

PAR-3 mediates the initial clustering and apical localization of junction and polarity proteins during *C. elegans* intestinal epithelial cell polarization

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SUMMARY

The apicobasal polarity of epithelial cells is critical for organ morphogenesis and function, and loss of polarity can promote tumorigenesis. Most epithelial cells form when precursor cells receive a polarization cue, develop distinct apical and basolateral domains and assemble junctions near their apical surface. The scaffolding protein PAR-3 regulates epithelial cell polarity, but its cellular role in the transition from precursor cell to polarized epithelial cell has not been determined *in vivo*. Here, we use a targeted protein-degradation strategy to remove PAR-3 from *C. elegans* embryos and examine its cellular role as intestinal precursor cells become polarized epithelial cells. At initial stages of polarization, PAR-3 accumulates in cortical foci that contain E-cadherin, other adherens junction proteins, and the polarity proteins PAR-6 and PKC-3. Using live imaging, we show that PAR-3 foci move apically and cluster, and that PAR-3 is required to assemble E-cadherin into foci and for foci to accumulate at the apical surface. We propose that PAR-3 facilitates polarization by promoting the initial clustering of junction and polarity proteins that then travel and accumulate apically. Unexpectedly, superficial epidermal cells form apical junctions in the absence of PAR-3, and we show that PAR-6 has a PAR-3-independent role in these cells to promote apical junction maturation. These findings indicate that PAR-3 and PAR-6 function sequentially to position and mature apical junctions, and that the requirement for PAR-3 can vary in different types of epithelial cells.

KEY WORDS: PAR-3, PAR-6, Epithelial, Mesenchymal, Polarity, *C. elegans*

INTRODUCTION

Polarized epithelial cells perform vital roles in organ morphogenesis and function. Most types of epithelial cells form when precursor cells respond to a polarizing cue, develop distinct apical and basolateral surfaces and assemble apical junctions with neighboring cells (Giepmans and van Ijzendoorn, 2009; Shin et al., 2006). Mutations that disrupt the apicobasal polarity of epithelial cells cause defects in tissue morphogenesis and integrity, impair organ function and can lead to unchecked proliferation and tumorigenesis (Bilder, 2004; Dow and Humbert, 2007; Wodarz and Nathke, 2007). Therefore, there is considerable interest in learning how cells polarize to form epithelial cells and how polarity is lost in certain types of cancer.

Many proteins that regulate epithelial cell polarity have been identified through genetic studies in model organisms. One important polarity regulator is the multi-PDZ domain protein PAR-3. First identified for its role in polarizing the *C. elegans* one-cell embryo (Etemad-Moghadam et al., 1995; Kempthues et al., 1988), PAR-3 is now known to help polarize a wide variety of animal cell types, including epithelial cells (Goldstein and Macara, 2007). PAR-3 localizes asymmetrically at the cortex of polarized cells and can interact with a variety of polarity proteins. Within many polarized cells, PAR-3 associates with PAR-6 (a PDZ and CRIB domain protein) and its binding partner aPKC (atypical protein kinase C), which regulates downstream effectors by

phosphorylation (Izumi et al., 1998; Joberty et al., 2000; Lin et al., 2000). These findings suggest that PAR-3 serves as a scaffold that recruits other polarity proteins to set up an asymmetric cortical signaling center.

The role of PAR-3 in polarizing epithelial cells has been investigated most extensively in the *Drosophila* blastoderm. In contrast to most epithelial cell types, blastoderm epithelial cells polarize as they form by cellularization, which occurs when membrane furrows invaginate from the embryo surface to separate cortical nuclei. As the cellularization furrows grow inward, Bazooka (Baz), the PAR-3 homolog in *Drosophila*, accumulates within spot-like lateral clusters just below the apical surface (Harris and Peifer, 2004; Harris and Peifer, 2005; McGill et al., 2009). Clusters of E-cadherin (Shotgun) form independently at the apical surface and travel to the apicolateral region, where they are trapped by Baz and eventually form adherens junctions (McGill et al., 2009). Although both Baz and E-cadherin are required for the polarization of blastoderm epithelial cells, Baz appears to act upstream and is required for E-cadherin localization (Harris and Peifer, 2004).

