoco/GPR domains, to recruit additional spindle-positioning proteins to the membrane.

5.3.3 LIN-5 (Mud/NuMA)

Another important component of the cortical pulling force complex in *C. elegans* is the LIN-5 protein, which is the functional equivalent of mammalian NuMA (nuclear mitotic apparatus) and *Drosophila* Mud (mushroom body defect). The *lin-5* gene was defined by "lineage-abnormal" mutations (Albertson 1978). Homozygous *lin-5* mutants are sterile, but when derived from heterozygous parents complete normal embryonic development. Postembryonic cell divisions fail in mitosis in these mutants; chromosomes may not fully align at the metaphase plate; anaphase and cytokinesis do not occur, yet cells exit from mitosis at the normal time and enter the next round of DNA synthesis, centrosome duplication, and mitotic entry (Albertson 1978; Sulston and Horvitz 1981; Lorson et al. 2000). Dependent on the lineage, postembryonic blast cells in *lin-5* mutants continue abortive mitoses and become highly polyploid. Embryonic development in these mutants is driven by maternal product, as knockdown of *lin-5* by RNAi and temperature shift of *lin-5(ev571*ts*)* mutants cause complete embryonic lethality (Lorson et al. 2000).

The extended central domain of the LIN-5 protein is predicted to form an α-helical coiled-coil structure (Lorson et al. 2000). While the amino acid sequence

provided little functional information, immunopurification followed by mass spectrometry revealed that LIN-5 and GPR-1/GPR-2 form part of a protein complex (Srinivasan et al. 2003). The strong overlap in phenotype also supports joint functions: *gpr-1*/*gpr-2* and *lin-5* RNAi each causes reduced spindle elongation, lack of posterior movement of the spindle in anaphase, and failure to undergo normal asymmetric division of the zygote (Gotta et al. 2003; Srinivasan et al. 2003). The characteristic oscillations and flattening of the posterior spindle pole that normally occur during spindle migration are also absent after *gpr-1*/*gpr-2* and *lin-5* knockdown, and the spindle does not rotate in the P1 blastomere. Chromosome segregation and cytokinesis continue for two or three rounds of cell division, giving rise to dead embryos with a few highly polyploid nuclei. The physical association between LIN-5 and GPR-1/GPR-2, as well as interaction between GPR-1/GPR-2 and Gα, and the strong resemblance in *lin-5*, *gpr-1*/*gpr-2*, and *goa-1*/*gpa-16* RNAi phenotypes all supported a model in which the encoded proteins act together to control the mitotic spindle position. In addition, *lin-5* is also required for meiotic spindle rotation, independently of *gpr-1*/*gpr-2* and *goa-1*/*gpa-16* Gα (Lorson et al. 2000; van der Voet et al. 2009).

5.3.4 The Trimeric Gα–GPR–LIN-5 Complex Recruits Dynein to the Cortex

The subcellular localizations of LIN-5 and GPR-1/GPR-2 show strong overlap and mutual dependence. The proteins are present at the spindle poles, in the cytoplasm, at the cell cortex, and, specifically in metaphase, at kinetochore microtubules. GPR-1/GPR-2 fails to localize in the absence of LIN-5, and LIN-5 loses its cortical localization when GPR-1/GPR-2 or Gα is gone (Lorson et al. 2000; Srinivasan et al. 2003; van der Voet et al. 2009). The combined data support that Gαi/o·GDP, GPR-1/GPR-2, and LIN-5 form a trimeric complex needed for spindle positioning in *C. elegans* (Fig. 5.4). Similarly, *Drosophila* Pins associates with Gαi and the LIN-5/NuMA-related protein Mud in NBs and epithelial cells (Izumi et al. 2004; Bowman et al. 2006; Siller and Doe 2009). Moreover, mammalian LGN recruits the NuMA protein to the cell cortex and simultaneously interacts with Gαi (Du and Macara 2004). Each of these complexes is critical for spindle positioning and orienting cell division, in mammals in particular in the developing skin and brain (Lechler and Fuchs 2005; Williams et al. 2011). Thus, an evolutionarily conserved mechanism appears to control the positioning of the cell division plane in all animals.

Observations in the one-cell *C. elegans* embryo provided additional functional insights. It was found that the Gα–GPR–LIN-5 complex is needed for the generation of pulling forces that act from the cortex at astral microtubules. Such external forces can be made visible by means of spindle midzone severing with a UV laser (Grill et al. 2001). Following spindle severing, the spindle poles move outward with

increased speed toward the cell periphery. Importantly, the posterior pole moves with a higher velocity and over a larger distance than the anterior pole. This indicates asymmetry in pulling forces, which depends on A/P polarity; *par-2* mutant embryos are "anteriorized" and show anterior and posterior pole movements with the same low peak velocity as the wild-type anterior pole. Vice versa, *par-3* mutant embryos are "posteriorized" with both sides showing high pulling forces (Grill et al. 2001). The knockdown of Gα, *gpr-1*/*gpr-2*, or *lin-5* largely eliminates these pulling forces (Nguyen-Ngoc et al. 2007), while specific loss of LIN-5 from spindle poles has no effect (van der Voet et al. 2009). Thus, cortical localization of LIN-5, through Gα–GPR-1/GPR-2 interaction, is needed for the pulling forces that position the spindle in mitosis.

Gα–GPR–LIN-5 contributes to cortical pulling forces through the recruitment of a dynein motor complex to the cell periphery (Couwenbergs et al. 2007; Nguyen-Ngoc et al. 2007). Dynein anchored by Gα–GPR–LIN-5 attaches microtubule plus ends to the cell cortex, while depolymerization of the microtubules ends is thought to be largely responsible for force generation (Kozlowski et al. 2007; Nguyen-Ngoc et al. 2007; Laan et al. 2012). Myristoylation of the Gα subunit allows membrane attachment of the complex, and, based on the analysis of human NuMA, the N-terminal part of LIN-5/NuMA mediates dynein interaction (Kotak et al. 2012). While these molecular interactions are conserved in the animal kingdom, variations are used in development. In meiosis of the *C. elegans* female pronucleus, LIN-5 and dynein are needed to rotate the meiotic spindle in order to expel the polar bodies. Instead of Gα–GPR-1/GPR-2, a complex of ASPM-1 (abnormal spindle-like, microcephaly associated) and calmodulin anchors LIN-5 and dynein at the spindle poles to mediate this rotation (van der Voet et al. 2009). In planar cell polarity, the Frizzled receptor and Dishevelled effector orient the spindle and division plane. Dishevelled interacts with Mud in *Drosophila* SOP cells and with NuMA during zebra fish gastrulation to engage the dynein motor complex in this process (Morin and Bellaïche 2011). Thus, the LIN-5/Mud/NuMA coiled-coil protein acts as a general dynein adaptor in spindle positioning. This adaptor also functions as an important target of spatiotemporal regulation of spindle-pulling forces, as discussed in the next section.

5.4 Regulation of Cortical Force Generation in the *C. elegans* One-Cell Embryo

The asymmetric localization of PAR proteins in the *C. elegans* embryo causes the spindle to migrate off-center in mitosis. As compared to *Drosophila* NBs and SOP cells, the distribution of cortical pulling force proteins is more dynamic and less asymmetric in the *C. elegans* zygote, and a combination of several factors may determine the plane of cell cleavage. Below, we review the mechanisms that have

been proposed to contribute to asymmetric pulling forces in the one-cell *C. elegans* embryo.

5.4.1 Gα Regulators

During delamination of the *Drosophila* NB, the polarity of the neuroepithelium is maintained, and apically localized PAR3–PAR6–aPKC recruits Inscuteable to the apical side of the cell. Pins and Gαi accumulate at the same side, presumably recruited by Inscuteable, thus cell polarity corresponds directly to the asymmetry in cortical force generators. By contrast, the GOA-1 and GPA-16 Gαi/o subunits show uniform localization at the cortex of the *C. elegans* zygote (Colombo et al. 2003; Gotta et al. 2003; Srinivasan et al. 2003). Nevertheless, the regulation of the active versus inactive state of the heterotrimeric G protein could create asymmetry in pulling forces. The activity of G proteins depends on the associated guanosine nucleotide, GTP or GDP. As for canonical G-protein signaling, GEF and GAP proteins have been identified that affect GOA-1 and GPA-16 Gαi/o activity in spindle positioning. However, GPR-1/GPR-2 associates specifically with Gαi/o·GDP, behaves as a GDP-dissociation inhibitor (GDI), and competes with Gβγ in Gα·GDP binding. While the regulation of the GTP binding and hydrolysis cycle is clearly critical in pulling force generation, it remains puzzling if and how this level of Gα regulation contributes to the spatiotemporal control of spindle positioning.

5.4.1.1 The Gα GTPase Cycle Is Essential for Pulling Force Generation

An important regulator of Gα in spindle positioning is the "resistant to inhibitors of cholinesterase 8" (RIC-8) protein. The *ric-8* gene was identified in a genetic screen for factors that mediate neurotransmitter release, a process regulated by heterotrimeric G-protein signaling (Miller et al. 1996). Remarkably, *ric-8* and *goa-1* showed closely related functions during spindle positioning in the early embryo (Miller and Rand 2000). Insight in the molecular function of RIC-8 came from studies that identified mammalian RIC-8A and RIC-8B as binding partners of Gαi/o/q (Tall et al. 2003). Further analysis revealed that RIC-8A exhibits potent GEF activity and associates with the Gα·GDP monomer and nucleotide-free transition state, but not with Gα·GTP or the trimeric Gα·GDP–Gβγ complex. RIC-8 interacts with GOA-1 as well as GPA-16 Gα in *C. elegans* and is a GEF for GOA-1 in vitro. The RIC-8 protein is present uniformly in the cytoplasm and weakly at the cortex. Strong inactivation of *ric-8*, by combined mutation and RNAi, causes loss of pulling forces in the early *C. elegans* embryo and resembles *goa-1*/*gpa-16* double RNAi or knockdown of other components of the LIN-5 complex (Afshar et al. 2004). Thus, RIC-8 acts as a general positive regulator of Gα-mediated spindle positioning.

It appears paradoxical that the RIC-8 GEF and GPR-1/GPR-2 GDI proteins display opposite biochemical activity while both promote spindle-pulling forces. GEF requirement may indicate that the Gα subunit needs to go through the GTP binding/hydrolysis cycle in this process. Mammalian RIC-8A does not activate Gα·GDP–Gβγ trimers, but catalyzes nucleotide exchange of free Gα·GDP and Gα·GDP in complex with a GoLoco/GPR motif protein and NuMA (Tall and Gilman 2005). Thereby, RIC-8 triggers the dissociation of the Gα·GDP–LGN–NuMA complex. Thus, force generation may involve dissociation of the pulling force complex or generation of Gα·GTP. A different model came from observations in NBs and SOP cells in *Drosophila* and of RIC-8A and RIC-8B knockout mouse ES cells (David et al. 2005; Hampoelz et al. 2005; Gabay et al. 2011). These studies showed that RIC-8 is needed for plasma membrane association of newly synthesized Gα subunits. Acting as a molecular chaperone, RIC-8 might use the nucleotide switch to dissociate from the properly folded and ER membrane associated Gα·GTP molecule. *C. elegans* GOA-1 and GPA-16 show differential interactions with RIC-8. RIC-8 acts as a GEF for GOA-1 but not GPA-16, and RIC-8 controls the cortical localization and protein level of GPA-16, but not GOA-1 (Afshar et al. 2004, 2005). As *ric-8* RNAi resembles the *goa-1*/*gpa-16* double knockdown phenotype, both chaperone and GEF activities of RIC-8 may contribute to pulling force generation.

The contribution of a GTP binding/hydrolysis cycle in noncanonical G-protein signaling is further supported by the involvement of a possible Gα GAP (GTPase-activating protein). Examining all *C. elegans* proteins with "regulator of G-protein signaling" (RGS) GTPase activation domains, RGS-7 was recognized for its essential function in embryonic development (Hess et al. 2004). One-cell embryos lacking *rgs-7* function showed increased movement and rocking of the posterior spindle pole and exaggerated asymmetric division. The severing of the spindle midzone with a UV laser beam demonstrated that the increased posterior displacement of the spindle results from decreased anterior pulling forces in *rgs-7* mutant embryos. RGS-7 is present at the cortex, though only detectable from the two-cell stage onward (Hess et al. 2004). It remains unknown why the loss of an apparently uniformly distributed RGS-7 protein reduces only anterior pulling forces. However, genetic and biochemical experiments strongly support that RGS-7 acts as a GAP for GOA-1.

The Gβγ dimer is also an important negative regulator of pulling force generation, as it competes with GPR-1/GPR-2 for Gα·GDP. As mentioned above, RNAi of *gpb-1* and *gpc-2* causes abnormal centrosome movements and spindle orientation. Based on combined RNAi with Gα, this phenotype results from hyperactive Gα that is no longer sequestered by Gβγ, and not from loss of Gβγ-specific effector signaling (Tsou et al. 2003). Spindle-severing experiments showed that *gpb-1* RNAi increases spindle-pulling forces in the anterior (Afshar et al. 2004, 2005). Thus, the asymmetry in Gβγ could contribute to the asymmetry in pulling forces and posterior displacement of the spindle in mitosis. A recent study showed dynamic regulation of cortical GPB-1 levels and trafficking through both early endosomes and recycling endosomes (Thyagarajan et al. 2011). In metaphase of the

zygote, trafficking rates are higher in the anterior than the posterior, and more GPB-1 remains present in endosomal vesicles in the posterior. Thus, a larger fraction of the uniformly distributed Gα protein may be available for GPR-1/GPR-2 association in the posterior. This provides a potential mechanism for spatiotemporal regulation of spindle positioning.

5.4.2 GPR-1/GPR-2 Regulators

Since the discovery of the GPR-1/GPR-2 proteins, asymmetry in their localization has been proposed to be responsible for asymmetric pulling forces (Colombo et al. 2003; Gotta et al. 2003). However, our group did not detect statistically relevant posterior enrichment of GPR-1/GPR-2 (or LIN-5) in metaphase, while spindle-pulling forces are clearly higher in the posterior at that stage (Srinivasan et al. 2003; Galli et al. 2011; Berends et al. 2013). The localization of GPR-1/GPR-2 is quite dynamic and likely subjected to regulatory mechanisms. GPR-1/GPR-2 and LIN-5 become anteriorly enriched during polarity establishment and prophase of the first mitotic division, which contributes to pronuclear centration (Tsou et al. 2003; Park and Rose 2008). Subsequently, GPR-1/GPR-2 and LIN-5 redistribute to become higher at the posterior cortex in mitosis. This enrichment is limited but detectable in anaphase; however, it may either follow or cause asymmetry in pulling forces (Colombo et al. 2003; Gotta et al. 2003; Srinivasan et al. 2003; Galli et al. 2011; Berends et al. 2013). Moreover, asymmetry in cortical dynein localization has not been detected during any phase of *C. elegans* zygotic division (Nguyen-Ngoc et al. 2007). Thus, the question whether asymmetric distribution of the pulling force complex is responsible for asymmetric spindle positioning has not been conclusively answered.

The localization of GPR-1/GPR-2 requires not only Gα but also LIN-5 (Gotta et al. 2003; Srinivasan et al. 2003). A mechanism proposed for mammalian LGN may explain this dual dependence (Du and Macara 2004). The N-terminal TPR domains of LGN and C-terminal GoLoco/GPR motifs engage in intramolecular interactions that cause a closed protein conformation. The binding of either Gαi or NuMA abolishes this intramolecular interaction and allows for simultaneous binding of the other partner (Du and Macara 2004). Structural studies indicate that the LGN GoLoco domains interact in tandem with the LGN TPR repeats (Pan et al. 2013). Given that GPR-1/GPR-2 has only few confirmed TPR and GoLoco/GPR motifs, it is unclear whether the conformational switch model proposed for LGN should apply to GPR-1/GPR-2 as well. The dependence on both Gα and LIN-5 supports that GPR-1/GPR-2 uses a related mechanism for its cortical localization.

An additional regulatory protein might induce asymmetry in GPR-1/GPR-2 function. LET-99 is a DEP (Dishevelled/EGL-10/Pleckstrin) domain protein that antagonizes cortical localization of Gα–GPR-1/GPR-2 (Tsou et al. 2003; Bringmann et al. 2007; Park and Rose 2008). The *let-99* mutant phenotype

resembles aspects of the *gpb-1* RNAi phenotype and indicates negative regulation of cortical Gα–GPR-1/GPR-2. Both anterior and posterior PAR proteins inhibit LET-99 at the cortex, which restricts LET-99 localization to a cortical band at about 60 % of the long axis of the one-cell embryo. The identification of the LET-99 band has resulted in a cortical force model with three instead of two (anterior and posterior) domains. Negative regulation of force generation in the posterior LET-99 band (possibly in combination with asymmetric distribution of cortical GPR-1/GPR-2) results in net anterior pulling forces during pronuclear centration and net posterior pulling forces from metaphase onwards (Tsou et al. 2003; Couwenbergs et al. 2004; Krueger et al. 2010). This indicates the possibility that not anterior–posterior GPR-1/GPR-2 asymmetry per se, but rather reduced pulling on astral microtubules that reach the LET-99 lateral band, is responsible for a net increase in posterior pulling forces and spindle displacement in mitosis.

An RNAi screen for defects in pronuclear and spindle movements identified casein kinase-1 (CSNK-1) as a pulling force regulator (Panbianco et al. 2008). CSNK-1 is membrane associated and enriched in the anterior of the *C. elegans* zygote in a PAR-dependent manner. CSNK-1 negatively regulates the localization of GPR-1/GPR-2–LIN-5, possibly indirectly by confining the PIP2-generating PIP2 kinase PPK-1 to the posterior. PPK-1 or PIP2 may positively regulate the localization of GPR-1/GPR-2–LIN-5, although neither GPR-1/GPR-2 nor LIN-5 has a known PIP2-binding domain (Panbianco et al. 2008). The protein phosphatase PPH-6 and its associated subunit Sit4p-associated protein-1 (SAP-1) also promote GPR-1/GPR-2 localization and spindle-pulling forces in anaphase (Afshar et al. 2010). Co-depletion of CSNK-1 and PPH-6 resembles the PPH-6/SAPS-1 depleted phenotype of decreased cortical GPR-1/GPR-2 localization and spindle forces. Thus, CSNK-1 may act on PPH-6 to inhibit GPR-1/GPR-2–LIN-5 localization in the anterior. While the exact molecular mechanisms remain unclear, both kinases and phosphatases contribute to regulation of GPR-1/GPR-2 localization.

5.4.3 LIN-5 Regulators

Because Gα–GPR–LIN-5 form a trimeric complex, the regulation of Gα and GPR-1/GPR-2 levels described above also applies to cortical localization of LIN-5. In addition, LIN-5 is also subjected to specific controls. Immunopurification followed by mass spectrometry showed extensive phosphorylation of LIN-5 at 25 different residues (Galli et al. 2011). Stable isotope labeling combined with kinase knockdown and quantitative phosphopeptide analysis revealed that four LIN-5 serine residues are phosphorylated directly by the atypical protein kinase C 3 (PKC-3), in a cyclin-dependent kinase 1 (CDK-1)-dependent manner. PKC-3 is part of the anterior PAR complex, while CDK-1 is a key positive regulator of mitotic entry, thus establishing a direct connection between the LIN-5 complex, PAR polarity, and cell cycle progression. Phosphorylation of LIN-5 by PKC-3 occurs in the anterior, peaks in metaphase, and then disappears rapidly (Galli

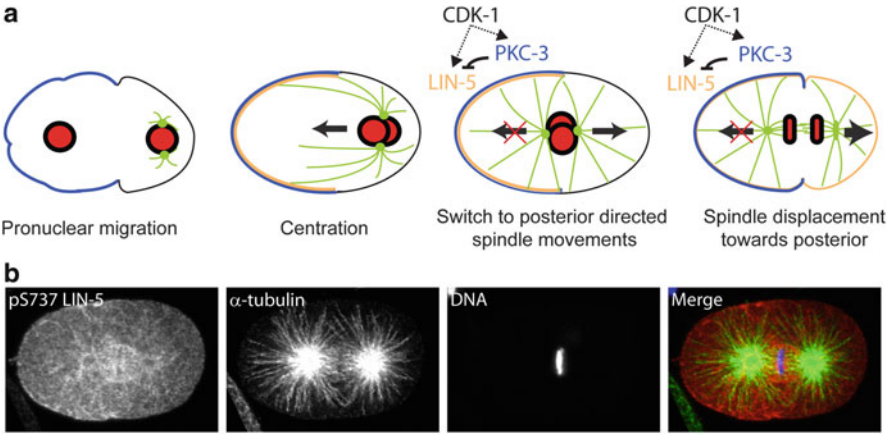

Fig. 5.5 PKC-3 phosphorylates LIN-5 to regulate cortical pulling forces. (**a**) Different phases of the first mitotic division of the *C. elegans* embryo, illustrating the positioning of the spindle apparatus (*green*) and localization of PKC-3 (*blue*) and LIN-5 (*yellow*). The schemes indicate the interactions between LIN-5, PKC-3, and (possibly) CDK-1. The *arrows* in the figures signify the direction of pulling forces. (**b**) Immunohistochemical staining of a one-cell embryo in metaphase, with antibodies recognizing LIN-5 phosphorylated on S737 (*red*), α-tubulin to mark the spindle (*green*), and DAPI to visualize DNA (*blue*). pS737 LIN-5 is clearly enriched anteriorly during this mitotic phase. Figure adapted after Galli et al. (2011)

et al. 2011). Spindle midzone severing experiments in combination with non-phosphorylatable and phosphorylation-mimicking LIN-5 mutations demonstrated that the PKC-3-specific phosphorylation of LIN-5 inhibits pulling forces. This spatiotemporal regulation coincides with the switch from anterior-directed movement of the pronuclei–centrosomal complex during centration to posterior migration of the spindle in metaphase/anaphase (Fig. 5.5).

To promote symmetric cell division, apically localized aPKC antagonizes spindle-pulling forces in polarized *Drosophila* and mammalian epithelial cells (Hao et al. 2010; Guilgur et al. 2012). Madin–Darby canine kidney (MDCK) cells in 3D culture form organized cysts with a single luminal epithelial layer. To instruct a planar spindle position, aPKC phosphorylates LGN/Pins, which creates a 14-3-3 protein binding site and prevents association with Gαi (Hao et al. 2010). Thus, phosphorylation of pulling force complex components by aPKC provides a direct way to coordinate the cell division plane with cell polarity in worms, flies, and mammals. Similarly, mitotic kinases are likely to couple cell cycle progression and pulling force generation through phosphorylation of LIN-5/NuMA or GPR/LGN. The LIN-5 phosphorylation by PKC-3, described above, depends indirectly on CDK-1 (Galli et al. 2011). CDK-1 phosphorylation possibly activates PKC-3 or might prime LIN-5 for subsequent phosphorylation by PKC-3. Direct phosphorylation of NuMA by CDK-1 has been implicated in dynein localization in mammalian cells grown in culture (Kiyomitsu and Cheeseman 2013; Kotak et al. 2013). NuMA phosphorylation by CDK-1 during metaphase negatively affects cortical

dynein levels and is antagonized by the continuously active protein phosphatase PPP2CA. This mechanism explains an observed increase in cortical dynein levels from metaphase to anaphase, following inactivation of CDK-1. Additional phosphorylations by cell cycle and polarity kinases will probably contribute to spatiotemporal control of cortical pulling forces.

