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).

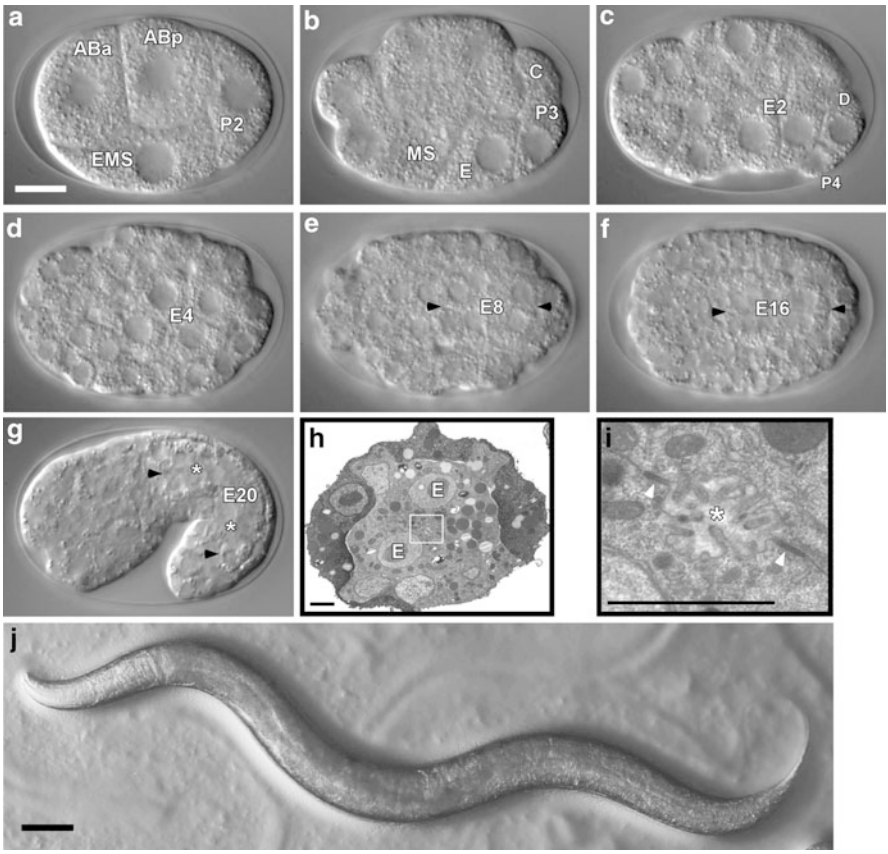

Fig. 2.1 *C. elegans* embryogenesis and development of the intestine (E lineage, the midgut, or endoderm). (**a**–**c**) Generation of five somatic founder cells (AB, MS, E, C, and D) and the primordial germ cell P4. (**a**) 4-cell stage; (**b**) 12-cell stage: the intestinal precursor cell, the E cell, 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**)

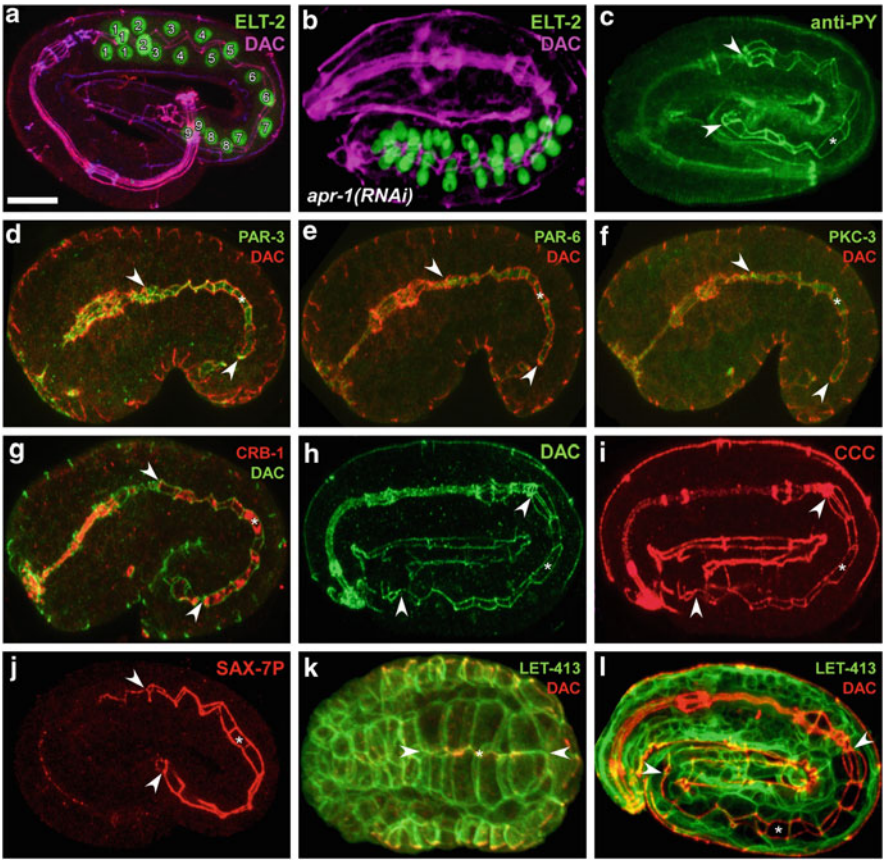

Fig. 2.2 Distribution of intestinal markers during *C. elegans* embryogenesis. (**a**, **b**) Wild-type embryo (fourfold stage, Table 2.1) and *apr-1*(*RNAi*) embryo (threefold stage) stained against the nuclear ELT-2 GATA-factor (anti-GFP fluorescence) and the junctional DLG-1/Discs large–AJM-1 complex (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