5.4.4 Dynein

The recruitment of cytoplasmic dynein appears the most important function of the LIN-5 complex. Dynein acts in association with various regulator and adaptor proteins, including the multiple subunit dynactin complex (Raaijmakers et al. 2013). Components of cytoplasmic dynein as well as dynactin are conserved between *C. elegans* and mammals and include potential adaptors for LIN-5 association as well as other regulators of localization and activity (O'Rourke et al. 2007; Raaijmakers et al. 2013). For example, the dynein adaptor lissencephaly 1 (LIS-1) is required for cortical pulling forces (Nguyen-Ngoc et al. 2007). In vitro studies have shown that LIS-1 promotes dynein's continued association with microtubules (Huang et al. 2012). Continued attachment to depolymerizing microtubule ends is likely needed for pulling force generation.

Dynein acting independently of the LIN-5 pulling force complex has been proposed to contribute to centration movements (Kimura and Kimura 2011). The dynein light chain protein, dynein roadblock (DYRB-1), anchors organelles for transport along microtubules. Because of the resistance of the viscous cytoplasm, this generates a dragging force at centrosomes. This mechanism may contribute to centration because microtubules extending toward the anterior are longer, and the majority of organelles are anterior of the maternal and paternal pronucleus at the time of meeting (Kimura and Kimura 2011). Another mechanism proposed to contribute to the centration of the pronuclei–centrosomal complex is the sliding of microtubules along the cortex, when microtubules are not attached end-on but laterally by a cortical LIN-5/dynein complex (Gusnowski and Srayko 2011). Lateral sliding is more prominent during centration than during anaphase and also more evident in the anterior than the posterior part of the embryo. It is unclear whether this sliding is actively regulated or a consequence of the geometry of the spindle and angle under which microtubules reach the cortex. Pushing forces of microtubules that buckle up against the cortex without being captured by dynein may further promote centration of the pronuclei–centrosomal complex (Laan et al. 2012).

5.4.5 *Microtubule Dynamics*

The generation of cortical pulling forces depends strongly on the dynamic instability of microtubules. This is supported by the finding that pulling force generation is lost after the administration of microtubule-stabilizing drugs (Nguyen-Ngoc et al. 2007). Microtubule plus tips grow with an estimated speed of ~0.5 μm/s and depolymerize with a speed of ~0.84 μm/s during catastrophe (Kozlowski et al. 2007). Live observations of embryos expressing plus-end tracking EBP-2::GFP have shown that catastrophe rarely occurs before a growing microtubule reaches the cortex. When reaching the cortex, however, catastrophe follows within 1–2 s (Kozlowski et al. 2007). Microtubules often reach the cortex as bundles of multiple filaments, which can be captured by pulling force complexes. In vitro experiments have indicated that a single depolymerizing microtubule generates six to ten times as much force as a motor protein (~50 pN, a single dynein motor ~7 to 8 pN) (Grishchuk et al. 2005; Kozlowski et al. 2007). It is possible that dynein motor activity is only required to maintain contact with the depolymerizing microtubule (Laan et al. 2012).

Force generators that are anchored in a more rigid cortex are less able to maintain contact with the depolymerizing microtubule and thus produce less force (Kozlowski et al. 2007). So far, there is no experimental evidence showing an asymmetry in spindle geometry between the anterior and posterior part of the *C. elegans* zygote. However, F-actin is enriched at the anterior cortex when the zygote enters mitosis. This most likely increases cortical rigidity and must therefore decrease the forces generated by anterior pulling force complexes. Indeed, several groups observed substantially increased pulling forces in the anterior after actin depletion by drug treatment (Afshar et al. 2010; Redemann et al. 2010; Berends et al. 2013). Thus, in addition to the regulation of the pulling force complex, a difference in the cortical rigidity caused by actin accumulation provides a possible cause of pulling force asymmetry.

5.5 Concluding Remarks

Through controlled spindle positioning, polarized cells decide between symmetric and asymmetric cell division and create daughter cells of the correct sizes at the appropriate positions. Studies in worms, flies, and mammals have provided detailed descriptions of representative symmetric and asymmetric cell divisions. By now, most of the basic players in polarity establishment and spindle positioning may be identified. It remains incompletely understood, however, how cell and tissue polarity translates to the proper positioning of the mitotic spindle. While the one-cell *C. elegans* embryo offers a relatively simple and well-tractable model, providing a complete answer to this question has proven to be remarkably difficult. Polarity-dependent differences in pulling force component localization, phosphorylation,

antagonist association, and actin accumulation have all been described to contribute to the lower anterior compared to posterior pulling forces that position the spindle during asymmetric division of the *C. elegans* zygote. Importantly, the identified principles appear to be conserved and apply broadly to the regulation of the cell division plane in other cell types and organisms.

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Part III
Cell Polarity and Cancer

Chapter 6
Par Proteins in Tumor Formation and Progression

Melina Mescher and Sandra Iden

Abstract Proteins of the evolutionary conserved family of partitioning-defective (Par) genes have emerged as key regulators of polarity and thus of cell and tissue architecture. Par proteins mediate a variety of cellular processes and couple control of cell shape to crucial signaling pathways regulating growth and survival, metabolism, cell fate, and differentiation. Alterations in adhesion, polarity, and architecture of tumor cells are hallmarks of cancer and implicated in tumor growth, invasion, and metastasis. Seminal work in *Drosophila* and mammalian cell culture suggested a molecular connection between the regulation of polarity and oncogenic processes. Recent advance stems from different mouse models revealing a causal link between polarity protein dysfunction and the formation and progression of cancer starting to shed light into some underlying mechanisms. It has become apparent that polarity signaling impacts on a multitude of processes involved in tumor formation and progression and that significant context dependency exists.

This chapter provides an overview of physiological processes that when disturbed facilitate tumor formation. Recent evidence from model organisms implicating Par protein dysfunction in the onset and progression of cancer is summarized. As Par proteins are tightly connected to aPKC signalling, data regarding a role of aPKC in cancer is also presented. Important findings from invertebrate tumor models will be reviewed, though the chapter focuses on functional data derived from mammalian systems.

Keywords AMPK • Animal models • ATM • Aurothiomalate • Cell cycle control • Lung cancer • Mammary cancer • Metformin • Oncogene • Oriented divisions • Par proteins • Peutz–Jeghers syndrome • Ras • Senescence • Skin cancer • Tumor suppressor

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K. Ebnet (ed.), *Cell Polarity 2*, DOI 10.1007/978-3-319-14466-5_6

6.1 Introduction

6.1.1 *Polarity Proteins: Molecular Regulators of Cytoarchitecture*

Establishment of cell asymmetry and orchestrated tissue architecture are critical to development, organ homeostasis, and regeneration. Almost every cell type in our body at least transiently polarizes based on intrinsic cues or upon extrinsic stimuli. Research of the past two decades unraveled a variety of molecular regulators of cell polarity that contribute to processes like directed cell migration, apicobasal polarity, oriented cell divisions, or directed vesicular transport. Most of these regulators are evolutionary conserved, and studies in a range of model organisms including fly, nematodes, zebra fish, frog, mice, and men identified common but also unique functions in polarization processes of multicellular organisms. In a genetic screen for mutants affecting the first asymmetric division of *C. elegans* embryos, Kenneth Kemphues and colleagues (1988) identified six so-called *par*titioning-defective genes, Par-1 to Par-6, as critical players in this process. Subsequent analyses revealed that specific localization and function of individual Par proteins are required for asymmetric segregation of cytoplasmic and cortical factors (Kemphues 2000). To date, evolutionary conservation of all Par family members except for Par-2 has been demonstrated (Goldstein and Macara 2007). In mammalian epithelia, these proteins localize to distinct sites at intercellular adhesions or in the cytoplasm, and the activation of the Ser/Thr kinases Par1, Par4, and aPKC and subsequent phosphorylation of polarity proteins define Par protein localization and interaction with other proteins (Fig. 6.1). In *Drosophila*, other essential regulators of polarity such as Crumbs, Stardust/Pals1, and Scribble, Discs Large (Dlg) and Lethal Giant Larvae (Lgl) have been discovered (Knust 1994; Bilder 2004). Based on their physical or genetic interactions, polarity proteins are typically grouped into three classical polarity protein complexes, the Par3, Scribble, and Crumbs complexes. Owing to their recently acknowledged role in the regulation of cell polarity, a group of "novel" polarity proteins including Yurt, Coracle, Neurexin, and Na^+/K^+ ATPase has been added to the list (Tepass 2012). Finally, a common molecular link between the regulation of cell–cell adhesion, cytoarchitecture, and tumorigenesis becomes evident from the relevance of primary tumor suppressor genes or intercellular adhesion molecules, e.g., VHL, APC, ASPP2, PTEN, NF2/Merlin, and E-cadherin, in the regulation of various polarization processes. Please refer to Volume 1 of this book for a comprehensive overview of the different sets of conserved polarity proteins and to learn about the basic regulation of cellular asymmetry in different systems.

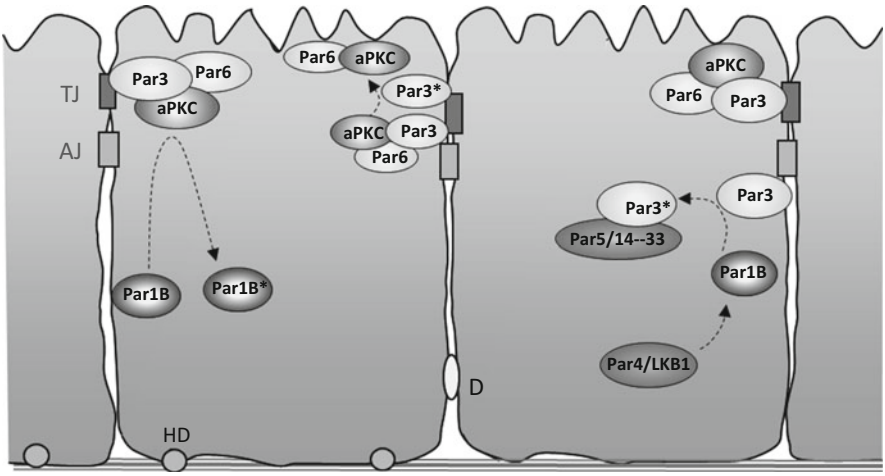

Fig. 6.1 Overview of mammalian Par proteins in simple epithelia. Par3 and Par6 are scaffold proteins that interact with the Ser/Thr kinase atypical PKC (aPKC). The Par3 complex localizes to tight junctions (TJs) and is required for proper TJ formation of simple epithelia. During apical domain assembly, Cdc42-mediated activation of aPKC results in phosphorylation of Par3 and dissociation of aPKC/Par6 to the apical plasma membrane. Other mammalian Par proteins include Par1/MARK, Par4/LKB1, and Par5/14-3-3. The Ser/Thr kinase Par1B is able to phosphorylate Par3, thereby regulating basolateral exclusion of Par3, which is subsequently bound by Par5/14-3-3 through phosphoserine recognition motifs in Par5. aPKC-mediated Par1B phosphorylation mediates the dissociation of Par1B away from the cortex. The tumor suppressor protein Par4/LKB1, a Ser/Thr kinase, phosphorylates a variety of downstream targets including Par1B and the tumor suppressor protein kinase AMPK (not shown), thereby controlling a range of cellular effects including intercellular adhesion, polarization, and energy metabolism. *TJ* tight junction, *AJ* adherens junctions, *D* desmosomes, *HD* hemi-desmosomes, *asterisks* phosphorylated protein

6.1.2 Tissue Homeostasis: Sore Points at Risk of Transformation

In complex organisms, tissue homeostasis requires the coordination of self-renewal, differentiation, and elimination of damaged cells, while the overall tissue function is preserved. Disturbances in tissue homeostasis may result in a failure to repair and renew and subsequent degeneration or in hyperplastic growth and development of cancer.

6.1.2.1 Hallmarks of Tumorigenesis and Cancer

Most human cancers are of epithelial origin and thus derived from cells that typically exhibit pronounced apicobasally polarized cytoarchitecture. Epithelial features such as tight intercellular adhesion, contact inhibition, or apicobasal polarity are often progressively lost in cancer. Tumorigenesis represents a multistep

process in which genetic aberrations—e.g., mutations or deletions in tumor suppressor genes and activating mutations or gene amplification of oncogenes—may cause initial benign hyperproliferation, accompanied by inhibition of cell death and enhanced stress resistance. The transition of benign cells to fully transformed malignant tumor cells typically requires secondary events and can involve aberrant cell–matrix adhesions, increase in migratory potential, invasion, and finally spreading to distal body sites where tumor cells extravasate and metastasize. Tumor cell invasion is frequently accompanied by epithelial-to-mesenchymal transition (EMT), a transcriptionally regulated process reflecting a switch in cellular programs toward decreased cell cohesion and increased motility. Tumor progression, however, can also be accompanied by collective cell invasion with maintained epithelial intercellular adhesions.

In an effort to provide a general concept illustrating the diversity of neoplastic disease, Hanahan and Weinberg (2000, 2011) defined major principles as hallmarks of cancer: (1) sustaining proliferative signaling, (2) evading growth suppressors, (3) resisting cell death, (4) enabling replicative immortality, (5) inducing angiogenesis, (6) activating invasion and metastasis, and recently added to these (7) reprogramming energy metabolism and (8) evading immune destruction. These hallmarks disclose the complex nature of cellular and environmental factors contributing to cancer. Importantly, to date polarity proteins have been implicated in the vast majority of above processes, and a future challenge will be to dissect specific mechanisms underlying the formation of cancer upon deregulation of cell polarity.

6.1.2.2 Cell Polarity and Growth Control

The establishment of cell polarity is coordinated with cellular growth control. In epithelial cells, growth is inhibited once cells reach a critical density that is sensed by molecules of intercellular adhesions (McClatchey and Yap 2012). When cell polarity is disrupted, cells may become insensitive to growth inhibition, favoring neoplasia. As became apparent more recently, polarity proteins can however also promote cellular growth signaling either indirectly by ensuring proper cytoarchitecture or more directly through interaction with critical mitogens and localizing them to specific cellular sites. The precise mechanisms as well as tissue specificity of cross talk between polarity and oncogenic signaling largely remain to be clarified.

Oncogene expression is often a consequence of activating mutations in genes normally controlling growth and mitotic signaling. Common proto-oncogenes are, e.g., secreted factors of the Wnt family; growth factor receptor tyrosine kinases (RTKs) such as EGFR or ErbB2; cytoplasmic signaling mediators like Ras, Raf, and Src; or transcription factors such as c-myc. During development or at homeostatic conditions, polarity proteins of the Par family impinge on several of these branches, and aberrant polarity protein function can cooperate with specific

oncogenes in tumor formation and progression as demonstrated in various tumor models outlined later in this chapter.

6.1.2.3 Tumor Suppression Through Checkpoint Controls and Senescence

The genome is constantly threatened by intrinsic or extrinsic insults, and DNA damaging events occur frequently in each cell. Similarly, mitotic defects may result in genome instability. Incorrect repair of DNA damage can give rise to manifestation of mutations, and subsequent alterations in gene function may promote cancer development. DNA repair defects are indeed linked to cancer susceptibility, as evidenced by a variety of congenital syndromes. Persistent DNA lesions instead cause cell cycle arrest followed by induction of apoptosis or cellular senescence. Though this serves to prevent tumorigenesis, these processes may lead to tissue dysfunction and degeneration. The maintenance of genome integrity is thus an important principle underlying tissue homeostasis, and multicellular organisms have evolved several checkpoints that control cellular responses upon damage. Though molecular features of tumor suppressor proteins are multifaceted, it is undisputed that many of them serve indispensable roles in distinct steps of cell cycle control, e.g., p16, p21, and p27, or DNA repair like BRCA1 and BRCA2. p53 represents a central component of the DNA damage response machinery and upon activation stops cell from growing through induction of senescence, initiates DNA repair, or, in case of irreparable damage, triggers apoptosis. p53 is thus a key molecular guard at the crossroad of cancer and aging (Reinhardt and Schumacher 2012). About 50 % of all human cancers exhibit mutational inactivation of p53.

Senescence is an irreversible growth arrest phenotype induced upon significant cellular insults including DNA and oxidative damage. Senescence therefore represents an important tumor-suppressive mechanism that permanently arrests cells at risk for malignant transformation. Therapeutic strategies aim at (re)activation of this pathway to counteract cancer cell growth. Recent work however has also revealed that senescent fibroblasts in the tumor microenvironment through secretion of pro-inflammatory mediators are able to promote tumor progression, a phenomenon termed "senescence-associated secretory phenotype (SASP)" (Coppé et al. 2010; Velarde et al. 2013). Though at first glance not coupled to the regulation of cytoarchitecture, evidence accumulates implicating polarity proteins and their kinases in cellular and systemic DNA damage responses, cell cycle arrest, and senescence (see below).

6.1.2.4 Regulation of Oriented Division and Cell Fate

Homeostasis of proliferating tissues requires a concerted ratio of stem cell maintenance and generation of daughter cells that differentiate and fulfill specific tissue functions. Stem cells can be the cells of origin of a variety of cancers, and defective

fate decisions that result in an increase or abnormal behavior of stem cells may thus favor tumorigenesis. One way to balance self-renewal and differentiation is to generate differential cell fate through asymmetric cell division (ACD). Disturbances in ACD toward symmetric divisions may either fuel neoplastic growth and tumorigenesis or result in the loss of self-renewal capacity. In the past decade, studies particularly in *Drosophila* neuroblasts and *C. elegans* embryos identified core molecular players of mitotic spindle orientation and asymmetric cell divisions. These include Par3 (Bazooka in flies), aPKC, and Par6, which localize apically in dividing neuroblasts, and a spindle orientation complex consisting of Gαi, LGN (Pins), and NuMA (Mud). This complex together with dynactin is thought to exert pulling forces on astral microtubules to align the mitotic spindle with apical polarity cues transmitted by the Par3 complex (Williams and Fuchs 2013). Though it is not yet well understood to what extent these mechanisms are conserved, oriented divisions in mammals have been observed in multiple tissues and organs including the central nervous system, skin epidermis, mammary gland, lung bud epithelium, intestine, and satellite cells of skeletal muscle. Among these, a role for mammalian Par3 as well as LGN, mIns, NuMA, and dynactin in oriented divisions and cell fate determination has been proposed (Cicalese et al. 2009; Quyn et al. 2010; Poulson and Lechler 2010; El-Hashash and Warburton 2011; Williams et al. 2011; Brack and Rando 2012; Lancaster and Knoblich 2012). Evidence is mounting that impaired oriented divisions are involved in mammalian cancer development. In early-stage dividing colon cancer stem cells (CSCs), for instance, tumor-suppressive miRNAs have recently been demonstrated to target Notch, thereby directing cell fate decisions toward differentiation and preventing the expansion of CSCs (Caruso et al. 2012; Bu et al. 2013). Consequently, the modulation of oriented divisions by proteins of the Par protein network has recently been implicated in tumorigenesis.

6.1.3 Animal Disease Models: Important Tools to Understand Human Cancer

In vivo models are of tremendous importance to understand the genetic, cellular, and molecular processes underlying neoplastic growth, invasion, and metastasis in the context of the tissue. Principle pathways known to induce human malignancies can be selectively activated or inhibited to assess underlying events. A screen for mutants affecting embryonic epithelial architecture in *Drosophila* was instrumental to identify tumor suppressors of the Scribble complex (Bilder 2000; Bilder 2004). Similarly, the power of *Drosophila* genetics was important to reveal mutations and oncogenes that cooperate with Ras in development and invasive behavior of tumors of the eye imaginal disk (Brumby and Richardson 2003; Pagliarini and Xu 2003; Uhlirova and Bohmann 2006; Brumby et al. 2011). While for some time neglected, also *C. elegans* reflects a promising system to analyze oncogenic processes in a

well-defined organism with conserved programs that control, e.g., growth factor signaling, differentiation, maintenance of genome stability, or cell death pathways (Kirienko et al. 2010). As vertebrates with short life span, zebra fish has proven advantageous for functional investigations of different tumors relevant for human disease, including melanoma, neuroblastoma, and various leukemias (Mione and Trede 2010; van der Velden and Haramis 2011). A variety of findings discussed in this chapter have been obtained in autochthonous or orthotopic experimental mouse models that to a certain degree mimic the genetic causes, signaling processes and histopathology of human cancer.

6.2 Par Proteins in Tumor Formation

6.2.1 Disturbed Polarity Protein Function and Its Consequence for Tumor Formation

Loss of polarity is considered a prerequisite for tumor formation and progression. In *D. melanogaster*, mutations in genes of the Scribble complex, Scrib, Dlg, and Lgl, cause loss of apicobasal polarity and neoplastic outgrowth when mutated or combined with Ras mutations (Bilder 2004). Whether polarity proteins however also contribute to mammalian malignancies remained to be demonstrated. In vitro data derived from mammalian cell cultures indicated that in the presence of oncogenes or after the loss of tumor suppressors, polarity protein dysfunction fosters proliferation and motility. Whether this holds true for the in vivo tissue context is often unclear. Particularly proteins of the Par3 and Scribble complexes have been implicated in control of growth and survival signaling, though no common theme exists. Human Scribble complex proteins likely act as tumor suppressors, whereas the function of the Par3 complex appears more diverging with clear evidence also for pro-oncogenic functions. This is reflected by expression in human cancers: Scribble complex proteins as well as Par4/LKB1 show mainly reduced protein levels, whereas Par3, aPKC, and Par6 can be both up- and downregulated in carcinoma (Huang and Muthuswamy 2010). Beyond expression, also mislocalization of polarity proteins is frequently observed in human cancer (Ellenbroek et al. 2012). Pro-oncogenic functions have been particularly proposed for aPKCλ/ι as it is required for the transformation and tumorigenesis of pancreatic and lung cancer cells (Murray et al. 2012). Several recent publications now unraveled that disturbed Par polarity protein functions indeed impact tumor formation and progression in various tissues (Fig. 6.2). Noteworthy, not all effects caused by loss of Par proteins are directly ascribed to disturbed cytoarchitecture. Instead, a variety of pathologies is likely caused by primary changes in metabolic programs or stress signaling.

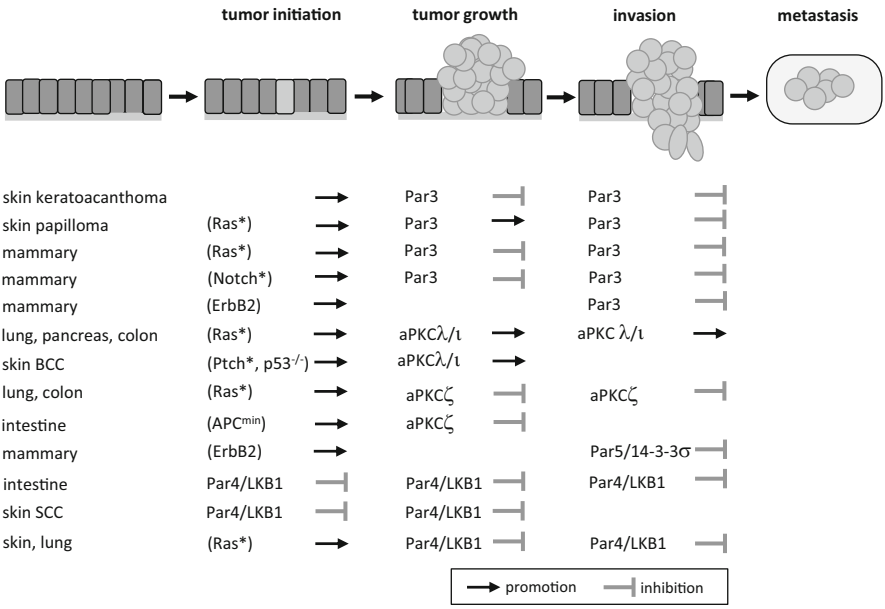

Fig. 6.2 In vivo consequence of disrupted Par or aPKC function on initiation, growth, invasion, and metastasis of mammalian cancers. Examples shown are based on experimental animal models as described in the text. Oncogenic mutants are indicated by *asterisks*. Inactivation of LKB1 is sufficient to initiate tumors and mimics Peutz–Jeghers tumor susceptibility syndrome. Loss of function of other Par proteins or aPKC seems insufficient to induce tumors; however, a range of oncogenes including mutant Ras, Notch, and ErbB2 cooperate with impaired polarity protein function to promote tumor growth, invasions, and/or metastasis. Details are outlined in the text

6.2.1.1 Par3, aPKC, and Par6

Initial evidence for a role of Par3 complex proteins in neoplasia comes from *Drosophila*. Mutations in these components alone were not sufficient to drive tumorigenesis (Pagliarini and Xu 2003). In fact, Brumby, Richardson, and colleagues (2011) reported that overexpression of aPKC cooperates with active Ras, suggesting that activation rather than loss of apical polarity proteins facilitates tumorigenesis.

The role of proteins of the Par3 complex in mammalian tumorigenesis has recently been addressed in several studies using mouse models and assessing patient specimen. They pointed out that dependent on the tissue context, members of the Par3–aPKC complex may act both as pro-oncogene and as tumor suppressors. Strikingly, genetic ablation of aPKCλ/ι promotes Ras-driven tumorigenesis in the lung, colon, and pancreas, with accumulating evidence that aPKC acts through stimulating Rac and MEK/ERK (Murray et al. 2009; Regala et al. 2009; Scotti et al. 2010). Moreover, chemical aPKC inhibition is able to suppress Smo/Gli-mediated BCC growth (Atwood et al. 2013) (Fig. 6.2). In correlation with this, aPKCλ/ι is often overexpressed in primary and metastatic tumor tissue (Murray

et al. 2012). The function of the closely related aPKCζ isoform in cancer seems less clear. Interestingly, aPKCζ serves to counteract rather than promote lung tumors through regulation of IL6 upon nutrient deprivation (Galvez et al. 2009). Moreover, aPKCζ prevents intestinal tumorigenesis likely through regulation of metabolic signaling as aPKCζ deficiency facilitated metabolic reprogramming of cancer cells to sustain the starving environment in the tumor tissue (Ma et al. 2013) (Fig. 6.2). aPKCζ may also target and regulate c-myc expression through phosphorylation critical in the suppression of prostate tumors (Young et al. 2013). Incongruent with above findings, aPKCζ is also able to inhibit apoptosis and to reduce sensitivity of cancer cells toward chemotherapeutic agents, indicating for a context-specific role of aPKCζ (Rimessi et al. 2012).

In invertebrates, the Par3 complex has been implicated in asymmetric divisions. As disturbed oriented divisions of stem/progenitor cells in mammals are considered to contribute to cancer, an important measure will identify if defective division processes and fate decisions are causal for tumors elicited by polarity protein dysfunction. In skin epidermis, ACD promotes epidermal stratification (Lechler and Fuchs 2005) and is also observed in adult tissue homeostasis (Niessen et al. 2012). Importantly, epidermal VEGF has recently been implicated in the promotion of stemness, increase in symmetric divisions of CSCs accompanied by expansion of CSCs (Beck et al. 2011), thus linking oriented divisions to the development of skin cancer. It is therefore tempting that mice with impaired polarity protein function are prone to skin tumors as consequence of altered division processes. Loss of aPKCλ/ι in the epidermis however results in abundant ACD and a loss of stem/progenitor cells with age rather than predominant symmetric divisions and amplification of stem cells (Niessen et al. 2013). One would therefore predict that loss of aPKCλ/ι and subsequent increase in ACDs oppose skin tumorigenesis. In the hematopoietic system, aPKCζ and aPKCλ/ι are even dispensable for stem cell function and fate determination (Sengupta et al. 2011). Interestingly, in glioblastoma cells, loss of the tumor suppressor PTEN stimulates aPKC-mediated phosphorylation and inactivation of the basolateral polarity protein Lgl, thereby maintaining tumor-initiating cells undifferentiated (Gont et al. 2013). This supports a view that at least in some systems, aPKC promotes tumorigenesis through determination of cell fate. The recent identification of a cancer-associated somatic aPKC mutation affecting binding of aPKC to the tumor suppressors Lgl1/2 provided first evidence that interaction of aPKC with other polarity proteins may actually serve to prevent cancer (Linch et al. 2013). Importantly, aPKCλ/ι has been recently also implicated in the suppression of cellular senescence of breast and glioblastoma cell lines, adding another field of action of aPKC. This function of aPKCλ/ι appears to be selective for cancer cells, does not require substantial DNA damage, but instead is reported to involve defective mitosis (Paget et al. 2012). Above findings underline that future efforts should aim at identifying specific interaction profiles of aPKC under physiologic versus oncogenic conditions.

Recently, three studies provided insight into the in vivo role of Par3 in mammalian tumorigenesis using skin and mammary tumor models. Conditional deletion of Par3 in mouse epidermis did not elicit skin tumors. When combined with a

two-stage skin carcinogenesis approach that is based on induction of oncogenic mutations of the endogenous Ras locus, epidermal Par3 deletion however resulted in substantial reduction of papilloma formation and growth (Fig. 6.2). This was accompanied by reduced proliferation, reduced cortical localization of Sos2 and Ras, as well as loss of junctional aPKC and redistribution to the cytoplasm. Par3 deficiency caused impaired MEK/ERK activity, whereas apoptotic cell death was increased (Iden et al. 2012). Artificial targeting of aPKC to the membrane rescued impaired ERK activity observed in Par3-deficient cells. These data indicate that Par3 exhibits pro-oncogenic function in papillomagenesis likely by coordinating the precise localization of Ras signaling components and aPKC to intercellular adhesions and thereby promoting efficient downstream mitogenic signaling via MEK/ERK (Iden et al. 2012) (Fig. 6.3). The identical system however also revealed tumor-suppressive features of Par3 in the skin as epidermal Par3 deletion predisposed mice to formation of keratoacanthomas, aggressively growing cutaneous tumors frequently diagnosed in humans. In a significant subset of cancer patients receiving BRaf inhibitor therapy, these tumors develop probably due to paradoxical ERK activation downstream of endogenous CRaf (Oberholzer et al. 2012). Interestingly, such activation also occurs in Par3-deficient keratoacanthoma (Iden et al. 2012), suggesting that Par3 usually serves to restrict CRaf function. Though not yet formally shown, keratoacanthoma and papilloma are thought to derive from different cellular origin (Perez-Losada and Balmain 2003), each with different localization and function of Par3. Future studies are required to uncover the contribution of cellular context and tumor microenvironment in this system.

Further insight into the complex role of Par3 in mammalian cancer comes from two recent studies addressing the function of Par3 in breast cancer. In different transplantation-based mouse mammary tumor models, loss of Par3 in the context of oncogenic Notch, Ras, or ErbB2 predominantly affected invasion and metastasis (see below; McCaffrey et al. 2012; Xue et al. 2013). Par3 deficiency affected primary mammary tumor growth only in the presence of Notch and Ras oncogenes (McCaffrey et al. 2012), whereas epidermal loss of Par3 resulted in reduced growth of Ras-mediated skin tumors (Iden et al. 2012) (Fig. 6.2). These findings further support the idea that Par3 function in tumor growth is subject to oncogene and/or cell- or tissue-specific parameters.

It is currently mostly unclear how Par6 contributes to the tumor phenotypes described above. In contrast to Par3, Par6 is mostly overexpressed in breast cancers (Huang and Muthuswamy, 2010). In vitro data indicate that Par6 promotes MEK/ERK activation through interaction with aPKC and Cdc42, whereas overexpression of Par6 did not affect polarization and morphogenesis of mammary epithelial cells (Nolan et al. 2008). Mammals exhibit three Par6 genes, Par6A, Par6B, and Par6C, which complicates loss-of-function approaches. Alan Fields and coworkers previously characterized an inhibitor of PB1 domain interactions that affects the binding of aPKC to Par6 (Stallings-Mann et al. 2006). This gold compound aurothiomalate (ATM) efficiently blocks oncogenic functions of aPKC in non-small cell lung cancer (Murray et al. 2012), suggesting that aPKC-bound

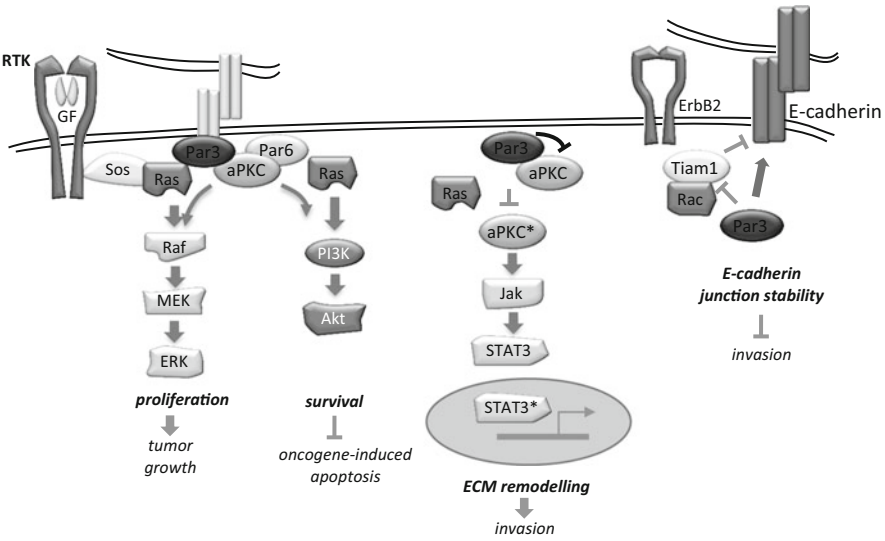

Fig. 6.3 Polarity protein signaling impinges on central cascades that influence tumor formation and progression. Four different signaling axes that are modulated by Par3/aPKC are shown. From *left* to *right*: In epidermal keratinocytes, Par3 serves to recruit aPKC and Ras signaling components to the plasma membrane resulting in efficient downstream signaling toward Ras/Raf/MEK/ERK promoting proliferation and ultimately growth of skin papillomas. Moreover, Par3 and aPKC are able to promote cell survival by stimulation of PI3K–Akt signaling, thereby counteracting oncogene-induced apoptosis. In mammary epithelial cells expressing Ras or Notch oncogenes, loss of Par3 results in aberrant aPKC activation and subsequent JAK/STAT3 activation and transcriptional regulation of ECM components, which promote tumor cell invasion. In ErbB2-overexpressing mammary epithelial cells, Par3 has been reported to control Tiam1–Rac signaling and to stabilize junctional E-cadherin and cell–cell cohesion, which prevents tumor cell invasion and metastasis. *Asterisks* activated protein. Details see text

Par6 takes part in downstream oncogenic signaling. It remains to be demonstrated though how loss of Par6 isoforms affects tumorigenesis in vivo.

6.2.1.2 Par4/LKB1 and Par1/MARK

Liver kinase B1 (LKB1) is the human homologue of *C. elegans* Par4 and thus far the only true tumor suppressor gene among the Par proteins. Par4/LKB1 encodes a serine/threonine kinase involved in metabolism, polarity, cytoskeleton organization, and proliferation. Par4/LKB1 directly phosphorylates and activates at least 14 downstream kinases. Among these is AMP-activated protein kinase (AMPK), a master regulator of cellular and organismal energy metabolism. AMPK controls lipid, glucose, and cholesterol metabolism in specialized metabolic tissues, such as liver, muscle, and adipose tissues, rendering AMPK-activating compounds a key therapeutic strategy for diabetic patients. Other kinases activated by Par4/LKB1

include a family of Par1 homologues termed microtubule affinity-regulating kinases (MARKs) in humans (Fig. 6.1).

Mutations in LKB1 on human chromosome 19p13 were identified as cause for Peutz–Jeghers syndrome (PJS), a disorder characterized by the development of benign polyps in the gastrointestinal (GI) tract and increased predisposition to many malignancies, including those of colon, breast, ovarian, pancreatic, and lung tissues (Shackelford and Shaw 2009). Moreover, somatic LKB1 mutations are frequently found in human lung (Ji et al. 2007) and cervical cancers (Wingo et al. 2009). Various mouse models with conditional gene deletion are beginning to shed light into tissue-specific functions of Par4/LKB1 and relevant downstream pathways (for comprehensive overview, see Ollila and Mäkelä 2011). Constitutive deletion of Par4/LKB1 in mice revealed a crucial role in embryonic development with severe vascular and neural defects of LKB1−/− embryos. Heterozygous germ line mutation of Par4/LKB1 in mice recapitulates PJS with polyp formation in the GI tract. Impaired spindle orientation accompanied by significant mislocalization of the Par4/LKB1 target AMPK has been observed in epithelial cells of the GI (Wei et al. 2012), opening the possibility that PJS is at least in part caused by abnormal regulation of oriented divisions and cell fate. Moreover, epidermal loss of Par4/LKB1 results in spontaneous squamous cell carcinomas, whereas heterozygous loss of Par4/LKB1 cooperates with Ras-induced tumors of the lung and skin (Gurumurthy et al. 2008; Joshi et al. 2008), further substantiating the role of Par4/LKB1 as mammalian tumor suppressor (Fig. 6.2). In zebra fish, interestingly, Par4/LKB1 is dispensable for embryonic survival but becomes essential under conditions of energetic stress (van der Velden et al. 2011).

Despite convincing data of tumor-suppressive roles of Par4/LKB1, a major question often remains as to what extent tumors resulting from loss of Par4/LKB1 are due to abnormal metabolic regulation, altered cytoarchitecture, or other causes. In several tissues, Par4/LKB1-mediated activation of AMPK is responsible for tumor suppression, whereas polarity defects in the pancreas caused by loss of Par4/LKB1 suggested that at least in certain tissues, Par4/LKB1-mediated polarity signaling toward Par1/MARK isoforms is prevailing (Hezel et al. 2008). Also other AMPK-independent routes of Par4/LKB1-mediated tumor suppression have been proposed that involve inhibition of Akt (Joshi et al. 2008) or inhibition of YAP, a transcriptional co-activator controlling tissue homeostasis in a density-dependent manner (Nguyen et al. 2013).

6.2.1.3 Par5/14-3-3

Par5, a 14-3-3 family protein, has also been implicated in tumorigenesis. 14-3-3 proteins bind to specific phosphoserine/phosphothreonine motifs on a wide variety of interacting proteins including transcription factors, biosynthetic enzymes, cytoskeletal proteins, signaling molecules, apoptosis factors, and tumor suppressors. In humans, the 14-3-3 family comprises seven isoforms. Of these, 14-3-3σ is considered a G(2)-M checkpoint control gene and tumor suppressor, whereas the other

isoforms appear to promote tumorigenesis. Upon DNA damage, 14-3-3σ is upregulated by p53 and BRCA1 and elicits growth arrest (Aprelikova et al. 2001). Silencing of 14-3-3σ is implicated in tumor progression of malignant melanoma through loss of cell cycle control, impaired induction of cellular senescence, and increased migratory capacity (Schultz et al. 2009) and associated with multiple other human malignancies including lung and breast cancer as well as hepatocellular carcinoma. Genetic ablation of 14-3-3σ in mice resulted in accelerated onset and increased progression of mammary and salivary tumors in experimental ErbB2-based tumor models, whereby in mice harboring one conditional allele epigenetic inactivation of the remaining intact allele was frequently observed (Ling et al. 2012). Interaction of 14-3-3σ with Par3 previously detected in mammary epithelial cells (Ling et al. 2010) seems to contribute to such tumor-suppressive effect, as 14-3-3σ mutant mice harbored mislocalized Par3, impaired intercellular adhesions, and increased invasive phenotypes, which could be restored upon re-expression of 14-3-3σ in the presence of the ErbB2 oncogene (Ling et al. 2012) (Fig. 6.2).

In contrast, ubiquitously expressed 14-3-3ζ bears tumor-promoting functions through its interaction with various oncogenes including B- and CRaf, Bad, p85PI3K, and FoxO transcription factors (Freeman and Morrison 2011). 14-3-3ζ is upregulated in prostate cancer and activates proliferation, cell survival, and androgen receptor transcriptional activity (Murata et al. 2012). 14-3-3ζ binding to the p85 regulatory subunit of PI3K and subsequent activation of mitogenic PI3K–Akt signaling are thought to contribute to its pro-oncogenic effect (Neal et al. 2012). Moreover, high 14-3-3ζ expression is associated with recurring disease and poor survival of patients with oral squamous cell carcinomas, breast cancer, and non-small cell lung carcinomas (Neal and Yu 2010), and elevated levels of 14-3-3ζ have been linked to drug resistance to certain anticancer therapies (Freeman and Morrison 2011).

Together, similar to the dual and mostly opposing role of aPKCζ and aPKCλ/ι in cancer, also individual proteins of the family of 14-3-3 proteins bear heterogeneity concerning their specific role in mammalian cancer.

6.3 Par Proteins in Tumor Progression

As apparent from certain examples above, disturbed polarity protein function next to primary tumor formation often also affects distinct events during tumor cell invasion and metastasis. These processes are diverse in nature and may include cohesion between cells, cell–ECM interactions, cellular or metabolic reprogramming, sensitivity toward microenvironmental measures, cytoskeletal reorganization fueling cell motility, regulation of survival and growth signaling outside the primary tumor niche, and induction of anoikis.

6.3.1 Par3, aPKC, and Par6

In a *Drosophila* genetic screen, Pagliarini and Xu (2003) uncovered mutations in polarity proteins that caused noninvasive tumors of the eye disk to become metastatic. This study revealed that the fly orthologs of various polarity proteins and their interacting proteins such as Par3, Scribble, Pals1, and Cdc42 act as metastasis suppressor in Ras-induced tumors.

Loss of Par3 expression but overexpression and cytoplasmic accumulation of aPKCλ/ι is frequently associated with progression of different human cancers such as cervical, skin, and breast cancer and hepatocellular carcinoma (Huang and Muthuswamy 2010; Iden et al. 2012; McCaffrey et al. 2012; Jan et al. 2013; Liu et al. 2013). Recent loss-of-function approaches in mouse tumor models revealed that mammalian Par3 indeed serves to prevent invasion of skin and mammary tumors (Iden et al. 2012; McCaffrey et al. 2012; Xue et al. 2013) (Fig. 6.2). In contacting keratinocytes as well as mammary epithelial cells, Par3 serves to localize aPKC to sites of intercellular adhesions, and Par3 deficiency causes abnormal cytoplasmic localization of aPKC (McCaffrey and Macara 2009; Iden et al. 2012). Above studies in murine mammary tumor models provided important insight into the role of Par3 in breast cancer progression (see also Part 3, Chap. 8). The group of Ian Macara demonstrated that RNAi-mediated loss of Par3 combined with expression of oncogenic Ras or Notch (McCaffrey et al. 2012) results in increased invasion and metastasis. In the presence of oncogene, aPKC was hyperactive and caused activation of JAK/STAT signaling and increased MMP9-mediated ECM destruction (McCaffrey et al. 2012) (Fig. 6.3). Moreover, loss of Par3 is frequently observed in human breast cancer and correlates with elevated MMP9 expression (McCaffrey et al. 2012). Increased tumor cell invasion and metastasis upon loss of Par3 could also be demonstrated in another study that used an ErbB2-driven breast cancer model (Xue et al. 2013), although impaired E-cadherin junction stability and decreased cellular cohesion were considered the primary causes for this phenotype. Par3 is able to interact with the Rac-GEF Tiam1 in different cell types, although the outcome of this on Rac signaling seems context dependent (Iden and Collard 2008). Loss of Par3 in breast cancer cell lines resulted in constitutive, Tiam1-mediated Rac stimulation and uncontrolled Arp2/3 activity, whereas pharmacological inhibition of Tiam1–Rac signaling restored E-cadherin junction stability and decreased cell invasion and scattering (Xue et al. 2013) (Fig. 6.3). This suggests that Par3 serves to tightly control and restrict Tiam1–Rac action in mammary cells. In addition, Par3 may also dictate the mode of tumor cell migration as a requirement of Par3 and Par6, and interaction with DDR1 has recently been demonstrated for collective migration of squamous cell carcinoma cells (Hidalgo-Carcedo et al. 2011). Together, these studies indicate that Par3 in breast cancer acts as invasion and metastasis suppressor likely through support of cellular cohesion and/or control of aPKC activity. Several functional studies indeed demonstrated that aPKCλ/ι promotes tumor progression. Constitutive active aPKC (aPKCλ/ι-CAAX) increased intestinal tumor metastasis, whereas loss of aPKCλ/ι

had the opposite effect (Murray et al. 2009) (Fig. 6.2). aPKCλ/ι has further been shown to induce invasive phenotypes of non-small cell lung cancer cells, where aPKC-mediated phosphorylation of Par6 occurs downstream of TGFß receptor signaling to promote EMT, invasion, and metastasis (Regala et al. 2009; Gunaratne et al. 2013). Similar effects have been reported for gastric cancer, where aPKC expression correlates with lymphatic invasion and poor prognosis (Yoshihama et al. 2013), and esophageal cancer, where aPKC may confer resistance to anoikis through activation of Akt-dependent survival signaling (Liu et al. 2011) (Fig. 6.3). Together, data derived from these animal models highlight that the Par3–aPKC axis does not solely promote or prevent tumor progression and metastasis but that fine-tuned expression, localization, and activity of these polarity proteins are crucial for proper tissue homeostasis, whereas imbalances in this axis may promote malignancy.

6.3.2 Par4/LKB1 and Par5/14-3-3

Similar to Par3, Par4/LKB1 is able to suppress tumor cell invasion and metastasis. Homozygous inactivation of LKB1 cooperates with KRas, with LKB1-deficient tumors resulting in a higher tumor spectrum and increased metastasis of mutant Ras lung tumors (Ji et al. 2007) (Fig. 6.2). Inactivation of Par4/LKB1 has recently also been reported to promote esophageal cancer cell migration and invasion in vitro (Gu et al. 2012), and Par4/LKB1 is shown to phosphorylate Smad4, thereby preventing DNA binding of Smad4 and counteracting TGFß-induced EMT of breast epithelial cell lines (Morén et al. 2011). Whether Par1/MARK proteins serve as PAR4/LKB1 target in tumor progression is currently not known.

Targeted disruption of 14-3-3σ in mice indicated that 14-3-3σ prevents metastasis of ErbB2-driven breast cancer likely through maintaining intercellular adhesions (Ling et al. 2012). In contrast, other 14-3-3 isoforms seem to promote EMT and cancer progression, e.g., through direct binding to Snail and TGFß receptor 1 (Freeman and Morrison 2011). These examples demonstrate again that individual components of the Par protein network may have antagonistic effects on processes underlying tumor initiation and progression.

6.4 Therapeutic Outlook

Fundamental research in invertebrate and vertebrate model organisms identified key molecules required for coordination of cell polarity, and a variety of human pathologies has been associated with disturbed polarity signaling. Typically, however, decades pass before any of such basic discoveries enter the arena of diagnostic value, clinical application, and therapy. Animal models with defective polarity signaling may serve as in vivo systems to better understand molecular events

underlying specific pathologies, e.g., Peutz–Jeghers syndrome in mice with loss of LKB1 or keratoacanthoma formation upon epidermal Par3 deletion. To date several studies have evaluated the significance of altered polarity protein expression, localization, or activity of polarity-related kinases in human malignancies to explore whether these parameters may serve as prognostic markers. In hepatocellular carcinoma, for instance, Par3 overexpression is a risk factor of extrahepatic metastasis and associated with decreased 5-year survival (Jan et al. 2013). Moreover, high expression of aPKCλ/ι correlates with poor survival of pancreatic cancer patients (Scotti et al. 2010), and reduced apical localization with cytoplasmic accumulation of aPKC correlates with cervical cancer progression (Liu et al. 2013).