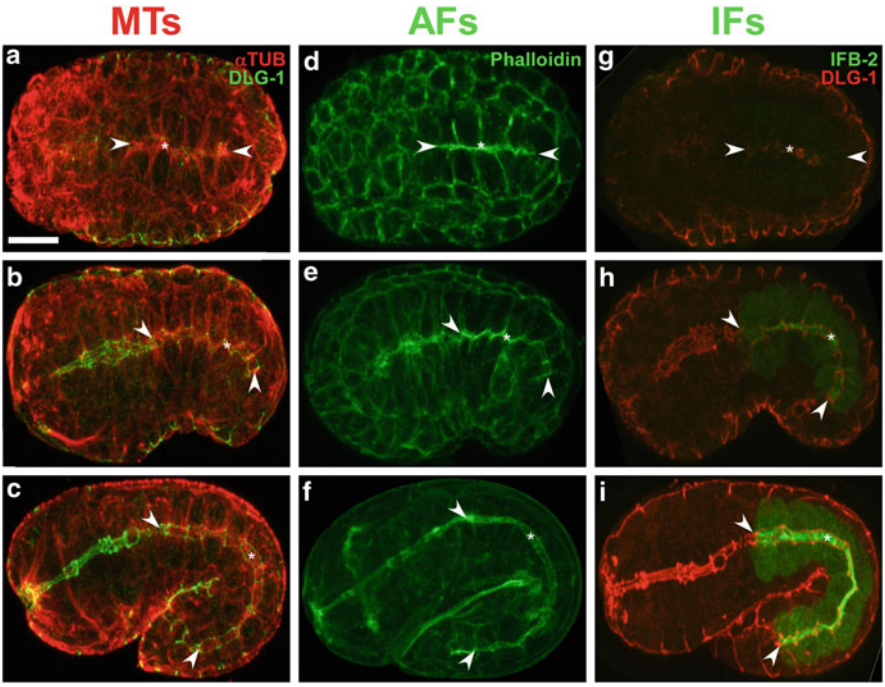

Fig. 2.3 Distribution of the three major cytoskeletal networks during development of the *C. elegans* intestine. (**a**–**c**) Tubulin-based microtubules (MTs, *red*, anti-α-tubulin fluorescence, mab4A1; Piperno and Fuller (1985)). (**d**–**f**) Actin-based microfilaments (AFs, *green*, phalloidin staining). (**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

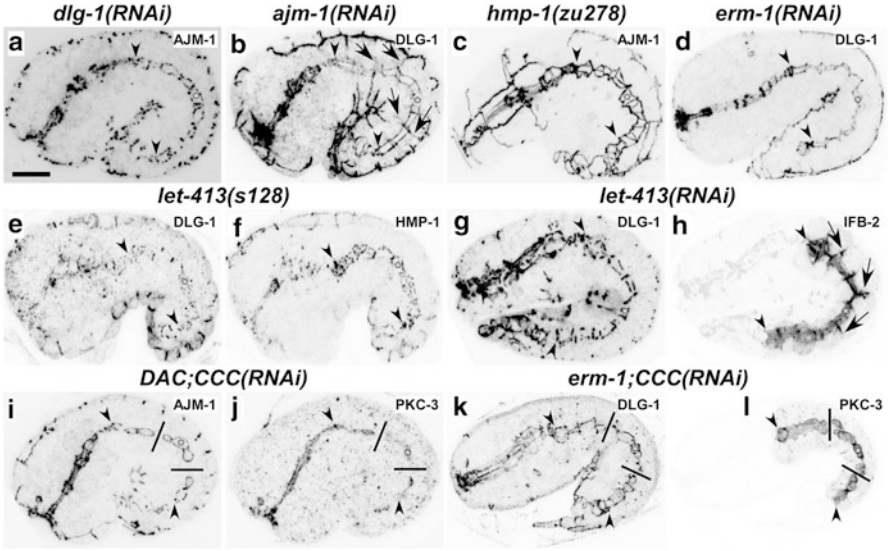

Fig. 2.4 Distribution of junctional and apicoluminal markers in *C. elegans* RNAi and mutant embryos. (**a**, **b**) RNAi against the DAC (DLG-1/Discs large–AJM-1 complex). Note that AJM-1 puncta do not consolidate into a mature apical junctional belt (**a**, compare to Fig. 2.2c), whereas DLG-1 spreads 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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