Importantly, also therapeutic targeting of the polarity machinery for the prevention of human cancer is worthwhile as intervention strategies centered around two critical kinases linked to the Par polarity network, AMPK and aPKC, have been excelled. Metformin, a first-line antidiabetic drug introduced into the clinic more than 50 years ago, acts through inhibition of gluconeogenesis via activation of the LKB1/AMP-activated protein kinase pathway in the liver. Administration of metformin is associated with reduced cancer incidence and increased life span of patients, and the tumor-suppressive potential of metformin has been functionally confirmed (Ben Sahra et al. 2010). For instance, in two-stage skin carcinogenesis in mice, metformin administration antagonizes TPA-mediated tumor growth. Although metformin-induced responses in different systems not always involve AMPK, prevention of TPA-induced papilloma growth involved AMPK activation and decreased downstream signaling of mTORC1 and p70S6K (Checkley et al. 2013). Secondly, pharmacological inhibition of aPKC functions is currently assessed clinically. A phase I dose escalation study of the PKCι inhibitor ATM for treatment of advanced non-small cell lung cancer, ovarian cancer, and pancreatic cancer has very recently been completed successfully (Mansfield et al. 2013). Next to aPKC-mediated oncogenic signaling toward Rac–PAK–MEK–ERK, which is discussed as predominant signaling axis in ATM-sensitive tumors, inhibition of aPKC may also reestablish sensitivity toward chemotherapy through induction of apoptosis (Murray et al. 2012; Rimessi et al. 2012) or toward cellular senescence (Paget et al. 2012). Inhibition of aPKC signaling may thus represent an important concept for prevention and/or therapy of human cancer.

Together, these examples highlight that targeting polarity protein signaling has entered the clinical level, and application of above compounds in larger cancer patient cohorts will hopefully prove their long-term success.

6.5 Conclusion and Future Challenges

Research in the past few years has revealed exciting advances that elucidated the vast role of Par polarity proteins in fundamental processes underlying the prevention or induction of cancer. Different animal models were instrumental to assess the in vivo role of these proteins in the onset and progression of cancer in the

organismal context. They could confirm and detail certain functions previously indicated by cell culture approaches or have been important to uncover novel and unexpected features of polarity proteins in cancer. These include the modulation of and response to microenvironmental factors, which can only be sufficiently assessed in model organisms. A future challenge will be the identification of the primary events within polarity or non-polarity processes that initiate or prevent tumorigenesis and malignancy upon polarity protein dysfunction. In addition, deeper insight into tissue-specific molecular programs linking cell polarity, oncogenic signaling, and tumorigenesis is required for translational approaches. Moreover, and though already on a good track, development and application of small molecule inhibitors targeting Par proteins and their signaling network will further our understanding of polarity protein functions in tumor-initiating events and metastatic processes. These may hopefully also prove useful for the design of new strategies for therapies of human cancer.

Acknowledgments We are grateful for the financial and/or infrastructural support by the Excellence Initiative by the German federal and state governments (CECAD Cologne), the German Research Foundation (CRC829 and CRC832), Köln Fortune, the Stiftung Kölner Krebsforschung (EI—Exzellenz Initiieren), and the Center for Molecular Medicine Cologne (CMMC).

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Chapter 7
Cell Polarity: A Key Defence Mechanism Against Infection and Cancer Cell Invasion?

Yihua Wang and Xin Lu

Abstract It is now emerging that a number of cellular targets of pathogens are involved in the establishment and/or maintenance of epithelial cell polarity. Increasing evidence also suggests that cancer-causing pathogens such as *Helicobacter pylori* (*H. pylori*) and human papilloma virus (HPV) may induce oncogenesis by disrupting cell polarity. This is mainly achieved through their ability to deregulate the function of cell polarity components and/or regulators. Hence cell polarity represents the first line of defence against infection. Interestingly, EGFR/RAS oncogenic signals also induce cancer cell invasion by inducing epithelial to mesenchymal transition (EMT). Since the loss of cell polarity is a prerequisition of EMT, cell polarity also represents the last line of defence against cancer cell invasion. As such we argue that cell polarity may be a key defence mechanism against infection and cancer cell invasion. The potential role of cell polarity as a gatekeeper against cancer through its ability to regulate asymmetric cell division and tumour suppression has been discussed in a number of recent reviews. In this review we will focus on the role of cell polarity as a potential target of infection and cancer cell invasion.

Keywords Cancer • Bacteria *H. pylori* • Cell polarity • Cancer-causing virus HPV • Infection • RAS oncogene

7.1 Introduction

There are over 260 cell types in our body, and around 200 different types of cancer have been reported that are derived from approximately 60 different organs. To a large extent, cancers derived from different organs and cell types have distinct features and distinct genetic mutation signatures. This heterogeneous nature contributes to the complexity and difficulty in treating cancers. However, one common feature of cancer is excessive cell growth due to enhanced cell proliferation and/or

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K. Ebnet (ed.), *Cell Polarity 2*, DOI 10.1007/978-3-319-14466-5_7

reduced cell death. Additionally, over 80 % of human cancers originate from epithelial cells. Therefore, it is critical to study the common features of epithelial cancers as it will help us to understand why most cancers derive from epithelial cells and will also guide us to develop more effective treatment for epithelial cancers.

Epithelial cells are widely distributed, lining the surface of the animal body and internal cavities (e.g. digestive tract and circulatory system) and form many glands. They are involved in diverse functions including secretion, absorption and barrier functions. There are three main epithelial cell types: 1) squamous epithelial cells that are mainly found in the skin, oral cavity and oesophagus; 2) cuboidal epithelial cells that are located in ductal tissues such as the mammary gland and the prostate; and 3) columnar epithelial cells that are mainly located in the stomach and intestine. The common features of epithelial cells are their adherence to each other via tight junctions, desmosomes and adherence junctions, and their polarity. Epithelial cells have planar cell polarity and apical–basal polarity, and they both play crucial roles in the development and maintenance of epithelial tissue homeostasis. Apical–basal polarity is most prominent in cuboidal and columnar epithelial cells, and it is the focus of this review. It is defined by the apical membrane facing the outside surface of the body, or the lumen of internal cavities, and the basolateral membrane oriented away from the lumen. The basolateral membrane acts as a scaffold for the epithelial cells to join the underlying connective tissue and cell–cell junctions. Tight junctions (TJs) are located at the apical side of the adherens junction (AJs) and are often partially localised with the apical polarity complex. Gap junctions, desmosomes and hemi-desmosomes are located at basolateral membranes of epithelial cells and regulate communication between cells and with the extracellular matrix (ECM). TJs are crucial for epithelial barrier function providing a tight seal between the membranes of the neighbouring cells, while AJs use the forces that are generated by the actin cytoskeleton to keep the cellular membranes of neighbouring cells together (Suzuki and Ohno 2006). TJs and AJs also limit the paracellular permeability of fluid and ions between the lumen and the interstitium (Hartsock and Nelson 2008).

Three groups of proteins play a central role in the establishment and maintenance of apical–basal cell polarity: the Crumbs–PALS1 (Stardust)–PATJ (Crumbs complex) and Par3 (Bazooka)–Par6–aPKC (Par complex) complexes, which are found apically, and the lethal giant larvae (Lgl)–Scribble (Scrib)–discs large (Dlg) proteins (Scribble complex) which localise at the basolateral membrane (Margolis and Borg 2005; Suzuki and Ohno 2006). Both the Par and Crumbs complexes promote apical membrane identity, whereas the Scribble complex promotes basolateral membrane identity by antagonising the other two (Bilder 2004).

Excessive cell growth and the loss of cell polarity have long been used by pathologists as hallmarks for cancer diagnosis (Hanahan and Weinberg 2011). Extensive studies carried out by human cancer genome projects in the past 10 years have clearly illustrated that regulators of the cell cycle are frequently mutated in human cancer, emphasising the importance of cell cycle regulation in tumourigenesis. The mutation rate of genes that encode the key components of cell

polarity complexes is relatively low in human cancers, in contrast with genes involved in cell cycle regulation. Instead, the genes involved in cell–cell adhesion represent an emerging class of mutated genes found in human epithelial cancers (Royer and Lu 2011; Berx and van Roy 2009). Unanswered questions still remain. Are these passenger or driver mutations? Is the observed loss of cell polarity a consequence of excessive cell growth or is it an evidence of cell polarity's tumour-suppressive function? Additionally, RAS and p53 remain to be one of the most frequently mutated oncogene and tumour suppressor pathways found in most human cancers. Therefore an intriguing question is whether the cell polarity machinery (i.e. the regulators and components of cell polarity complexes) could be regulated by oncogene and tumour suppressor pathways, in particular the RAS and p53 pathways? Epidermal growth factor receptor (EGFR)/RAS is arguably the best characterised oncogenic signalling pathway that disrupts cell polarity and cell–cell adhesion, and it induces epithelial to mesenchymal transition (EMT) and cancer cell invasion. Consistent with this, tensin homolog (PTEN), which negatively regulates the phosphatidylinositol 3-kinase (PI3K) pathway by dephosphorylating the PI3K product, phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P3], and producing phosphatidylinositol (4,5)-bisphosphate [PtdIns (4,5)P2], is a tumour suppressor that regulates cell polarity. During polarisation of epithelial cells, PTEN is targeted to the future apical membrane domain, where it generates PIP_2, which facilitates recruitment of annexin 2 (Anx2), Cdc42 and the apical Par6–Par3–aPKC complex (Martin-Belmonte et al. 2007). As apical accumulation of PIP_2 is dependent on apical targeting of PTEN and as membrane targeting of Par3 is mediated by direct binding to phosphoinositide lipids, PTEN may be instrumental in the apical localisation of Par3 (Feng et al. 2008; Dow et al. 2008; Wu et al. 2007; Martin-Belmonte et al. 2007). Recent studies have also illustrated that p53 mutation often occurs in metastatic cancers and that mutant p53 facilitates EMT and cancer cell invasion (Zhang et al. 2011). Therefore, cell polarity may play an active role during tumour genesis.

Intriguingly, apoptosis-stimulating protein of *p*53-2 (ASPP2), a known tumour suppressor and an activator of p53 (Samuels-Lev et al. 2001), has recently been identified as a binding partner of Par3, a regulator of apical polarity and cell–cell adhesion in vitro and in vivo (Sottocornola et al. 2010; Cong et al. 2010). ASPP1 and iASPP together with ASPP2 make up the ASPP family of proteins. ASPP1 and ASPP2 stimulate, whereas iASPP inhibits, p53's apoptotic function (Trigiante and Lu 2006). They are also characterised as ankyrin repeats, an SH3 domain and a proline-rich-region-containing proteins (ASPP). A number of transgenic mouse studies have established ASPP2 as a haploinsufficient tumour suppressor (Kampa et al. 2009; Vives et al. 2006; Tordella et al. 2013). It also acts an activator of the RAS oncogene, and the identified ASPP2–RAS interaction mediates the tumour-suppressive function of RAS oncogene including the induction of cellular senescence in primary cells and apoptosis in cancer cells (Wang et al. 2012, 2013a, b). RAS activation also induces ASPP2 translocation from cell–cell junctions to the cytosol and nucleus (Wang et al. 2013a). Importantly, ASPP2 has been identified as a cellular target of the cancer-causing pathogens *Helicobacter pylori* (*H. pylori*)

(Buti et al. 2011) and human hepatitis C virus (HCV) (Cao et al. 2004). These new findings suggest that regulators of cell polarity, such as ASPP2, may connect cell polarity to oncogenes and tumour suppressor pathways as well as to infection and tumourigenesis.

A number of cellular targets of cancer-causing pathogens are involved in the establishment and/or maintenance of epithelial cell polarity. Increasing evidence also suggests that non-cancer-causing viruses (such as influenza, dengue, tick-borne encephalitic viruses, rabies, severe acute respiratory syndrome (SARS) coronavirus and human immunodeficiency virus (HIV)) target components of cell polarity or TJs/AJs to enable efficient replication and spread of pathogens (Mothes et al. 2010; Javier and Rice 2011; Bonazzi and Cossart 2011). Hence, cell polarity represents the first line of defence against infections. Importantly, cancer-causing pathogens such as *H. pylori*, human papilloma virus (HPV), human hepatitis B virus (HBV), HCV (Moudgil et al. 2013; Thomas 2013) and human T-cell lymphoma virus (HTLV) may induce tumourigenesis by disrupting cell polarity (Fig. 7.1). This is mainly achieved via their ability to deregulate the function of cell polarity components and/or regulators. Therefore, cell polarity could be a first line of defence against cancer-causing infections and may represent a prime target of cancer-causing pathogens (Javier and Rice 2011). Interestingly, one of the most frequently mutated oncogenic signalling pathways, EGFR/RAS/PI3K, induces cancer cell invasion by inducing EMT. The loss of cell polarity is a prerequisite of EMT (Thiery 2003; Thiery et al. 2009) and an initial step of epithelial cancer metastasis. Many cancer-causing pathogens such as *H. pylori* also induce EMT upon infection. Hence cell polarity may represent the last line of defence against cancer cell invasion. By reviewing the recent studies in this area of research, we would like to present a hypothesis that cell polarity may be a key defence mechanism against infection and cancer cell invasion.

7.2 Cell Polarity: The First Line of Defence Against Infections

A unique feature of epithelial cells is their ability to form a barrier. External barriers protect organisms from water leakage. Both external and internal barriers also prevent unwanted substances from entering the skin or other organs and therefore causing cell and tissue damage. Perhaps one of the most important functions of the barrier is its ability to protect us from infections. The epithelial barrier is achieved through the ability of epithelial cells to adhere to each other through the formation of TJs sealing the epithelial sheet in a highly polarised manner. Correct orientation of highly polarised epithelial cells is also crucial for secretion and absorption. Hence, the integrity of cell polarity and cell–cell adhesion is vital in the establishment and maintenance of epithelial tissue homeostasis and organogenesis. It is therefore not surprising that cellular targets of pathogens are often involved in the

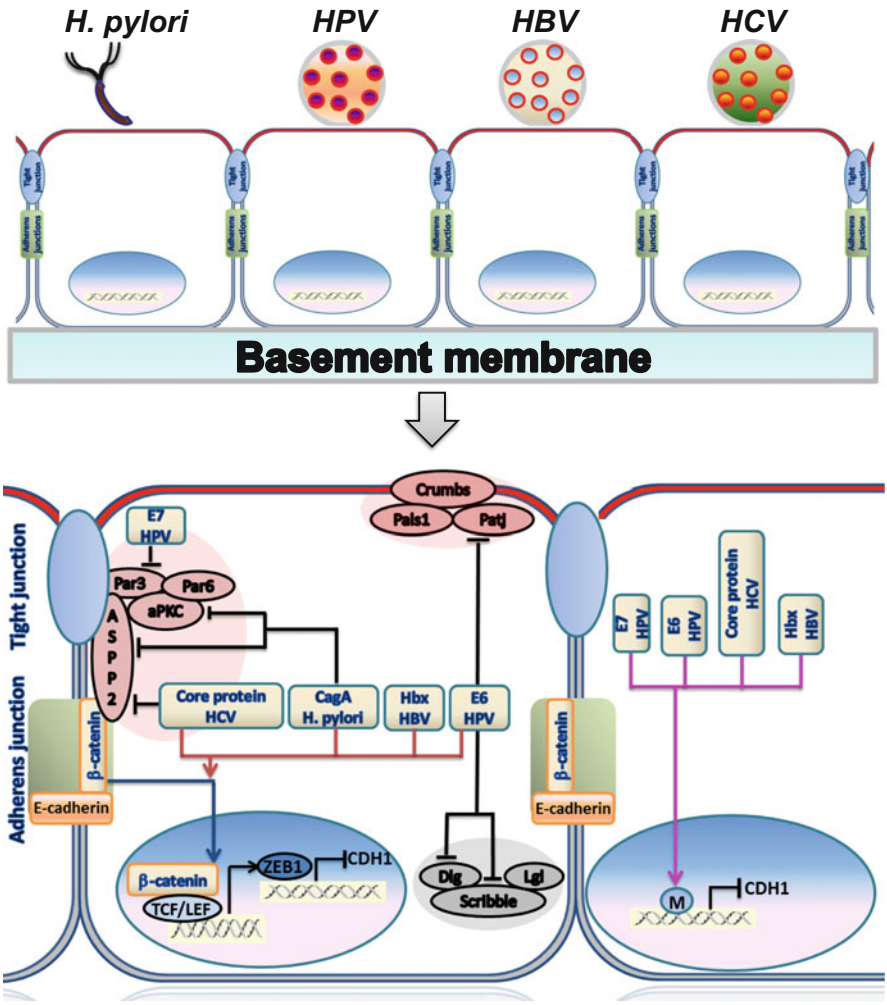

Fig. 7.1 Cell polarity is the first line of defence against infections. Epithelial cells form a barrier, which protects us from infections. Human papilloma virus (HPV) E6 and E7 proteins promote degradation of Scribble, discs large homolog (Dlg), PALS1-associated tight-junction protein (PATJ) and Par3. Apoptosis-stimulating of p53 protein 2 (ASPP2) is inhibited by *Helicobacter pylori* (*H. pylori*) CagA protein and human hepatitis C virus (HCV) core protein. *H. pylori* CagA also inhibits the function of atypical protein kinase C (aPKC), which causes junctional and polarity defects. These pathogens induce epithelial mesenchymal transition (EMT) by controlling the expression levels of E-cadherin either directly or indirectly via β-catenin, a binding partner of E-cadherin at adherens junctions and also an activator of the Wnt signalling pathway. Nuclear β-catenin complexes with T-cell and lymphoid enhancer (TCF/LEF) to transactivate the expression of ZEB1, which represses the expression of E-cadherin. Human hepatitis B virus (HBV), Hbx protein HPV (E6/E7) and HCV (core protein) downregulate E-cadherin expression directly, usually via inducing methylation (M) of the E-cadherin promoter (CDH1). *H. pylori*, HPV, HBV and HCV also enhance the nuclear accumulation of β-catenin via various mechanisms. *Hbx* HBV X protein. *Arrows* (➡) indicate activation and *T-junctions* (⊣) indicate inhibition

establishment or maintenance of cell polarity and cell–cell adhesions. In particular, PDZ domain-containing proteins (PSD95/Dlg/ZO-1) (Kennedy 1995) play essential roles in most aspect of cellular homoeostasis including control of cell–cell adhesion, cell polarity and cell migration. These proteins have recently been identified as cellular targets of pathogenic viruses (Javier and Rice 2011). The PDZ domain is a common structural domain of 80–90 amino acids found in the signalling proteins of bacteria, yeast, plants, viruses and animals (Ponting et al. 1997). For example, PDZ-containing basal–lateral polarity complex Dlg1 is a target of adenovirus, influenza, HPV, HTLV, HIV and rabies (Javier and Rice 2011). Additionally, cancer-causing pathogens such as the gram-negative bacteria *H. pylori*, DNA tumour virus such as adenovirus, HPV, HBV and HCV, as well as the tumour-causing retrovirus HTLV are all able to bind and deregulate the function of regulators or components of cell polarity in order to achieve their oncogenic properties (Javier and Rice 2011; Javier 2008; Banks et al. 2012). All these suggest that cell polarity represents a first line of defence against infection and is a prime target of cancer-causing pathogens (Fig. 7.1).

7.2.1 *Par Complex and ASPP2 Are Targeted by Cancer-Causing* H. pylori*, Papilloma Virus and Hepatitis Viruses*

H. pylori is a bacterial species that specifically colonises gastric epithelium and is associated with peptic ulcer disease, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma. *H. pylori* strains belong to two types. Type I strains contain in their genome the cytotoxin-associated gene pathogenicity island (Cag PAI) and express CagA protein, whereas Type II strains are CagA negative. *H. pylori* CagA-positive strains are associated with gastritis, ulcerations and gastric adenocarcinoma (Peek and Blaser 2002). CagA is delivered into gastric epithelial cells (Segal et al. 1999) and is able to induce the formation of an elongated cell shape (Bagnoli et al. 2005). In polarised epithelial cells, CagA causes loss of apical–basolateral polarity (Amieva et al. 2003; Bagnoli et al. 2005). *H. pylori* CagA specifically interacts with Par1 kinase, which has an essential role in epithelial cell polarity. Association of CagA inhibits Par1 kinase activity and prevents atypical protein kinase C (aPKC)-mediated Par1 phosphorylation, which dissociates Par1 from the membrane, collectively causing junctional and polarity defects (Saadat et al. 2007). Recently, it has been demonstrated that CagA interacts with ASPP2 (Buti et al. 2011; Nešić et al. 2014), a haploinsufficient tumour suppressor (Kampa et al. 2009; Vives et al. 2006) and a binding partner and a regulator of Par3 (Cong et al. 2010; Sottocornola et al. 2010). In mammalian cells, ASPP2 associates with Par3, a complex crucial for the formation and localization of the apical–junctional complex (AJC). ASPP2-depleted cells are defective in the formation of TJs and acquire a migratory phenotype (Sottocornola et al. 2010; Cong et al. 2010).

Interestingly, the CagA–ASPP2 interaction facilitates the formation of the CagA–ASPP2–p53 complex and consequently results in proteasomal degradation of p53. How this CagA–ASPP2 interaction leads to enhanced p53 degradation is currently unknown. Nonetheless the identification of ASPP2 as a prime cellular binding partner of CagA in addition to Par1 extended the list of cell polarity components as cellular targets of CagA.

It has been well established that ASPP2 functions as a tumour suppressor by enhancing the transcriptional activity of p53 (Samuels-Lev et al. 2001). Additionally, ASPP2 acts as a regulator of cell polarity and cell adhesion in a p53-independent manner. This property of ASPP2 is evolutionarily conserved. Drosophila ASPP (dASPP) localises at AJs and regulates the activity of C-terminal kinase (dCsk). Loss of function of dASPP increases cell spreading and apoptosis (Langton et al. 2007, 2009). The fact that ASPP2 may suppress tumour growth via both p53-dependent and p53-independent pathways places it as an ideal cellular target of oncoproteins derived from cancer-causing pathogens, such as CagA from *H. pylori*. Consistent with this, ASPP2 was also identified as a cellular target of the core protein of HCV. Hepatocytes are highly polarised and have basolateral and apical poles, separated by TJs in contact with blood vessels and bile ducts respectively (Perrault and Pecheur 2009). Chronic infections with HBV and HCV are associated with 80 % of hepatocellular carcinomas (HCCs) worldwide. Both HBV and HCV express proteins that have transforming potential and which directly affect cell polarity. This binding of HCV core protein to ASPP2 blocks the interaction between p53 and ASPP2 and inhibits p53-mediated apoptosis (Cao et al. 2004). HCV core protein is also known to be involved in disrupting cell polarity and cell–cell adhesion upon infection (Awad et al. 2013). Thus it is tempting to speculate that HCV core protein may disrupt cell polarity and cell–cell adhesion through its ability to interact with ASPP2.

It was shown recently that loss of the Par3 promotes breast tumourigenesis and metastasis (McCaffrey et al. 2012). ASPP2 is known to function as a tumour suppressor by enhancing the transcriptional activity of p53. ASPP2 and Par3 also form a protein complex that regulates cell polarity independently of p53. The junctional localisation of ASPP2 and Par3 is interdependent, and the interaction between ASPP2 and Par3 controls the integrity of cell polarity and cell–cell adhesion in vitro and in vivo (Sottocornola et al. 2010; Cong et al. 2010). Thus targeting either ASPP2 or Par3 could inactivate the ASPP2–Par3 complex and disrupt cell polarity and/or cell–cell adhesion. Like many other components of cell polarity complexes, Par3 contains a PDZ domain. Interestingly, Par3 is a target of E7 oncoprotein of rhesus papilloma virus (RhPV1), a virus closely related to HPV16, and it causes cervical cancer in the rhesus macaque (Tomaic et al. 2009). The PDZ-binding motif (PBM) of E7 interacts with Par3 and targets it for proteasome-mediated degradation (Tomaic et al. 2009). Thus, RhPV1 may cause cervical cancer in the rhesus macaque by deregulating both p53-dependent and p53-independent tumour-suppressive functions of ASPP2. p53 degradation mediated by HPV protein, E6, compromises the apoptotic function of the ASPP2–p53 complex. By targeting Par3 for proteasome-mediated degradation, E7 disrupts the

ASPP2–Par3 complex. Thus, oncogenic papilloma viruses could cause an increased resistance to apoptotic stimuli on one hand and induce EMT on the other, to impose the aggressive and invasive phenotype.

7.2.2 Crumbs and Scribble Complexes Are Targeted by Cancer-Causing Viruses

HPV is the most studied cancer-causing virus and causes a diverse range of epithelial lesions that are the causative agents of a number of human cancers, the most prominent being cervical cancer. Cervical cancer occurs following persistent infection with a high-risk viral subtype (HPV16 or 18) and is characterised by continued expression of the viral oncoproteins E6 and E7. The E6 and E7 proteins derived from high-risk HPV are able to bind and inactivate tumour-suppressive functions of p53 and Rb, respectively, to drive cell proliferation. E6 binds p53 and targets it for E6AP-mediated proteasomal degradation (Talis et al. 1998), whereas E7 binds Rb and prevents it from inhibiting the transcriptional activity of E2F and cell cycle entry (Moody and Laimins 2010). It is now emerging that E6 and E7 exert a co-ordinated attack on PDZ domain-containing proteins that are components or regulators of Crumb or Scribble cell polarity complexes.

Analysis of the sequences of E6 proteins derived from the cancer-causing mucosal HPV types reveals a remarkable conservation of amino acid sequences at the extreme carboxyl termini of the proteins. All of these E6 proteins have a class I PDZ (PSD95/Dlg/ZO-1)-binding motif (x-T/S-x-L/V) (PBM) at their carboxyl termini (Songyang et al. 1997). E6 oncoprotein has been shown to interact, through this motif, with a large number of PDZ domain-containing cellular targets (Banks et al. 2012; Pim et al. 2012; Thomas et al. 2008). The PBM of HPV E6 is able to interact with discs large (Dlg) and Scribble from the Scribble complex and PALS1-associated tight-junction protein (PATJ) of the Crumb complex. Crumb is an apical polarity complex whereas Scribble is a basolateral polarity complex (Kiyono et al. 1997; Lee et al. 1997; Nakagawa and Huibregtse 2000; Gardiol et al. 1999; Thomas et al. 2005; Storrs and Silverstein 2007). In each case, E6, by recruiting cellular ubiquitin-protein ligases, can target distinct pools of these proteins for proteasome-mediated degradation (Gardiol et al. 1999; Nakagawa and Huibregtse 2000; Storrs and Silverstein 2007; Tomaic et al. 2009; Watson et al. 2003). In many of the model systems that have been analysed, the capacity of E6 from the high-risk HPV types to retain PDZ-binding capacity has been shown to contribute towards its transforming activity, both within tissue culture model systems (Kiyono et al. 1997; Watson et al. 2003) and also in transgenic mouse models (Nguyen et al. 2003).

Like HPV, human adenovirus infects epithelial cells. The oncoprotein E4-ORF from human adenovirus targets both the Crumb and Scribble complexes. This is achieved by binding to the PDZ domains of Dlg1 and PATJ. Additionally, HTLV infects T cells that do not have an apical cell polarity complex. TAX oncoprotein

from HTLV only targets the Scribble complex as it binds the PDZ domain of Dlg1, Dlg4 and Scribble. All these argue for the importance of cell polarity in virus-induced malignancy.

7.2.3 *Cancer-Causing Pathogens such as* H. pylori, *Papilloma Virus and Hepatitis Viruses Can Induce EMT*

For a primary epithelial cancer cell to metastasise, it must first disrupt cell–cell adhesion to allow it to detach from the primary cancer site and to migrate to a new site. Disruption of cell–cell adhesion is often associated with EMT. Epithelial cells adhere to each other via TJs and AJs at the apical side and via gap junctions and desmosomes at the basolateral side. ZO1, ZO2, occludin and claudin are the main components of TJs, whereas E-cadherin and β-catenin are main components of AJs (Dejana 2004). Importantly, TJs and AJs often overlap with the apical polarity complex Par3–Par6–aPKC. Par3 and ASPP2 control the initial formation of TJs and AJs in vitro (Cong et al. 2010; Sottocornola et al. 2010). Reduced E-cadherin expression, loss of cell polarity and disruption of cell–cell adhesions are a prerequisite of EMT.

Interestingly, the PDZ-containing protein ZO2 is a cellular target of human adenovirus E4-ORF1 (Glaunsinger et al. 2001). HCV also targets TJ proteins claudin-1 and occludin to allow its cellular entry (Perrault and Pecheur 2009; Mee et al. 2008; Benedicto et al. 2009; Meertens et al. 2008; Evans et al. 2007). The actual roles of claudin-1 and occludin in HCV cell entry remain unclear, but interestingly a direct interaction between the HCV envelope glycoproteins and occludin has been shown (Benedicto et al. 2008). Furthermore, knockdown of occludin in a cell–cell fusion assay, where fusion activity depends on cell surface expression of the HCV glycoprotein complex, gave rise to diminished fusion activity, suggesting that occludin may be implied in the HCV fusion process (Benedicto et al. 2009). During infection, HCV disrupts TJs and cell polarity in various in vitro systems (Mee et al. 2010; Benedicto et al. 2008; Wilson et al. 2012). It was shown that HCV infection promotes vascular endothelial growth factor (VEGF) expression that depolarises hepatoma cells, promoting viral transmission and lymphocyte migration into the parenchyma that may promote hepatocyte injury (Mee et al. 2010). HCV glycoproteins also perturb TJ and AJ protein expression and increase hepatoma migration. This is achieved by stabilising hypoxia-inducible factor 1α (HIF-1α), which upregulates the expression of EMT-inducing transcription factors such as Snail and Twist (Wilson et al. 2012).

Perhaps the best characterised mechanism that many cancer-causing pathogens use to induce EMT is through their ability to control the expression levels of E-cadherin either directly or indirectly via β-catenin. Both HPV E6 and E7 are able to downregulate E-cadherin expression (D'Costa et al. 2012; Caberg

et al. 2008); the E-cadherin promoter is repressed in cells expressing E6, resulting in fewer E-cadherin transcripts. Mechanistically this is not caused by either an increase in histone deacetylase activity or a binding of trans-repressors to the E-cadherin promoter Epal element. In contrast, E6 expression induces DNA methyltransferase (DNMT) activity (D'Costa et al. 2012). It was also shown that HPV16 E7 silencing may induce E-cadherin re-expression via AP-2 transcription factor (Caberg et al. 2008). The HBV X protein (Hbx) is able to induce methylation of E-cadherin promoter to reduce expression (Lee et al. 2005). Similarly the core protein of HCV also induces E-cadherin promoter methylation to downregulate E-cadherin expression (Arora et al. 2008).

Most cancer-causing pathogens control E-cadherin expression by regulating the expression levels and cellular localisation of β-catenin, a binding partner of E-cadherin at AJs and activator of the Wnt signalling pathway (Fig. 7.1). Nuclear β-catenin forms complexes with the T-cell and lymphoid enhancer (TCF/LEF) and transactivates the expression of ZEB1 (Sanchez-Tillo et al. 2011). In turn, ZEB1 represses the expression of E-cadherin (Peinado et al. 2007). HPV16 E6 oncoprotein enhances the nuclear accumulation of β-catenin, and this effect requires an intact E6 PDZ-binding domain (Bonilla-Delgado et al. 2012). As a result, increased nuclear β-catenin represses the expression of E-cadherin (Bonilla-Delgado et al. 2012). Hbx also increases the expression levels of β-catenin by perturbing the interaction between β-catenin and the tumour suppressor, adenomatous polyposis coli (APC) (Hsieh et al. 2011). Hbx competitively binds APC to displace β-catenin from its degradation complex. This results in upregulation of nuclear β-catenin and activation of Wnt signalling (Hsieh et al. 2011). However the mechanisms with respect to the role of Wnt-5a in HBV-associated hepatocellular carcinoma (HCC) need further investigation. In addition, mutations in the C-terminus of Hbx upregulate Wnt-5a expression (Liu et al. 2008) and induce nuclear β-catenin. One of the nonstructural proteins of HCV, NS5A, stabilises β-catenin by activating PI3K (Street et al. 2005). Mechanically, NS5A interacts with the SRC homology 3 (SH3) domains of members of the SRC family of tyrosine kinases and modulates kinase activity. Finally CagA of *H. pylori* physically interacts with E-cadherin, and this interaction impairs the complex formation between E-cadherin and β-catenin, causing cytoplasmic and nuclear accumulation of β-catenin (Murata-Kamiya et al. 2007). Additional pathways, including those that are mediated by the transactivation of EGFR (Polk and Peek 2010) or PI3K/AKT(Suzuki et al. 2009; Sokolova et al. 2008; Nakayama et al. 2009), have been demonstrated to regulate β-catenin activation in response to *H. pylori* infection. Activation of PI3K and AKT leads to the phosphorylation and inactivation of glycogen synthase kinase 3β (GSK3β), permitting β-catenin to accumulate in the cytosol and nucleus. All these examples demonstrate that by enhancing nuclear β-catenin expression, cancer-causing pathogens are able to induce cell proliferation on one hand and disrupt cell–cell adhesion and induce EMT on the other. Thus, the ability of cancer-causing pathogens to directly target and perturb cell polarity and cell–cell adhesion makes them potent inducers of EMT, which is likely to contribute to their oncogenic properties.

7.3 Cell Polarity: The Last Line of Defence Against Cancer Cell Invasion

Correct establishment and maintenance of cell polarity are required for the development and homeostasis of all metazoans. Reduced expression of components or regulators of cell polarity in human cancers has been reported and reviewed extensively, supporting a tumour-suppressive role of cell polarity (Royer and Lu 2011; Muthuswamy and Xue 2012). Importantly, loss of normal cell polarity and tissue architecture is a defining characteristic of cancer malignancy. Malignant transformation can be induced by the abnormal activation of various oncogenic and growth factor signalling pathways, which not only stimulate cell proliferation but also result in disruption of apical–basal polarity, cell–cell adhesion and EMT. The co-operation between the loss of cell polarity and oncogene activation resembles the actions of cancer-causing pathogens since many oncoproteins of cancer-causing pathogens can disrupt cell polarity on one hand and activate oncogenic signalling on the other. Therefore, we will focus on the evidence supporting a positive role of cell polarity in defending cancer cell invasion.

7.3.1 Enhanced Growth Factor Signalling Targets Cell Polarity Complex to Induce EMT and Cancer Metastasis

Deregulation of growth factor signalling such as an elevation of transforming growth factor β (TGFβ) signalling promotes EMT during normal development and tumour progression (Thiery 2003). This is partly achieved by the ability of TGFβ signalling to target Par6, a component of the apical cell polarity complex (Fig. 7.2). Type II TGFβ receptor, TGFβRII, is a receptor tyrosine kinase, which binds and phosphorylates Par6. Phosphorylated Par6 is required for TGFβ to induce EMT in mammary gland epithelial cells, and it is also required for Par6–Smurf1 interaction. Smurf1 is an E3 ubiquitin ligase, and it targets the small guanosine triphosphatase (GTPase) RhoA for degradation. RhoA is crucial for the maintenance of the actin cytoskeleton and stabilisation of AJCs. Thus, activation of TGFβ signalling ultimately results in the destabilisation and loss of AJCs and the initiation of EMT (Ozdamar et al. 2005). In addition, TGFβ signalling activation induces the expression of three families of transcription factors: the Snail, ZEB and bHLH families, either through a Smad-dependent mechanism (in the case of Snail proteins) or indirectly through activation of other transcription factors or relief of repression (Xu et al. 2009). Upon activation, these transcription factors in turn repress epithelial marker gene expression and concomitantly activate mesenchymal gene expression (Peinado et al. 2007).

Abnormal activation of the receptor tyrosine kinase ErbB2 (also known as human epidermal growth factor receptor 2, HER2 or Neu), an oncogene that has

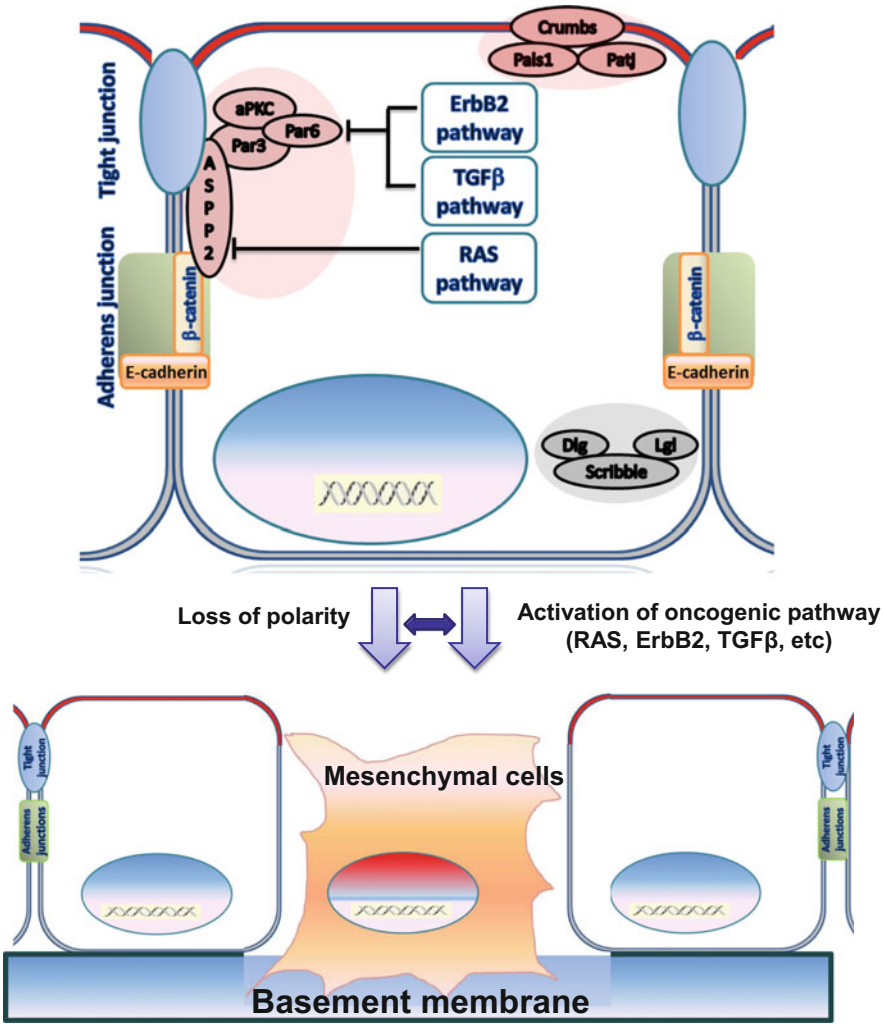

Fig. 7.2 Cell polarity is the last line of defence against cancer cell invasion. Loss of cell polarity co-operates with activation of oncogenes to facilitate epithelial mesenchymal transition (EMT) and to promote cancer cell invasion and metastasis. Activation of TGFβ signalling results in phosphorylation of Par6, and this is required for TGFβ-dependent EMT. Activation of ErbB2 signalling results in disruption of the apical Par6–Par3–aPKC polarity complex by promoting dissociation between Par3 and Par6–aPKC. This function is crucial for ErbB2-mediated disruption of cell polarity. RAS activation induces ASPP2 translocation from cell–cell junctions to the cytosol and nucleus. *T-junctions* (⊣) indicate inhibition

been implicated in human breast, ovarian, gastric, oesophageal and endometrial cancers, can directly disrupt cell polarity and inhibit apoptosis by binding to the Par6–aPKC protein complex (Aranda et al. 2006) (Fig. 7.2). Inhibition of this association restores correct cell polarity and abrogates the anti-apoptotic effects

of ErbB2 but does not affect its role in the stimulation of cell proliferation suggesting that cell polarity may be linked to apoptotic functions. Again, enhanced ErbB2 signalling promotes EMT and cancer invasion. Interestingly, ErbB2 is known to co-operate with mutant p53 to increase tumourigenesis in mice (Li et al. 1997). Furthermore in breast cancers, mutant p53 status in combination with high ErbB2 expression is associated with a very poor prognosis (Rahko et al. 2003). However, reactivation of wild-type p53 by nutlin3 (through inhibition of MDM2) both normalised the sphere-forming activity of the ErbB2-induced mammary tumour cells and concomitantly reduced their tumour-initiating activity (Cicalese et al. 2009).

7.3.2 Loss of Cell Polarity and Activation of RAS Oncogene Induce EMT and Cancer Metastasis

Loss of cell polarity when combined with activation of signals such as RAS oncogene may promote the formation of metastatic tumours (Pagliarini and Xu 2003; Igaki et al. 2006; Brumby and Richardson 2003; Langton et al. 2007, 2009) (Fig. 7.2). For example, Scrib-deficient mutants co-operate with oncogenes to mediate transformation in Drosophila. Normally, Scrib-deficient mutant clones, in the eye imaginal discs, are eliminated by c-Jun N-terminal kinase (JNK)-dependent apoptosis. However, in the presence of activated oncogenic pathways such as RAS or Notch, apoptosis is inhibited and neoplastic tumours occur (Brumby and Richardson 2003). Again in Drosophila, oncogenic RAS, when expressed within clonal patches of tissue in the eye disc, induces hyper-proliferation, but the transformed cells do not invade into other tissues. Cells lacking components of polarity complex, such as dASPP, Scribble, Dlg, Lgl and Bazooka (equivalent to human Par3), are disorganised in the affected tissue but they also do not invade. However when combined with oncogenic RAS, these defects promote formation of metastatic tumours (Pagliarini and Xu 2003; Igaki et al. 2006; Brumby and Richardson 2003; Langton et al. 2007, 2009). Some of the observations in Drosophila were confirmed in mammals recently. Loss of Par3 in primary mammary epithelial cells (MECs) co-operates with oncogenic HRAS to promote tumourigenesis in mouse (McCaffrey et al. 2012). E-cadherin was almost undetectable in Par3-depleted tumours in the presence of activated RAS oncogene (McCaffrey et al. 2012). Additionally, KRAS activation and Scrib loss co-operate to facilitate prostate tumour progression (Pearson et al. 2011). Pearson and colleagues generated male mice in which Scrib loss and hyperactivated KRAS (LSL-KRAS *G12D*) were specifically induced in the prostate (Pearson et al. 2011). It was demonstrated that Scrib loss and oncogenic KRAS co-operate to accelerate disease progression in mice, illustrating the multistep nature of prostate cancer progression and providing evidence to support published studies on Drosophila in vivo and mammalian cells in vitro (Dow et al. 2008; Brumby and Richardson 2003).

Consistent with the notion that loss of cell polarity is a hallmark of epithelial cancers, expression of Par3 was significantly reduced in primary oesophageal squamous cell carcinoma (ESCC) compared with their non-tumour counterparts. This reduced expression was associated with positive lymph node metastasis and poor differentiation (Zen et al. 2009). Interestingly, ASPP2, a binding partner of Par3, is often downregulated in metastatic tumours. Reduced ASPP2 expression associates with poor prognosis in diffuse large B-cell lymphomas (Aranda et al. 2006), and ASPP2 expression is reduced in both invasive and metastatic cells compared with normal breast epithelium (Sgroi et al. 1999) and squamous cell carcinomas (SCCs) of the head and neck (Tordella et al. 2013). Importantly, a reduction in ASPP2 expression is sufficient to cause spontaneous development of poorly differentiated SCC in mice in vivo with some of the SCC exhibiting an invasive phenotype (Tordella et al. 2013). This is in agreement with a recent finding that ASPP2 is a molecular switch of EMT and its reverse mesenchymal to epithelial transition (MET), and an inhibitor of metastasis. This newly identified property of ASPP2 requires its ability to bind Par3 and β-catenin (Wang et al. 2014). Additionally activation of the RAS oncogenic signalling pathway, due to a mutation, is a common event in human SCC. It is therefore tempting to speculate that reduced Par3 and ASPP2 expression may co-operate with RAS oncogene activation to promote EMT, cancer invasion and cancer metastasis. Together, the existing evidence suggests that cell polarity may act as the last line of defence against cancer cell invasion.

7.4 Conclusions and Perspectives

The identification of increasing numbers of components or regulators of cell polarity complex as direct pathogenic targets argues strongly that cell polarity is likely to act as a first line of defence to guard us against infection. It is now emerging that components or regulators of cell polarity are binding partners of cellular oncogenes and tumour suppressors. They are also common cellular targets of cancer-causing pathogens. Loss of cell polarity often associates with oncogene activation in highly invasive and metastatic human cancers. This resembles the action of cancer-causing pathogens such as *H. pylori*, HPV and hepatitis viruses that are potent inducers of EMT and cancer invasion. All of these argue for a role of cell polarity as a last line of defence against cancer cell invasion. Future studies are needed to provide experimental evidence to demonstrate precisely how cell polarity acts as a barricade against infection and cancer cell invasion. Finally, cancer-causing pathogens will induce the inflammatory response. Cell remodelling and cell migration are fundamental cellular responses to inflammatory signalling. Therefore, future studies are also needed to examine the interplay between cell polarity and inflammation and cancer metastasis.

Acknowledgements We would like to thank the Ludwig Institute for Cancer Research Ltd. for supporting this work. Dr Yihua Wang is supported by the Medical Research Council [MR/J000930/1]. We also thank Dr Michael White, Dr Ludovico Buti and Dr Kimberley Bryon-Dodd for their critical reading of the manuscript.

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Chapter 8
Cell Polarity in Mammary Gland Morphogenesis and Breast Cancer

Carlis Rejon and Luke McCaffrey

Abstract Epithelial cells form organized structures such as tubes and alveoli, the organization of which is controlled by conserved complexes of polarity proteins. Mammary gland development requires extensive epithelial remodeling coordinated with proliferation and apoptosis to generate the highly branched epithelial ductal network that extends into a complex fatty stroma. Mammary epithelial cells dynamically regulate cell polarity during branching morphogenesis, and therefore the mammary gland represents a unique model to understand epithelial remodeling during normal development. Moreover, the mammary gland undergoes extensive remodeling during the progression of breast cancer, which is associated with a loss of apical-basal polarity and epithelial organization. In this chapter, we present the mammary gland as a model to understand unique roles for polarity proteins in epithelial remodeling. We also discuss novel signaling pathways regulated by polarity proteins that function in normal mammary gland development, as well as breast cancer initiation, invasion, and metastasis.

Keywords 3D culture • EMT • Epithelial • Invasion • Metastasis • Stem cell

8.1 Overview of Mammary Gland Development

The mammary gland consists of a treelike structure of epithelial ducts embedded in a complex stroma, consisting of fat cells, fibroblasts, and immune cells (Fig. 8.1) (Sternlicht 2006). The ducts are formed from an epithelial bilayer: an inner luminal layer that lines the ductal cavity and an outer myoepithelial layer that contacts the basement membrane. Although these two major cell types are both epithelial, their organization and functions are quite distinct; luminal cells are polarized cuboidal epithelial cells with distinct apical and basolateral domains (apical-basal polarity) that are separated by tight junctions (Fig. 8.1). These cells form E-cadherin-based adherens junctions that maintain ductal integrity. Luminal epithelial cells are also

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K. Ebnet (ed.), *Cell Polarity 2*, DOI 10.1007/978-3-319-14466-5_8

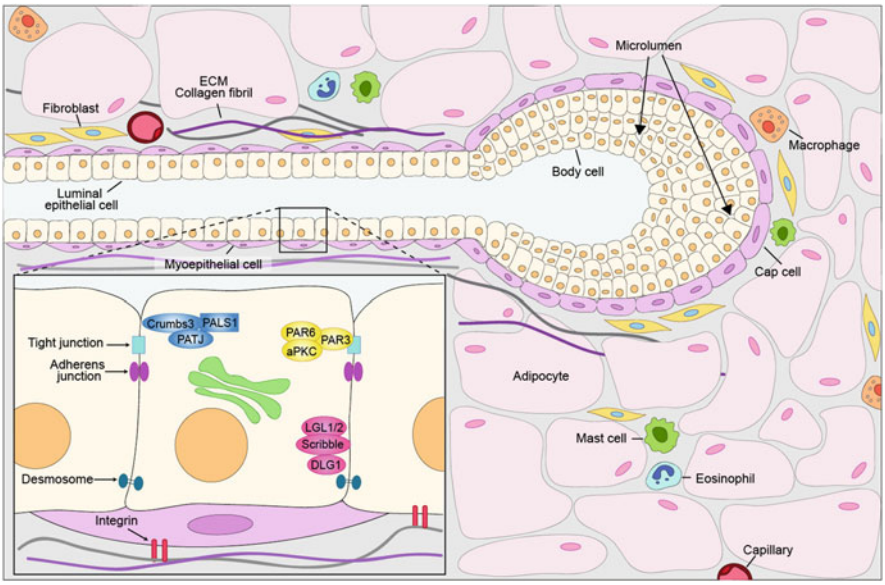

Fig. 8.1 Overview of the mammary gland. Diagram of a bilayered mammary duct with inner luminal epithelial cells and outer myoepithelial cells. The terminal end bud is multilayered with microlumen (*arrows*) and is covered by a cap cell layer that contains mammary stem cells. Components of the mammary stroma include adipocytes, immune cells, and fibroblast. An enlarged region of a duct shows the location of polarity and adhesion complexes in luminal epithelial cells with apical-basal polarity

characterized by the expression of intermediate filament cytokeratins 8/18 (CK8/18), whereas myoepithelial cells express distinct cytoskeletal markers, including cytokeratin 5 and 14 (CK5 and CK14), as well as α-smooth muscle actin (α-SMA) (Williams and Daniel 1983). Myoepithelial cells do not contact the lumen but instead interact with the basement membrane through integrins. Furthermore, myoepithelial cells form cell-cell adhesions through P-cadherin, but do not form tight junctions (Chanson et al. 2011); therefore, they do not exhibit apical-basal polarity (Fig. 8.1).

The mammary gland is a unique epithelial tissue since most of the development occurs after birth. At mid-gestation, the murine mammary epithelium arises from the ectoderm to form a mammary bud, which undergoes limited growth and branching to form a rudimentary tree in the embryo (Sternlicht 2006). After birth the mammary gland grows isometrically with the animal; then at puberty, changes in circulating hormones initiate a program of enhanced growth and branching of the mammary epithelium (Sternlicht et al. 2006).

In the mouse, pubertal mammary morphogenesis initiates with the formation of bulbous epithelial structures at the distal tips of growing ducts, termed terminal end buds (Fig. 8.1). End buds are multilayered and highly dynamic structures, with higher rates of both proliferation and apoptosis compared to the subtending ducts

(Hinck and Silberstein 2005; McCaffrey and Macara 2009; Ball 1998). High proliferation likely generates the increased cell numbers necessary for growing the duct, and apoptosis is necessary for clearing the central lumen (Mailleux et al. 2007; Parsa et al. 2008). The leading outermost layer of the end bud consists of cap cells, which are enriched in stem cell activity during mammary gland development (Bai and Rohrschneider 2010; Kenney et al. 2001). At pregnancy, the mammary epithelium again undergoes extensive proliferation, branching, and differentiation into milk-producing alveolar structures (Oakes et al. 2008). After lactation is complete, an apoptotic program eliminates the bulk of alveolar cells, and the mammary gland returns to a state that is highly reminiscent of the virgin gland before pregnancy (Stein et al. 2007). Therefore, the mammary gland represents a highly dynamic epithelium for which to understand the role of cell polarity proteins during epithelial morphogenesis.

8.2 Mammary Epithelial Models for Studying Morphogenesis and Polarity

Polarity proteins have a wide range of biological effects across numerous species and tissue types, and remarkably, the same proteins can have distinct functions in different tissues or at different developmental stages within the same tissue (Thompson 2012; Tepass 2012; McCaffrey and Macara 2012; Nance and Zallen 2011; Martin-Belmonte and Perez-Moreno 2011). This highlights the fact that polarity proteins have multifaceted and complex interactions with numerous pathways that impinge on biological processes like survival, proliferation, apoptosis, and differentiation. Not surprisingly then, different experimental models used to evaluate polarity protein function may reveal different roles for those proteins. For example, while 2D cultures may capture some aspects of basic polarized cell biology, 3D cultures more closely model the in vivo environment (McCaffrey and Macara 2011; Page et al. 2012; Weaver et al. 2002). In addition, organotypic cultures that contain multiple cell types can reveal more complex signaling that occurs between different cell types (Ridky et al. 2010; Macias et al. 2011), and in vivo models can yield further insights into complex regulatory mechanisms such as stem cell renewal and differentiation, as well as tumorigenesis and metastasis (Shackleton et al. 2006; Cardiff et al. 2000).

The mammary gland represents an excellent system for understanding various aspects of polarity protein function with culture models that range from simple cell lines that are relatively easily maintained to more complex cultures of primary cells and organotypic cultures, which requires more expertise. For example, mouse mammary gland cell lines (e.g., NMuMG, EpH4) are capable of forming polarized cysts when cultured in 3D extracellular matrix (Hall et al. 1982; Niemann et al. 1998; Viloria-Petit et al. 2009). Human breast cells are also capable of forming cysts when cultured in 3D matrix. A commonly used human mammary

cell is the immortalized, but non-transformed, MCF10A cell line, which were derived from a fibrocystic breast patient (Debnath et al. 2003; Dow et al. 2007; Whyte et al. 2010). These cells express some luminal markers and are polarized in the sense that they form a central lumen and orient the Golgi apically between the nucleus and lumen; however, the apical determinant Crb3 is suppressed in these cells, and they do not form an apical membrane or functional tight junctions (Fogg et al. 2005). Therefore, MCF10A cells lack complete apical-basal polarity, which needs to be considered with interpreting polarity data obtained from these cells.

Another source for human mammary epithelial cells (HMECs) are primary cells isolated from breast reduction surgery (Lindley and Briegel 2010; Stampfer and Bartley 1988). A limitation of primary HMECs is that they have a limited life in culture; however this has been overcome by the generation of numerous immortalized and transformed derivatives (Yaswen and Stampfer 2001; Dimri et al. 2005). An advantage of primary mammary cells from mice or humans is that they can be cultured as "organoids," which are isolated from mammary glands as multicellular clusters and then embedded and grown in 3D extracellular matrix gels (Fig. 8.2). These are distinct from 3D cultures of cell lines in that they contain both luminal and myoepithelial cell types, with a bilayer organization that resembles the mammary gland in vivo (Pasic et al. 2011; Ewald et al. 2008; Macias et al. 2011). Remarkably, organoids undergo dynamic remodeling of polarity during lumen formation and branching morphogenesis in response to growth factor stimuli (Fig. 8.2) and therefore represent an excellent model for understanding epithelial morphogenesis in vitro (Akhtar and Streuli 2013; Ewald et al. 2008).

Mouse and human primary mammary epithelial cells can also be cultured in at low density as suspension cultures to enrich stem and progenitor cells. Under these conditions, differentiated cells die by anoikis, whereas stem and progenitor cells survive and grow into balls of cells called mammospheres (Dontu et al. 2004). Mammospheres contain differentiated cells, as well as a stem cell population that is

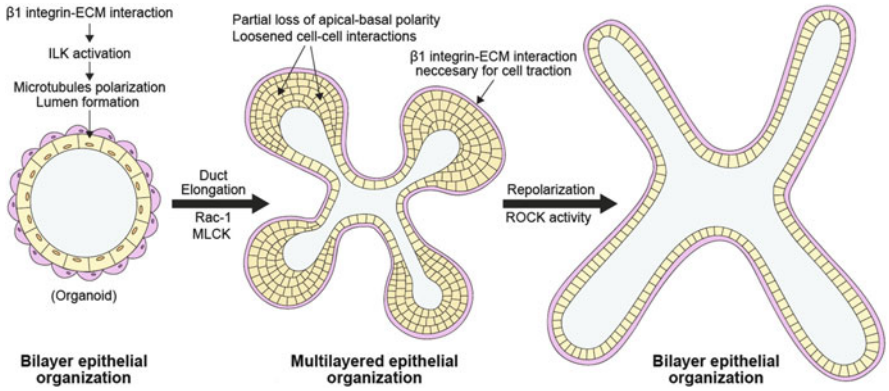

Fig. 8.2 Polarity is dynamically regulated during branching morphogenesis. Diagram showing formation of polarized organoids and transient multilayered luminal epithelia formed during mammary morphogenesis

maintained by asymmetric cell divisions and enables mammospheres to be sustained following serial passages (Cicalese et al. 2009).

Finally, in vivo mouse models are another important tool for understanding gene function in the mammary gland, particularly for understanding stem cell and tumorigenic functions. Several mammary gland-specific promoters are available to selectively express or knock out genes in the mammary epithelium. Commonly used promoters include the mouse mammary tumor virus long terminal repeat (MMTV), the whey acidic protein (WAP), and β-lactoglobulin (BLG) promoters, which predominantly target luminal epithelial cells (Borowsky 2011). In addition, the keratin 14 or keratin 5 promoters can be used to target myoepithelial cells (Van Keymeulen et al. 2011; Taddei et al. 2008); however, the keratin 14 promoter is active in stem cells during mammary embryogenesis, which results in expression in all mammary cell types (Van Keymeulen et al. 2011).

The ability to transplant mouse mammary gland stem cells into the mammary fat pad to regenerate the ductal epithelium is another useful tool for studying polarity in mammary morphogenesis (Deome et al. 1959; Daniel et al. 1968; Shackleton et al. 2006; Stingl et al. 2006). Mammary stem cells isolated from a donor are injected into the mammary fat pad of a prepubertal recipient mouse that has had the ductal epithelium removed surgically. *Lentivirus* can be used to express cDNA or shRNA in stem cells to study gene function, and when coupled with transplants, this represents a rapid and cost-effective alternative to generating transgenic and knockout mice (McCaffrey and Macara 2009).

8.3 Cell Polarity and Branching Morphogenesis

During pubertal development, the mammary epithelium undergoes dynamic changes in apical-basal polarity, particularly in the end bud. The terminal end bud is comprised of a mixture of polarized and non-polarized luminal epithelial cells; the polarized cells contact the developing central lumen, as well as microlumen, which may coalesce to form the primary lumen in subtending ducts and are surrounded by non-polarized cells (Fig. 8.1) (Ewald et al. 2012). For instance, whereas aPKCζ is usually localized to the apical membrane in polarized cells, it localizes with β-catenin, Scrib, and Numb at all cell membranes in non-polarized interior cells, and Par3 appears diffusely localized in the cytoplasm (Ewald et al. 2008; Ewald et al. 2012). As such, terminal end buds show characteristics of neoplastic hyperplasia, including a partial loss of apical-basal polarity and loosened cell-cell interactions. However, there is no invasion of these inner end bud cells into the highly organized extracellular matrix during ductal growth (Ewald et al. 2008).

Cellular and molecular mechanisms underlying branching morphogenesis have been investigated using 3D organotypic cultures (Fig. 8.2). In response to growth factors secreted by myoepithelial cells, luminal cells remodel to become multilayered, and then a process of collective migration pushes cells forward to initiate

branching (Ewald et al. 2008). Surprisingly, unlike some branched tissues like the *Drosophila* airway epithelium, collective movements during mammary branching do not have leader cells; instead, cells rearrange in a seemingly random order and in the absence of leading actin-rich protrusions (Ewald et al. 2008; Ewald et al. 2012). Diverse molecular pathways are involved in the control of mammary gland morphogenesis. For instance, Rac1 and MLCK activities are required for branching initiation, whereas ROCK is necessary to restore the bilayered epithelial architecture once branching has ceased (Ewald et al. 2008). Furthermore, the interaction between β1-integrins and laminin provides traction to the end buds and facilitates duct elongation (Klinowska et al. 1999). Association of β1-integrin with the basement membrane is also necessary for the establishment of apical-basal polarity in luminal cells and lumen formation (Akhtar and Streuli 2013). In contrast to MDCK cells, where Rac1 activation is required for the orientation of apical polarity (O'Brien et al. 2001; Yu et al. 2005), in mammary glands the integrin-linked kinase (ILK) acts downstream of β1-integrin to polarize microtubules along the apical-basal axis, control internal cell polarity, and drive lumen formation (Akhtar and Streuli 2013). Additionally, alterations in the expression, localization, or activity of diverse polarity proteins, including Scrib, Par4 (Lkb1), Llgl (Hugl1/2), and the Par3/aPKC complex, are associated with impairments in ductal morphogenesis, although the molecular mechanisms involved are not known (McCaffrey and Macara 2009; Whyte et al. 2010; Russ et al. 2012; Zhan et al. 2008; Partanen et al. 2012a).

The dynamic nature of the mammary gland is also evident during pregnancy, when the mammary epithelium remodels into a milk-secreting tissue in response to hormonal regulation by prolactin. During lactation the prolactin receptor (PRLR) locates preferentially in the basal membrane, where it activates a JAK2/STAT5 cascade that promotes proliferation and differentiation of milk-producing alveolar cells (Morales et al. 2012). The polarized distribution of PRLR to the basal membrane is dependent on NHERF1, a polarity scaffold that directly interacts with PRLR, as well as other proteins including Ezrin, and β-catenin. Interestingly, NHERF1 is localized to the apical membrane in luminal epithelial cell of mature virgin mammary ducts, but then undergoes a dynamic repositioning to the basal membrane during lactation, which is necessary for proper PRLR localization, alveolar differentiation, and milk production (Fig. 8.3b) (Morales et al. 2012; Stemmer-Rachamimov et al. 2001). Moreover, the interaction of mammary cells with the extracellular matrix is key for proper alveologenesis, since β1-integrin ablation prevents prolactin-induced differentiation of luminal epithelia due to defective STAT5 activation (Naylor et al. 2005). Together these studies highlight the importance of apical-basal polarity in positioning signaling modules, which is essential for hormonally regulated cell differentiation.

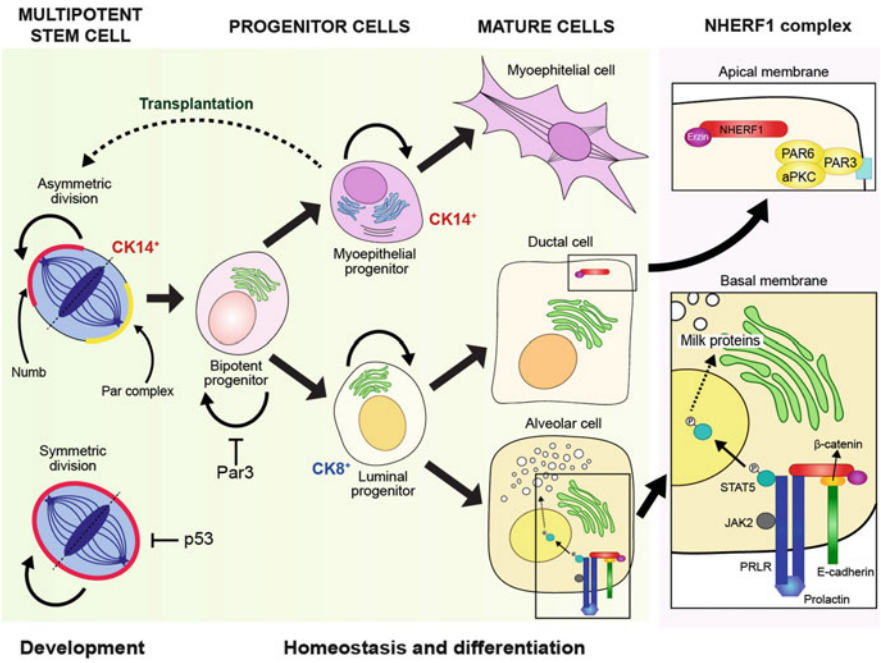

Fig. 8.3 Lineage specification in the mammary gland. (**a**) The mammary gland is hierarchally organized with mammary stem cells, bipotent progenitors, unipotent progenitors, and differentiated luminal and myoepithelial cells. Stem cells asymmetrically position Numb during divisions, which is dependent on p53. (**b**) Reorganization of a NHERF/Ezrin complex from the apical domain to the basal domain occurs during pregnancy. Basal NHERF/Ezrin positions the prolactin receptor basally, which is necessary for Stat5 activation and differentiation of milk-producing cells

8.4 Stem Cells and Mammary Gland Development

The mammary gland exhibits plasticity and grows extensively during puberty but also can cycle through multiple rounds of expansion and involution during pregnancy. Furthermore, transplantation of a single mammary epithelial cell into a cleared fat pad can regenerate the entire mammary ductal tree, indicating the presence of mammary stem cells (MaSC) with tremendous regenerative capability (Shackleton et al. 2006). The terminal end bud is a reservoir during development for active stem cells, which reside in a cap cell layer, the outermost layer of the end bud (Fig. 8.1) (Bai and Rohrschneider 2010). Furthermore, terminal end buds contain bipotent progenitor cells that express both luminal and myoepithelial markers, indicating that the terminal end bud may also represent a stem cell niche that regulates stem and progenitor cell differentiation (Kenney et al. 2001; McCaffrey and Macara 2009). This is in part regulated by Par3 since the depletion of Par3 from the mammary epithelium results in enlarged terminal end buds with an expanded pool of bipotent progenitors that are defective in differentiation and the ability to reorganizing into mature ducts (McCaffrey and Macara 2009).

Different cell surface markers have been used to isolate MaSC and lineage committed progenitors from the mammary gland [for a detailed review, see (Visvader 2009)]. At present there is no consensus whether a common progenitor can differentiate into all mature mammary epithelial cell lineages or if two different lineage-restricted precursor cells (luminal/alveolar and myoepithelial) are required during mammary tree expansion (Shackleton et al. 2006; Van Keymeulen et al. 2011). A unified hypothesis (Fig. 8.3a) suggests that unipotent progenitor cells are responsible of normal tissue maintenance and remodeling, while pluripotent cells participate in embryonic development or can be activated under certain circumstances (such as transplantation or tissue regeneration) to give rise to both luminal and myoepithelial lineages (Keller et al. 2011; Visvader and Lindeman 2011).

Regardless of the origin of the stem and progenitor cells, asymmetric cell divisions are necessary to establish the luminal and myoepithelial lineages while maintaining the pool of MaSCs (Fig. 8.3a) (Cicalese et al. 2009). Mammosphere assays show that p53 expression is required for asymmetric Numb segregation, via an unknown mechanism (Cicalese et al. 2009). This may involve the Par complex because in other cell types, aPKC and Par3 interact with Numb and aPKC directly phosphorylates Numb to control polarized distribution (Smith et al. 2007; Nishimura and Kaibuchi 2007). Asymmetric Numb may be important for mammary cell fate decisions; Numb is a negative regulator of Notch signaling, and Notch is involved in specifying luminal progenitors and the luminal cell fate (Pece et al. 2004; Gönczy 2008). In turn, Numb regulates p53 ubiquitination and degradation (Colaluca et al. 2008), creating then a feedback loop that might restrict the stem cell fate to only one of the daughter cells. Segregation of cell fate determinants and asymmetric cell division require establishment of a polarity axis (in response to external clues, such interaction between cellular integrins and ECM), in conjunction with cell polarization. In this sense, deletion of β1-integrin expression (Taddei et al. 2008) or alterations in the function of polarity proteins (Cdc42, Par3, aPKC, Pins/LGN) in mammary epithelial cells is associated with defects in mitotic spindle orientation or progenitor differentiation (Bray et al. 2011; McCaffrey and Macara 2009; Hao et al. 2010; Jaffe et al. 2008; Zheng et al. 2010).

8.5 Overview of Breast Cancer

Breast cancer is the most common cancer among women (Parkin et al. 1999). It progresses in a stepwise fashion through multiple stages including flat epithelial atypia (FAE), atypical ductal hyperplasia (ADH), ductal carcinoma in situ (DCIS), and invasive ductal carcinoma (IDC) (Bombonati and Sgroi 2011). FAE is considered a benign lesion and is characterized by ducts with nonuniform diameters, lined by single or multilayered epithelial cells (Schnitt 2003). The cells adjacent to the lumen retain at least some aspects of apical-basal polarity, whereas cells in the underlying layers do not contact the lumen and therefore lack apical-basal polarity.

Whereas ADH are small hyperplastic lesions that still retain some ductal structures, DCIS are larger and are characterized by uniform proliferative pre-malignant cells that have not invaded across the basement membrane (Ellis 2010; Zagouri et al. 2007). Up to 50 % of patients with DCIS will develop IDC, in which the carcinoma breaches the basement membrane. Therefore, mammary ductal and cellular organization changes throughout breast cancer progression, and loss of apical-basal polarity can occur at the earliest stages.

There are multiple breast cancer subtypes, which can be grouped based on the presence or absence of molecular markers and have distinct clinical outcomes. Luminal A breast cancers express markers of luminal epithelial cells, are estrogen receptor positive, and have the best prognosis (Rakha et al. 2008). Luminal B also express markers of luminal epithelial cells; however, they have reduced expression of genes associated with estrogen receptor signaling and have a poorer prognosis. HER2 breast cancers have amplified expression of the ErbB2 receptor tyrosine kinase and represent a poor prognosis subtype. Finally, triple-negative/basal-like breast cancers have the poorest prognosis and are negative for estrogen receptor, progesterone receptor, and ErbB2, but express markers of basal myoepithelial cells (Rakha et al. 2008). More recently, genome-wide expression profiling of breast cancers has led to a more refined classification system and the identification of an additional triple-negative cancer subtype called claudin low, which exhibits low expression of luminal differentiation markers (like claudins) and high expression of basal/mesenchymal and stem cell markers (Prat et al. 2010). Interestingly, the gene expression profile of the different breast cancer subtypes has a striking resemblance to expression profiles of cells along the stem/progenitor/differentiated cell spectrum, and it has been proposed that the cell of origin for the different breast cancer subtypes arises from distinct stem, progenitor, and differentiated cell populations (Visvader 2009). Strikingly, over 95 % of basal-like breast cancers have altered expression of at least one core component of the Crbs/Pals1/Patj, Par3/Par6/aPKC, or Scrib/Llgl/Dlg complexes, whereas they are disrupted in only 65 % of luminal A/B and 79 % of Her2-enriched tumors (TCGA 2012). Therefore, although the role of cell polarity proteins are best understood in luminal epithelial cells, we expect that they may have distinct functions in basal or stem cells, and therefore the functions of polarity proteins may be different depending on the cancer subtype.

8.6 Cell Polarity and Breast Cancer Progression

Cell polarity genes are essential regulators of epithelial organization that also function in growth control (Fig. 8.4). Furthermore, in some cases, loss of a polarity protein is sufficient for the development of benign or malignant lesions, indicating that they can function as tumor suppressors. For example, when Scrib is depleted from Comma-D cells (a mouse mammary cell line) and then transplanted orthotopically into the mammary fat pad, the glands exhibit epithelial overgrowth

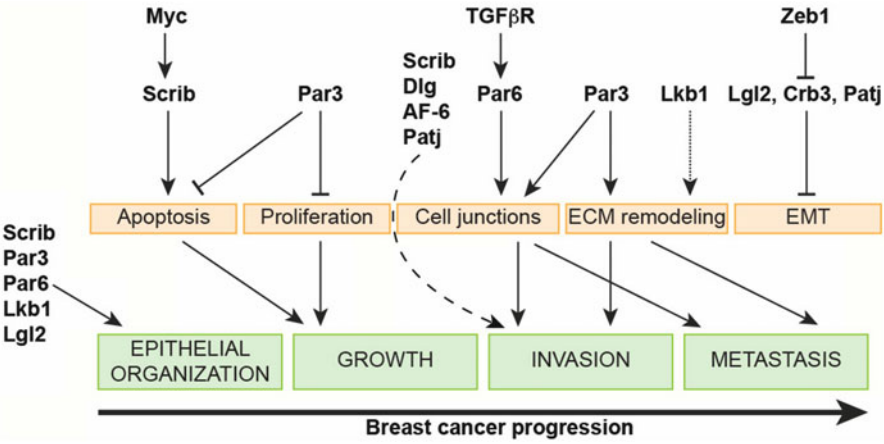

Fig. 8.4 Regulation of breast cancer progression by polarity proteins. Polarity proteins regulate diverse cellular processes of apoptosis, proliferation, cell junction remodeling, extracellular matrix (ECM) remodeling, and epithelial-mesenchymal transition (EMT). Each of these processes impinges on various stages of breast cancer progression from loss of epithelial organization and growth control, through invasion and metastasis. The *dashed line* represents that Scrib, Dlg, AF-6, and Patj regulate invasion through an undefined process. The *dotted line* signifies that Lkb1 regulates ECM remodeling, although it is not known whether this affects invasion and metastasis

with solid ducts that lack a central lumen (Zhan et al. 2008). This effect is, at least in part, due to fact that loss of Scrib protects cells from apoptosis (Zhan et al. 2008). Interestingly, 10 % of the Scrib-depleted mammary glands form palpable tumors with a well-differentiated glandular phenotype (Zhan et al. 2008). Given the low penetrance and long latency, cooperating events are likely necessary for tumor formation in Scrib-depleted glands.

The disruption of other polarity proteins can also induce early stages of tumor progression. When primary mouse mammary epithelial cells are depleted of the apical scaffold Par3 and transplanted orthotopically, the resulting ducts are dilated and multilayered, a phenotype that is reminiscent of the early stages of human breast cancers (McCaffrey and Macara 2009). Moreover, Par3-depleted ducts are significantly more proliferative than control ducts; however, they also have increased apoptosis, which offsets hyper-proliferation and limits tumor progression since palpable tumors are not observed (McCaffrey and Macara 2009; McCaffrey et al. 2012). These examples demonstrate that polarity proteins play a critical role in maintaining the ductal epithelium, and their loss can trigger early stages of cancer formation.

Given the low penetrance and long latency of tumors from Scrib-deficient cells, and the lack of palpable tumor formation in Par3-depleted mammary glands, it is likely that disrupted cell polarity cooperates with other events to promote tumorigenesis. For example, expression of Myc in the mammary gland is weakly tumorigenic, partly because Myc expression induces apoptosis through a pathway involving the GTPase Rac1, Jun N-terminal kinase (JNK), c-Jun, and the

proapoptotic protein Bim (Amundadottir et al. 1995; Zhan et al. 2008). Activation of this apoptotic pathway depends on the scaffold Scrib, and when Scrib is lost, Myc-induced apoptosis is short circuited enabling the formation of larger tumors (Zhan et al. 2008). Remarkably, mislocalization of Scrib is also able to enhance Myc-induced tumorigenesis (Zhan et al. 2008), supporting the view that polarity protein function can be disrupted by changes in expression or by changes in subcellular localization.

Disrupting other polarity proteins can also cooperate with Myc to promote mammary tumorigenesis. Homozygous deletion of *Lkb1* (also called Par4) from the mammary epithelium dramatically reduces tumor latency and increases both the number and size of tumors, compared to Myc alone (Partanen et al. 2012b). Interestingly, mice with heterozygous expression of *Lkb1* have an intermediate effect, indicating that even partial loss of Lkb1 expression sensitizes mice to Myc-induced tumors (Partanen et al. 2012b). However, unlike Scrib, loss of Lkb1 does not suppress Myc-induced apoptosis, indicating that polarity proteins can have diverse mechanisms in promoting Myc-driven tumors (Fig. 8.4). Instead, loss of Lkb1 leads to disrupted apical-basal polarity and cell junction defects, which caused mislocalization of the serine protease hepsin away from cell borders and compromised basement membrane organization (Lutzner et al. 2012). Importantly, low levels of Lkb1 correlate with elevated cytoplasmic hepsin in human breast cancers (Lutzner et al. 2012), indicating that the effects of loss of Lkb1 may be a general effect, independent of oncogenic functions of Myc specifically.

Disruption of Par3 also promotes oncogene-induced tumorigenesis and further promotes invasion and metastasis. Expression of the intracellular domain of the Notch receptor (NICD) in the mammary epithelium induces nonmetastatic tumors that are slow growing and retain E-cadherin and ZO-1 staining at cell-cell junctions (Hu et al. 2006; McCaffrey et al. 2012). However, depletion of Par3 from NICD-expressing tumors drastically reduces tumor latency, increases tumor growth, and promotes lung metastasis (McCaffrey et al. 2012). Interestingly, depletion of Par3 from an ErbB2 breast cancer model also promotes invasion and metastasis but does not affect primary tumor growth (Xue et al. 2012). Therefore, the growth-promoting effects of loss of Par3 may be dependent on the tumor context and the underlying signaling pathways regulating growth of that tumor. A striking example of this is that loss of Par3 in skin papilloma actually has the opposite effect and reduces tumor growth (Iden et al. 2012).

In some contexts, polarity proteins may act as oncogenes to promote breast tumorigenesis. For example, Par6 is overexpressed in hyperplastic benign breast lesions, and overexpression of Par6 in MCF10A cells induces hyper-proliferation, which acts through the MAPK pathway and is dependent on Par6 binding to Cdc42 and aPKC (Nolan et al. 2008). In addition, aPKCι itself is overexpressed in breast cancers, and in this case, expression correlates with more advanced tumors (Kojima et al. 2008; Paget et al. 2011). Screening of human breast cancer cell lines identified several with high levels of aPKCι activation; knocking down aPKCι expression in these cell lines reduced tumor cell proliferation and induced markers of senescence,

indicating that aPKCι overexpression may promote tumorigenesis by repressing senescence (Paget et al. 2011).

In addition to cooperating in parallel with oncogenes and tumor suppressors, polarity proteins can associate with oncogenes or tumor suppressors themselves to modulate tumor progression (Fig. 8.4). When bound to oncogenes, polarity proteins themselves may not act as classical oncogenes—where gain of function promotes tumorigenesis—but rather they function to enable oncogene-mediated tumor progression. For example, ErbB2 activation by induced dimerization promotes proliferation, inhibits apoptosis, and disrupts epithelial organization and apical-basal polarity (Aranda et al. 2006). Whereas Par6/aPKC has no role in ErbB2-mediated proliferation, they are necessary to both disrupt polarity and inhibit apoptosis. Interestingly, activated ErbB2 was shown to bind Par6/aPKC and displace Par3; therefore, activation of ErbB2 affects apical-basal polarity by disrupting the Par complex (Aranda et al. 2006). The contribution of the Par polarity complex in ErbB2-/Her2-positive breast cancers may occur at multiple levels. The 14-3-3σ tumor suppressor gene is frequently lost in ErbB2-amplified tumors, and genetic deletion of the 14-3-3σ locus in mice disrupts cell-cell junctions and apical-basal polarity and accelerates ErbB2-dependent tumor onset (Ling et al. 2010). Intriguingly, 14-3-3σ can bind Par3, and loss of 14-3-3σ mislocalizes Par3 from the plasma membrane (Ling et al. 2011; Ling et al. 2010), demonstrating another mechanism by which the Par complex can be disrupted in ErbB2 breast cancers.

8.7 Polarity and Epithelial-Mesenchymal Transitions (EMT) During Breast Cancer

Multiple mechanisms are employed by breast tumor cells to invade and disseminate from the primary tumor. At one end of the spectrum is collective migration, in which cells move as a group held together by cell-cell junctions. As described above, this is important for branching morphogenesis; however, collective invasion of groups of cells has also been observed in breast and other tumor cells (Friedl and Gilmour 2009; McCaffrey et al. 2012). At the other end of the spectrum is a single cell invasion, in which single cells can take various forms, such as amoeboid or mesenchymal, to invade through the extracellular matrix; interestingly, cells can dynamically change between invasion modes, demonstrating that invading cells exhibit remarkable plasticity (Friedl and Wolf 2009). Although invading cells have often, if not always, lost apical-basal polarity, they retain front-rear polarity, in which many apical-basal polarity proteins relocate to the leading edge to coordinate cytoskeletal remodeling (Godde et al. 2010).

Epithelial cells can be reprogrammed to become more mesenchymal, the so-called epithelial-mesenchymal transitions (EMT), which can promote a single cell mode of invasion. EMT is characterized by loss of E-cadherin and cell-cell junctions, loss of apical-basal polarity, and a switch in the expression of epithelial

cytoskeletal cytokeratins to mesenchymal cytoskeletal proteins like vimentin (Thiery et al. 2009). Furthermore, EMT confers stem cell-like properties to mammary cells, such as self-renewal and survival in low adhesion conditions (Mani et al. 2008; Morel et al. 2008).

EMT reprogramming is primarily driven by three families of transcription factors, Zeb, Twist, and Snail, which target the polarity machinery (Thiery et al. 2009). For example, Zeb1 suppresses expression of Crumbs3, Lgl2, and Patj (Fig. 8.4) (Aigner et al. 2007; Russ et al. 2012). Knockdown of Lgl2 induces a spindly "mesenchymal" phenotype; however, whether they undergo EMT is not clear since the expression of EMT markers was not reported. However, re-expression of Lgl2 is able to suppress Snail-induced EMT (Russ et al. 2012). However, simultaneously knockdown of two polarity proteins does not induce expression of mesenchymal markers, despite cells becoming invasive (Fig. 8.4) (Chatterjee et al. 2012). Moreover, knockdown of Par3 can induce invasion and metastasis, all in the absence of EMT (McCaffrey et al. 2012; Xue et al. 2012). Collectively, this indicates that loss of apical-basal polarity may be necessary for EMT; however, silencing of apical-basal polarity proteins is not sufficient to induce an EMT phenotype. These data support a model in which disrupted apical-basal polarity can induce invasion through diverse mechanisms that are independent of a mesenchymal mode of invasion (Fig. 8.4).

8.8 Cell Polarity and Invasion and Metastasis and Cell Polarity

The vast majority of breast cancer-related deaths result from metastasis to distant organs. Metastatic progression is a multistep process involving local invasion, entry into the circulatory or lymphatic system, exit at distant sites, and finally survival and growth of disseminated tumor cells. Although classically thought of as a late stage in tumor progression, there is substantial evidence that dissemination may occur early in the progression of breast cancer (Hüsemann et al. 2008; Podsypanina et al. 2008).

There is substantial evidence that disrupted apical-basal polarity can alter cell invasion. Whereas knocking down apical-basal proteins Scrib, AF-6, Patj, and Dlg alone had little effect on invasion of MCF10A cells, depletion of two proteins from different polarity complexes was sufficient to induce invasion (Fig. 8.4) (Chatterjee et al. 2012). This suggests that apical-basal polarity acts through multiple mechanisms or that polarity complexes can act redundantly to suppress cell invasion. It may also depend on the polarity protein targeted and how they affect other polarity components. For example, Par3 is mislocalized in the mammary epithelium of *Lkb1* knockout mice (Partanen et al. 2012b).

Interestingly, loss of a single polarity protein is sufficient to promote invasion when an oncogene is also present (Chatterjee et al. 2012; Dow et al. 2008). In MCF10A cells with activated ErbB2, loss of Scrib, Dlg, or AF-6 promoted invasion,

which was dependent on the ability of ErbB2 to interact with Par6/aPKC, again suggesting that multiple hits to the polarity machinery are necessary for invasion (Chatterjee et al. 2012). The cooperation of polarity in oncogene-mediated invasion is not limited to ErbB2 cancers. Depletion of Scrib in MCF10A cells expressing an activated Ras oncogene also induces extensive invasion (Dow et al. 2008). In this system, Scrib normally functions to suppress Ras-induced invasion, by blocking Raf-MEK-ERK signaling downstream of Ras (Dow et al. 2008).

In some contexts, signaling through polarity proteins is required for invasion, and disruption polarity signaling actually blocks invasion. For example, TGFβ is a potent inducer of invasion, and when mouse mammary epithelial cells are treated with TGFβ, they undergo robust invasion in 3D cultures (Viloria-Petit et al. 2009). However, expressing a Par6 mutant that no longer interacts with the TGFβ receptor disrupts signaling, and TGFβ no longer stimulates invasion (Viloria-Petit et al. 2009). This dependency on Par6 for growth factor receptor-mediated invasion is consistent with results from cells with active ErbB2 receptor; however the mechanisms are distinct. In response to TGFβ, Par6 is recruited to the receptor and phosphorylated, which then recruits an E3 ubiquitin ligase, Smurf1 (Viloria-Petit et al. 2009). However, in response to ErbB2 activation, Par6 is recruited to the receptor, but invasion acts through an Akt-dependent mechanism (Chatterjee et al. 2012). This demonstrates that polarity proteins may cooperate with distinct oncogenic signals through different effector pathways, with a similar end result of enhanced invasion.

Of the polarity complexes, only the Par complex has yet been shown to directly promote breast cancer metastasis in vivo. In addition to being necessary for TGF-β-dependent invasion, Par6 is also necessary for lung metastasis. Expressing a mutant that cannot be phosphorylated by the TGFβ receptor in the mouse mammary tumor EMT-6 cells blocks Par6 signaling, and both the incidence of metastasis and the number of metastatic colonies in the lungs are markedly decreased (Viloria-Petit et al. 2009). Furthermore, Par3 is frequently downregulated in human breast cancer, which correlates with metastatic progression, and two studies report that loss of Par3 promotes breast cancer metastasis (McCaffrey et al. 2012; Xue et al. 2012). Although tumorigenic, expression of NICD alone in the mammary epithelium does not progress to metastatic disease (Hu et al. 2006; McCaffrey et al. 2012). However, depletion of Par3 induces both local invasion and metastasis to the lungs. Mechanistically, loss of Par3 induces robust activation of Jak2/Stat3 signaling, an important mediator of immune function and breast cancer metastasis (McCaffrey et al. 2012; Ranger et al. 2009). Importantly, Stat3 activation is necessary for metastasis in Par3-depleted cells, because inhibiting Stat3 signaling with pharmacological inhibitors or shRNA reduces invasion in vitro and metastasis in vivo. Transcriptional profiling revealed that loss of Par3 upregulated MMP9, which induced remodeling of the extracellular matrix to enable invasion. Loss of Par3 causes mislocalization and activation of aPKC, which is necessary for Jak/Stat3 activation. Notably, cell-cell adhesions are retained, and cells do not undergo an overt EMT to become invasive (Fig. 8.4) (Macara and McCaffrey 2013; McCaffrey et al. 2012). Similarly, loss of Par3 increases invasion and

metastasis in an ErbB2 orthotopic transplant model (Xue et al. 2012). As has been reported previously, loss of Par3 induces global Rac1 activation by mislocalizing the Rac1 guanine nucleotide exchange factor Tiam1 (Chen and Macara 2005; Nishimura et al. 2005; Xue et al. 2012). Interestingly, altered Rac1 activity causes deregulated actin and E-cadherin dynamics at cell-cell junctions, thereby reducing cell cohesion and enabling invasion and metastasis (Xue et al. 2012). Furthermore, despite changes in E-cadherin dynamics, the cells do not show evidence of EMT (Xue et al. 2012). Therefore, loss of Par3 can cooperate with different oncogenes to induce metastasis, using complimentary mechanisms, but in the absence of EMT (Fig. 8.4).

8.9 Conclusions

Cell polarity is a dynamic event during epithelial morphogenesis and cancer progression in the mammary gland. A key function of apical-basal polarity proteins is to localize diverse signaling pathways at appropriate positions within cells to regulate cell proliferation, apoptosis, differentiation, and cell migration. Disruption of either expression or localization of the polarity machinery deregulates these events, which promotes cancer initiation as well as progression of invasive and metastatic breast cancer.

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Part IV
Pathogens Targeting Cell Polarity

Chapter 9
Cellular Polarity and Pathogenicity

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Abstract Cellular polarity is a fundamental mechanism involved in many common or specialized functions and shared by almost all cell types. Among the many cellular processes involving polarity are cell shaping, cell adhesion, cell migration, and cell division, as well as specialized functions like barrier formation, nutrient capture and directional transport in epithelia and endothelia, and signal transduction in neurons or phagocytosis in immune cells. Cell polarity is also involved in the compartmentalization of the organism and delimits the frontier between the external and internal environment. Bacteria, which live in close contacts with high eukaryotes, have evolved means to interact with host cell polarity, and this interaction may be either beneficial or detrimental for the host. In this chapter we will discuss the specific interactions of pathogenic bacteria with polarized epithelial or endothelial cells.

Keywords Pathogenic bacteria • Neisseria meningitidis • Pseudomonas aeruginosa • Polarity

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K. Ebnet (ed.), *Cell Polarity 2*, DOI 10.1007/978-3-319-14466-5_9

9.1 Cellular Polarity and Compartmentalization

Mucosal epithelia are the first line of defense of the organism. They cover the digestive tract (buccal/oral/esophageal, gastric, intestinal), the respiratory tract (nasal, buccal/oral/esophageal, pulmonary) and the genital tract. Mucosal epithelia allow the selective passage of nutrients and immune cells while restricting access to commensal and pathogenic flora. The mucosal barriers are actually a complex association of polarized epithelial cells with local specialized immune cells, secreting cells, lymphatic organs, and mucus flux. The selectivity of this barrier depends on the polarization of epithelial cells that form continuous layers with different membrane composition on their apical and basolateral sides.

Apical and basolateral membranes of both epithelia and endothelia display different composition in proteins, lipids, and phosphoinositides. Phosphoinositides are used as marker of polarity; in particular phosphatidylinositol 4,5-bisphosphate (PIP2) is specific for the apical membrane, and phosphatidylinositol 3,4,5-trisphosphate (PIP3) localizes at the basolateral membrane (Martin-Belmonte and Mostov 2008; Shewan et al. 2011). The phosphatidylinositol 3-kinase (PI3K), an enzyme responsible for the synthesis of PIP3, is a key regulator of cell polarity, acting through multiple signaling pathways such as the Rho GTPases, Rac1, and Cdc42. Indeed, PI3K is sufficient to induce the formation of basolateral membrane in epithelial cells (Gassama-Diagne et al. 2006). Apical and basolateral membranes are physically separated by the cell–cell junctions, i.e., adherens junctions (AJs) and tight junctions (TJs). Two complexes form AJs: the nectin–afadin and the cadherin–catenin complexes (for review (Niessen and Gottardi 2008)). Nectins and cadherins are homophylic transmembrane proteins that are required for the maintenance of cell–cell contact and are believed to be essential for the polarization of epithelial and endothelial cells (Ooshio et al. 2007). Nectin and cadherin are connected to the actin cytoskeleton through the afadin protein and catenin, respectively. TJs are localized apically from AJs. TJs regulate the paracellular flux of ions and nutrients and restrict microorganisms, such as commensal bacteria, in the lumen. TJs also block the diffusion of membrane lipids and proteins, thus maintaining the differential composition of apical and basolateral membranes. They contain several proteins such as JAM, claudins, and occludins that interact with their respective partners of the neighboring cells and are linked to the actin cytoskeleton through zonula occludens proteins ZO-1, ZO-2, and ZO-3. AJs and TJs are responsible for the barrier properties of the mucosal epithelia and that of the vascular endothelia.

Mechanisms of polarization of epithelium and endothelium involve three groups of highly conserved proteins called polarity complexes and designated PAR, Crumbs, and SCRIB, respectively (Martin-Belmonte and Mostov 2008). In mammalian cells, the master regulator complex is the PAR complex formed by Par3, Par6, and the atypical protein kinase C (aPKC). The PAR polarity complex is responsible for the establishments of the apical–basal border (Goldstein and Macara 2007). Which signal initiates the polarization remains unclear, but it seems that cell–cell contact initiates the recruitment and activation of the Par3/Par6/aPKC

complex, possibly through the nectin–afadin complex, a component of AJs, which binds directly Par3 (Ooshio et al. 2007). The PAR polarity complex coordinates the formation of AJs and TJs in both endothelial and epithelial cells (Martin-Belmonte and Mostov 2008; Baum and Georgiou 2011; Tyler et al. 2010; Lizama and Zovein 2013). The PAR polarity complex is directly recruited by the pleiotropic Rho GTPase Cdc42, which is a master regulator of cell polarity (Joberty et al. 2000; Etienne-Manneville 2004).

The PAR polarity complex recruits the Crumbs complex (CRB) that is formed by the proteins CRB, PALS1, and PATJ (Wang and Margolis 2007). This complex is responsible for the formation of the apical membrane. Another complex, designated SCRIB, which contains Scribble, Dgl, and Lgl, defines the basolateral membrane. Cell to matrix adhesion positively regulates the SCRIB complex. The SCRIB and PAR complexes are mutually exclusive and negatively regulate each other (Burute and Thery 2012).

9.2 Pathogens and Cellular Polarity

Many bacteria live as commensal on the mucosal epithelium. Only few pathogens are known to be able to disorganize or to cross these barriers. Invasive pathogenic bacteria that reach the blood circulation are also able to interact and cross highly polarized endothelial cells that form the vascular wall. The interaction of pathogenic bacteria with human cells leads to epithelium/endothelium disruption, thus favoring bacterial dissemination. In some circumstances, the bacterial interaction is responsible for modifications of host cell behavior. To achieve their goal, bacteria hijack several signaling pathways involved in cell survival, immune response, and cell polarity.

To date, few pathogens have been described for their ability to challenge cell polarity. In this chapter, we will discuss the different strategies pathogenic bacteria have evolved to affect cell polarity to promote their persistence and dissemination, using selected model organisms. *Chlamydia trachomatis* and *Streptococcus pneumoniae* modify the front–rear polarity, while *Pseudomonas aeruginosa* alter the apical/basal polarity. Other pathogens, such as *Listeria monocytogenes* and *Helicobacter pylori*, interact with AJs and/or TJs, whereas pathogens, *Neisseria meningitidis* or *Bacillus anthracis*, interact with endothelial cells and increase the vascular permeability. Finally, we will discuss the role of Cdc42, a key component of cell polarity, during host cell colonization.

9.2.1 Chlamydia trachomatis *and* Streptococcus pneumoniae *Alter Front–Rear Polarity*

Chlamydia trachomatis is one of the few bacterial pathogens that had been shown to actively modify host cell polarity during infection. This bacterium is an obligate intracellular pathogen that causes sexually transmitted diseases responsible for

urethritis, trachoma, and infertility. To support growth and replication, this bacterium has to organize an intracellular niche, i.e., membrane-bound vacuoles named inclusions, which intercept host vesicles from the Golgi apparatus to obtain nutrients (Heuer et al. 2009; Subtil 2011). Early during infection, *Chlamydia trachomatis* actively highjack the microtubule network. *Chlamydia trachomatis* inclusion bodies are transported by dynein along the microtubule network and then fused with each other at a host cell centrosome (Richards et al. 2013). Moreover, during infection, the Golgi apparatus is fragmented through the truncation of Golgi matrix protein golgin-84 (Heuer et al. 2009) and the implication of GTPase and that of Rab6 and Rab11 (Rejman Lipinski et al. 2009). While a role of the Golgi apparatus in directed secretion and cell polarity was described before (Bershadsky and Futerman 1994; Yadav et al. 2009), the *Chlamydia trachomatis*-induced Golgi apparatus fragmentation was only recently associated with cell polarity (Heymann et al. 2013). Heymann et al. showed that during *Chlamydia trachomatis* infection, the Golgi apparatus and the microtubule organization center (MTOC) are no longer relocalized during directional cell migration, leading to the inability of infected cells to migrate. This is only partly dependent on golgin-84 cleavage that causes Golgi apparatus fragmentation. Interestingly, the authors clearly showed that another factor, yet to be determined, affects motility. Thus, *Chlamydia trachomatis* have to interfere with the vesicular traffic to proliferate. This leads to a complete loss of cell polarity.

Streptococcus pneumoniae is another pathogen capable of altering cell polarity. It asymptomatically resides in the throat of healthy carriers. However, it is also a worldwide leading cause of pneumonia and meningitis. This bacterium adheres to cells via specific adhesins (Hammerschmidt 2006) and is able to transcytose through cells by the interaction of the pneumococcal surface protein C (or PspC) with the polymeric immunoglobulin receptor PIgR. This interaction leads to pathogen internalization through the activation of a PI3K/Cdc42 pathway (Zhang et al. 2000; Agarwal and Hammerschmidt 2009). In addition, *S. pneumoniae* also expresses an important toxin known as pneumolysin. This cholesterol-binding cytolysin is a major virulence factor of the pneumococcus. In vitro, pneumolysin forms ring-like pores of approximately 260 Å in cholesterol-enriched membranes. This property is responsible for the toxin-induced cell lysis (Bonev et al. 2000). However, at sub-lytic concentration, pneumolysin forms non-lytic ion channel-like pores (El-Rachkidy et al. 2008) that stabilize microtubules in a cholesterol- and Src-dependent manner (Iliev et al. 2009), thus inhibiting intracellular trafficking and dramatically altering host cell polarity.

9.2.2 Pseudomonas aeruginosa *Deals with the Apical–Basal Polarity of Epithelial Cells*

The Gram-negative rod *Pseudomonas aeruginosa* is an opportunistic pathogen of particular importance in nosocomial infections (Bereket et al. 2012). It has a tropism for injured tissues such as skin burns or surgical incisions and establishes

chronic lung infections in patients with cystic fibrosis. It has been shown that *P. aeruginosa* adhesion and invasion of epithelial cells are strongly reduced by cell polarization and cell–cell junction integrity (Plotkowski et al. 1999). *P. aeruginosa* possesses two major adhesins, the type IV pili (Tfp) and flagella, which promote two distinct binding mechanisms (Bucior et al. 2010; Bucior et al. 2012). Tfp mediate attachment onto host N-glycans localized at the apical surface of lung epithelial cells, while flagella bind to heparin sulfate proteoglycans (HSPGs) localized on the basolateral surface (Fig. 9.1). Both adhesins cooperate to optimize *P. aeruginosa* adhesion onto the epithelium. In addition, an alteration of the mucosal epithelium secondary to a wound modifies the polarity of the epithelium and uncovers a previously hidden receptor, thus allowing colonization of this niche by the pathogen.

Interestingly, basolateral membrane enrichment in PIP3 is likely to favor internalization of the pathogen into epithelial cells (Kazmierczak et al. 2004; Bridge et al. 2010). Kierbel and colleagues showed that initial adhesion of *P. aeruginosa* onto epithelial cells induces the activation of the PI3K and of the serine/threonine kinase Akt, leading to the local synthesis of PIP3 (Kierbel et al. 2005). This signaling pathway is responsible for a localized modification of the membrane polarity (Gassama-Diagne et al. 2006; Kierbel et al. 2007) (Fig. 9.1). This local transformation of apical membrane into basolateral membrane seems to be a key step in *P. aeruginosa* colonization of its niche.

After adhesion, the T3SS-dependent toxin ExoS of *P. aeruginosa* is injected into the host cell cytoplasm and induces a redistribution of the TJ proteins ZO-1 and occludin, leading to an increased cell permeability and to the transmigration of bacteria across a polarized epithelium (Soong et al. 2008).

Polarization of mucosal epithelium appears to be both the main line of defense against *P. aeruginosa* initial adhesion and the Achilles heel of these cells.

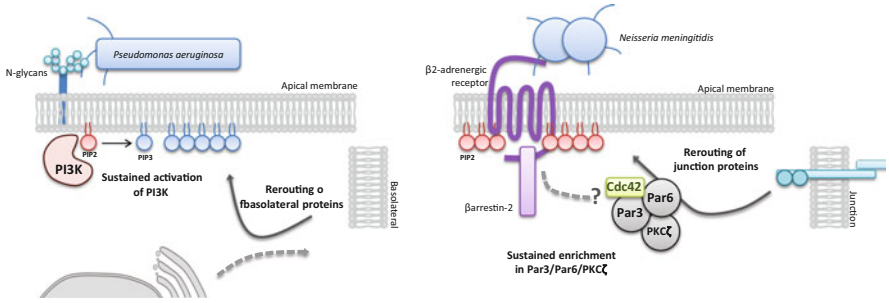

Fig. 9.1 Highlight on the modification of apical–basal polarity triggered by *Neisseria meningitidis* and *Pseudomonas aeruginosa*. *P. aeruginosa* adheres to the apical side of epithelial cells through interaction with N-glycans. Then, sustained activation of PI3K allows the local enrichment in PIP3. This is sufficient for relocation of basolateral membrane components by transcytosis. *N. meningitidis* activates a β2-adrenergic receptor/βarrestin-2 pathway that leads to membrane enrichment in PIP2 and the sustained recruitment of Cdc42 and that of the Par3/Par6/PKC-ζ polarity complex. Then, junction components are rerouted to the site of meningococcal adhesion. This is responsible for VE-cadherin depletion at the cell–cell junctions

9.2.3 *Pathogens Targeting Cell–Cell Junctions*

9.2.3.1 *Listeria monocytogenes*

Listeria monocytogenes is a Gram-positive facultative intracellular bacterium. It is a well-known opportunistic foodborne pathogen of humans and other mammals. *L. monocytogenes* causes listeriosis, a severe invasive disease, which occurs after food poisoning *or in* immunocompromised patients. This bacterium has a facultative intracellular life cycle into the intestine of its hosts. *L. monocytogenes* enters enterocyte cytoplasm, multiplies, and spreads into this compartment before infection of neighboring cells. To achieve this goal, *L. monocytogenes* possesses one major invasin, known as internalin A (InlA) (Bierne et al. 2007), that binds the AJ component E-cadherin and induces its own internalization into enterocytes (Bonazzi et al. 2009). Noteworthily, the InlA–E-cadherin interaction is species specific (Lecuit 2007), e.g., InlA interacts only with human E-cadherin but not with murine E-cadherin. This interaction is critical for the internalization of the bacterium; indeed, a mutant deficient for InlA is noninvasive in human enterocytes and avirulent in transgenic mice expressing the human E-cadherin (Disson et al. 2009). Upon InlA–E-cadherin interaction, activation of the Src kinase leads to a local actin reorganization involving dynamin, cortactin, and the Arp2/3 complex. In addition, the activation of the PI3K and Rho GTPases and the recruitment of α-catenin/β-catenin to the site of bacterial adhesion are all necessary for the invasion of these cells by *L. monocytogenes* (for review (Pizarro-Cerda et al. 2012)).

E-cadherin is normally hidden behind TJs and not accessible from the apical side of the epithelium, thus restricting *L. monocytogenes* entry. However, recent studies have shown that the intestinal epithelium displayed weaknesses (1) at the tip of intestinal villi, where removal of a dying cell is responsible for a local and transient defect of polarity (Pentecost et al. 2010), and (2) at the junction between enterocytes with goblet cells (Nikitas et al. 2011). At both sites, E-cadherin is accessible to InlA from the intestinal lumen, allowing internalization and proliferation of the bacteria.

9.2.3.2 *Helicobacter pylori*

Helicobacter pylori, a long helicoidally Gram-negative bacterium, is a well-known gastric pathogen associated with peptic ulcers and cancer. *H. pylori* possess a type IV secretion system (T4SS), a needle-like organelle capable of injecting toxins directly into the host cell cytoplasm. The CagA toxin is injected through the T4SS and acts at a different level on cell physiology. CagA activates the hepatocyte growth factor Met and causes the internalization of the AJ compound E-cadherin (Churin et al. 2003). CagA also associates with ZO-1 and JAM1 and recruits TJ component at the site of bacterial adhesion where they form an ectopic junction

(Amieva et al. 2003). These mechanisms provoke the degradation of existing AJs and TJs and a leakage of the epithelium.

9.2.3.3 Other Pathogenic Bacteria

Clostridium difficile, a Gram-positive rod, is an opportunistic pathogen responsible for gastrointestinal diseases and a leading cause of diarrhea in hospitalized patients. The diarrheic nature of *C. difficile* depends mainly on the expression of two toxins: TcdA and TcdB (Vedantam et al. 2012). The mechanism of secretion of these toxins is still unclear but seems to depend on the expression of a pore-forming protein (Tan et al. 2001). Once released, these toxins enter the host cell cytoplasm by receptor-mediated endocytosis (Voth and Ballard 2005). The acidification of the endosome is responsible for the activation of the toxins that will insert into the vacuole membrane and translocate to the cytoplasm (Davies et al. 2011). Once in the cytosol, TcdA and TcdB glycosylate and subsequently inactivate the GTPases Rho, Rac, and Cdc42, thus leading to the disruption of TJs and an increase in epithelium permeability (Feltis et al. 2000; Voth and Ballard 2005).

Interestingly, *Shigella flexneri*, a Gram-negative pathogen responsible for severe diarrheas and dysentery, has been described to actively stabilize adhesion of epithelial cell to the extracellular matrix and to reinforce cell–cell junction, leading to the inhibition of infected cell detachment. *S. flexneri* is transmitted by the fecal–oral route and infects humans via contaminated food or water. The polarity of epithelial cells forms a functional barrier since *S. flexneri* is not able to efficiently invade enterocytes from the apical membrane (Mounier et al. 1992). However, *S. flexneri* is able to cross the intestinal barrier by transcytosis through the M-cell (Sansonetti et al. 1996). Once in the basal compartment, bacteria invade epithelial cells, evade the vacuole (Ray et al. 2009), survive and move into the cytoplasm, and then travel from cell to cell using actin-based motility (Cossart and Sansonetti 2004; Schroeder and Hilbi 2008). Interestingly, during invasion of epithelial cells, the secretion of OspE through T3SS is responsible for the activation of the integrin-linked kinase (ILK). ILK activation increases the level of integrins at the membrane and reduces their recycling, thus stabilizing and promoting the formation of focal adhesions. This promotes stabilization and adhesion of infected cells to favor bacterial dissemination (Kim et al. 2009; Van Nhieu and Guignot 2009).

Finally, it has been recently shown that the Gram-negative pathogen *Neisseria gonorrhoeae*, a causative agent of the "clap" disease, targets TJ-associated protein β-catenin by an unknown mechanism. Infection of polarized epithelial cells with gonococci induces a reorganization of junctional proteins and a weakening of cell–cell junctions in order to promote bacterial attachment and transmigration through the epithelium (Edwards et al. 2013).

9.2.4 *Pathogenic Bacteria That interact With Endothelial Cells*

There are fewer pathogens interacting with the endothelial barrier. *Bacillus anthracis* and *Neisseria meningitidis*, two well-described pathogens that directly alter the cell–cell junctions and polarity, will be further described below.

9.2.4.1 *Bacillus anthracis*

Bacillus anthracis, also known as anthrax, is a deadly pathogen particularly known for its use as a bioweapon. This pathogen is responsible for different clinical presentations depending on the route of inoculation (skin edema, pneumonia, gastrointestinal disease, and hemorrhages). *B. anthracis* acts through a set of AB toxins with different effects. AB toxins are two component toxins secreted by pathogenic bacteria where the B component drives the correct delivery of the active compound A. Here, the B component, the protective antigen (PA), allows the delivery of two different A components, the lethal factor (LF) and the edema factor (EF). PA allows the receptor-mediated endocytosis of both LF and EF through the interaction with an integrin-like domain. The acidified endosome activates the PA that will form a pore into the membrane of the vacuole and allow the translocation into the cytosol of LF and EF (Mourez 2004). LF and EF act as a zinc-dependent endoprotease and an adenylate cyclase, respectively. LF and EF block different signaling pathways, including Notch, and disrupt endothelial barrier functions by preventing the proper targeting of molecules such as VE-cadherin at AJs (Guichard et al. 2010; Liu et al. 2012). Additionally, *B. anthracis* expresses and secretes the metalloprotease InhA that directly cleaves the TJs protein ZO-1 and increases the endothelial permeability (Mukherjee et al. 2011). These mechanisms are likely to be responsible for *B. anthracis*-induced hemorrhages. A last toxin, the anthrolysin O, has been proposed to disrupt epithelial permeability in the intestine through the reorganization of TJs (Bishop et al. 2010). The exact mechanism by which this toxin acts remains to be deciphered.

9.2.4.2 Hijacking of Host Cell Polarity by *Neisseria meningitidis*

Neisseria meningitidis (the meningococcus) is a Gram-negative diplococcus restricted to humans. This common inhabitant of the human nasopharynx is also responsible for two often fatal conditions: cerebrospinal meningitis and/or *purpura fulminans* (i.e., an extensive necrotic *purpura* with a massive vascular leakage and multiple organ failures). Indeed, in a small proportion of colonized people, the meningococcus invades the bloodstream and colonizes human vessels to cause meningitis and *purpura fulminans*. To cause meningitis, *N. meningitidis* cross the blood–brain barrier, a unique structure that tightly regulates the exchange of

nutrients from the blood to the central nervous system and to restrict the entry of blood-borne pathogen (Ballabh et al. 2004). The strategy of the meningococcus is to hijack endothelial cell polarity to open the paracellular pathway, thus allowing the crossing of the blood–brain barrier. The consequences of the interaction of *N. meningitidis* with host cells are not due to the injection of effectors into host cells via secretion systems but to the activation of signaling pathways following the recognition of host cell receptors by bacterial ligands.

First Step, Adhesion to Endothelial Cells

In vitro and in vivo experiments, using human skin grafted on SCID mice, revealed that the particular tropism of *N. meningitidis* for endothelial cells is mainly due to type IV pili (Tfp) (Pron et al. 1997; Dupin et al. 2012; Join-Lambert et al. 2013). Tfp consists of the multimeric assembly of the major pilin PilE that is continuously assembled into fibers from a platform in the inner membrane (for review see (Carbonnelle et al. 2009)). Three other minor pilins are associated into Tfp: ComP, PilX, and PilV. Each minor pilin is given a specific phenotype to the fiber, i.e., competence for DNA transformation, bacterial aggregation, and signaling to human cells, respectively (Winther-Larsen et al. 2001; Helaine et al. 2005; Mikaty et al. 2009; Brown et al. 2010). While the biology of Tfp is well known, the adhesion receptor on eukaryotic cells remains controversial. On the other hand, a signaling receptor, independent of a potent adhesion receptor, has been described in endothelial cells, i.e., the β2-adrenergic receptor (Coureuil et al. 2010; Lecuyer et al. 2012). The N-terminal domain of this receptor is targeted by the major pilin PilE and the minor pilus component PilV, resulting in the activation of a β-arrestin-mediated signaling pathway but not that of the heterotrimeric Gαs/adenylyl cyclase/cAMP pathway, a property referred to as biased activation (Drake et al. 2008; Coureuil et al. 2010). β-arrestins are scaffolding proteins involved in many cellular processes such as receptor internalization, MAP kinase activation, and actin polymerization (Scott et al. 2006; DeWire et al. 2007). The accumulation of β-arrestins appears to be critical during meningococcal-induced host cell response (Coureuil et al. 2010).

Neisseria meningitidis Recruits the Polarity Machinery to Cross the Endothelium

Following initial adhesion, *N. meningitidis*-activated β2-adrenergic pathway leads to the accumulation of a "raft-like" membrane domain enriched in cholesterol and PIP2 (Doulet et al. 2006; Mikaty et al. 2009) and then to the recruitment of a Cdc42 pathway. Noteworthily, the β2-adrenergic receptor is not internalized after infection by *N. meningitidis*, thus suggesting that the β2-adrenergic receptor is trapped in the "raft-like" structure. Once activated by the β2AR-βarrs pathway (Cant and Pitcher 2005; Coureuil et al. 2010), phosphorylated ezrin (which is recruited at

the site of meningococcal adhesion) sequesters transmembrane receptors, organizes actin filaments, and links the cortical actin network to the membrane (Doulet et al. 2006; Coureuil et al. 2010; Fehon et al. 2010). This process leads to the accumulation of many factors in a structure referred to as the cortical plaque (Merz et al. 1999), in which Rho GTPases, Src kinase, Arp2/3, and cortactin are activated and promote the formation of membrane protrusions (Eugene et al. 2002; Lambotin et al. 2005; Coureuil et al. 2009). These protrusions are associated with the shielding of growing microcolonies against shear stresses in the bloodstream (Mikaty et al. 2009). Interestingly, Arp2/3 and cortactin recruitment is dependent on Cdc42 and Par6/PKC-ζ while Src is recruited through its interaction with β-arrestins (Coureuil et al. 2010). The recruitment of Par6/PKC-ζ is followed by the Par6-dependent recruitment of Par3 and the subsequent delocalization of protein from the existing AJs (such as VE-cadherin, p120-catenin). These proteins are then sequestrated underneath bacterial colonies through their interaction with β-arrestins (Coureuil et al. 2009; Coureuil et al. 2010) (Fig. 9.1). Thus, the interaction of *N. meningitidis* with the apical membrane of endothelial cells leads to the formation of an "ectopic early junction-like domain," enriched in junction proteins. The formation of this junction-like domain at the site of bacterial cell interaction will eventually deplete the intercellular junction of VE-cadherin and β-catenins. This depletion opens the paracellular route and allows the crossing of the monolayer by *N. meningitidis*.

9.3 Is the Impact on Cell Polarity Underestimated *During Host–Pathogen Interaction*?

The interaction between pathogens and cellular polarity mechanisms is still a recent area of research. To date, most of the data concerning host–pathogen interaction are related to internalization and actin polymerization (for review see (Gouin et al. 2005)). However, several pathogens are also able to interact directly with Cdc42 itself, which is known to be essential for the establishment of cell polarity (Etienne-Manneville 2004). The impact of host–pathogen interaction on cell polarity has not been assessed (Fig. 9.2).

Salmonella enterica, the agent of typhoid fever, is able to invade non-phagocytic epithelial cell. Internalization is dependent on the type III secretion system (T3SS) of *S. enterica*, a needle organelle-like T4SS that allows the injection into the host cell cytoplasm of the effector SopE. SopE activates Rac1 and Cdc42, thanks to the functional mimicry with host GTPase exchange factors (GEFs) (Hardt et al. 1998). A second effector, SopB, is able to recruit the Arf GEF, ARNO, which will act in concert with SopE and leads to the recruitment of the actin nucleator WASP (Humphreys et al. 2012). Later, *S. enterica* also injects the third effector SptP that acts as a GTPase-activating protein (GAP) to inhibit Cdc42 (Rodriguez-Pachon et al. 2002).

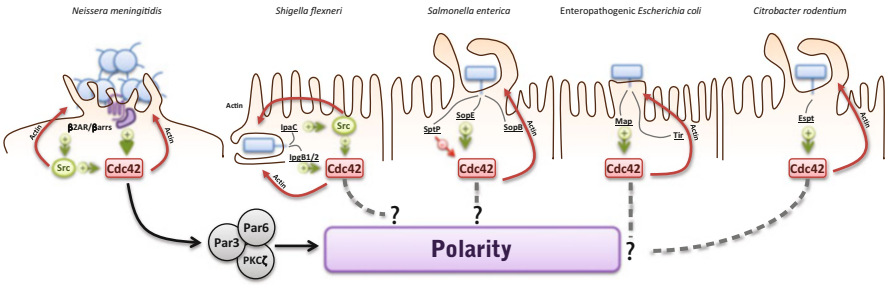

Fig. 9.2 Is Cdc42 an effector of cell polarity during infection? *N. meningitidis* interacts with the β2-adrenergic receptor and recruits β-arrestins through its type IV pili. This leads to the activation of Cdc42 and to the recruitment of the Par3/Par6/PKC-ζ polarity complex. *Shigella flexneri*, *Salmonella enterica*, enteropathogenic *Escherichia coli*, and *Citrobacter rodentium* all possess GEF-like effectors, injected via dedicated secretion systems that activate directly Cdc42 and promote actin polymerization. *S. flexneri* injects IpgB1/2 to activate Cdc42, while IpaC directly activates Src. *Salmonella enterica* injects SopE and SptP to activate and then inhibit Cdc42, respectively, while SopB directly activates actin polymerization. Enteropathogenic *E. coli* injects Map and Tir that activate Cdc42 and actin polymerization, respectively. *C. rodentium* injects Espt to activate Cdc42. The impact of pathogen-induced activation of Cdc42 on host cell polarity remains to be characterized

Similarly, *S. flexneri* has evolved a similar strategy to enter host cells by targeting Rac1 and Cdc42 through injection of IpgB1/2 and IpaC into the cytoplasm of the host cell. IpgB1/2 effectors also act as GEFs and activate Rac1 or Cdc42 (Ohya et al. 2005; Huang et al. 2009), while IpaC activates Cdc42 through the Src kinase pathway (Terry et al. 2008; Mounier et al. 2009).

Enteropathogenic *Escherichia coli* (EPEC), an intestinal Gram-negative pathogen responsible for diarrhea, activate actin dynamics through two different pathways. The first one is independent of Cdc42 and relies on the T3SS-secreted protein Tir. Tir leads to the formation of an actin-rich pedestal and favors firm adhesion of the bacterium. A second effector referred to as Map, a protein also secreted through the T3SS, possesses a GEF activity for Cdc42 (Kenny et al. 2002; Huang et al. 2009). Noteworthily, additionally to Map, two other T3SS toxins, EpsF and EpsG, act through an undefined mechanism to destabilize TJs and disrupt cell–cell junctions (Dean and Kenny 2004; Matsuzawa et al. 2005).

Finally, *Citrobacter rodentium* was recently described to inject the effector EspT into host cells to allow the formation of ruffles and lamellipodia through the direct activation of Cdc42 and Rac1 (Bulgin et al. 2009).

9.4 Conclusion

Host organisms have evolved to protect themselves from harmful microorganisms, and pathogenic bacteria have developed dedicated tools to circumvent epithelial and endothelial barriers. However, little is known about the subversion of polarity by pathogens. Only few pathogens were shown to actively alter host cell polarity.

On the other hand, the consequences of bacteria–host cell interaction(s) on host cell polarity are likely to be underestimated. Indeed, injection into the cytoplasm of effectors that impact the whole cell cytoskeleton are likely to modify cell polarity.

Acknowledgments We thank Dr. Alain Charbit for the helpful discussion and a careful reading of the manuscript.

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K. Ebnet (ed.), *Cell Polarity 2*, DOI 10.1007/978-3-319-14466-5