The role of PAR-3 in epithelial cells that form from unpolarized precursor cells is less clear. PAR-3 has been studied in cultured MDCK cells, which are derived from canine kidney cells that polarize by mesenchymal-to-epithelial transition *in vivo*. MDCK cells can be depolarized by removing calcium and subsequently repolarized by returning calcium to the medium (a calcium switch). PAR-3 localizes to tight junctions of MDCK cells (Izumi et al., 1998), and when its levels are reduced by siRNA treatment, the relocalization of tight junction and other apical proteins is severely delayed following a calcium switch (Chen and Macara, 2005; Horikoshi et al., 2009; Ooshio et al., 2007). Whether PAR-3 functions to concentrate junction components, analogous to its role

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in trapping clusters of adherens junction proteins in polarizing blastoderm epithelial cells, is not known. Because RNAi-mediated knockdown of PAR-3 in MDCK cells is incomplete, it is also unclear whether the ability of junctions to reform in PAR-3-depleted cells is due to residual PAR-3 or to the presence of a PAR-3-independent polarization mechanism. In vivo, PAR-3 has been shown to regulate the polarity of some mammalian epithelial cell types, although its cellular role during polarization is not known (Hirose et al., 2006).

In this study, we use a combination of live imaging and loss-of-function genetics to establish the in vivo cellular role of PAR-3 in *C. elegans* embryonic epithelial cells. *C. elegans* epithelial cells form during organogenesis, which begins during the middle stages of embryogenesis, and zygotically expressed PAR-3 localizes to apical junctions in these cells. Examining the function of PAR-3 in *C. elegans* epithelial cells has been hindered by the essential requirement of maternal PAR-3 in polarizing the one-cell embryo. Existing mutations in the single *par-3* gene eliminate maternal but not zygotic PAR-3 protein and cause a maternal-effect lethal phenotype (Aono et al., 2004; Etemad-Moghadam et al., 1995; Kempthues et al., 1988); mutant embryos have scrambled cell fates and fail to form organized epithelia. The function of PAR-3 in epithelial cells that form in larvae has been examined by feeding *par-3* double-stranded (ds) RNA to freshly hatched worms, reducing levels of zygotically expressed PAR-3. *par-3(RNAi)* larvae develop defects in the localization of several apical proteins within a subset of late-forming epithelial cells (Aono et al., 2004). This finding suggests that PAR-3 might have a broad role in epithelial polarity establishment or maintenance that could be studied if it were possible to remove both maternal and zygotic PAR-3 before epithelial cells form.

Here, we combine a targeted protein-degradation strategy and newly isolated *par-3* deletion alleles to eliminate PAR-3 from epithelial cells without disrupting polarization of the one-cell embryo. We show that at the initial stages of intestinal epithelial cell polarization, PAR-3 colocalizes with HMR-1 (E-cadherin), other adherens junction proteins, and with the polarity proteins PAR-6 and PKC-3 (aPKC) within cortical foci, which travel apically and cluster as polarization proceeds. Intestinal epithelial cells lacking PAR-3 mislocalize apical and junction proteins. Using live imaging, we establish that PAR-3 is required for the formation of HMR-1^{GFP} foci as intestinal epithelial cells polarize. Our results suggest that PAR-3 facilitates polarization by clustering polarity and junction proteins at the cell surface, which then accumulate at apical regions of the cell. Finally, we show that superficial epidermal cells can form apical junctions in the absence of PAR-3, and that PAR-6 has a PAR-3-independent role in these cells to promote junction maturation. These findings indicate that PAR-3 and PAR-6 have sequential roles in the positioning and maturation of apical junctions, and that some epithelial cell types can utilize PAR-3-independent mechanisms to form apical junctions.

MATERIALS AND METHODS

Strains

The following mutations and balancers were used. LGI: *hmr-1(zu389)* (Costa et al., 1998), *unc-101(m1)*, *par-6(zu222, tm1425)* (Totong et al., 2007; Watts et al., 1996), *hT2 [bli-4(e937) let-?(q782) qIs48]*; LGIII: *par-3(it71)* (Cheng et al., 1995), *unc-32(e189)*, *unc-119(ed3)*, *qC1[dpy-19(e1259) glp-1(q339)]*; LGIV: *him-8(e1489)*. The *par-3(tm2010)* and *par-3(tm2716)* alleles were obtained from the National BioResource Project for the Nematode *C. elegans* (Japan), outcrossed six times, and sequenced to confirm deletion breakpoints. *tm2010* removes nucleotides 5050 to 5457 of the *par-3* genomic sequence (start codon, +1) and *tm2716* removes

nucleotides 5773 to 6373. Both alleles failed to complement *par-3(it71)* for the maternal-effect lethal (Mel) phenotype (20/20 *tm2010/it71* and 7/7 *tm2716/it71* were Mel).

The following transgenes were used: *nals8 [Plim-7::mCherry]* (Voutev et al., 2009), *xnIs96 [hmr-1::gfp, unc-119]*, *xnIs123-127 [par-3s::gfp]*, *xnIs199 [yfp::par-3s]*, *zuls20 [par-3::zfl::gfp, unc-119]* (Nance et al., 2003), *zuls43 [Ppie-1::gfp::par-6::zfl, unc-119]* (Totong et al., 2007), *zuls73* and *zuls74 [par-3::gfp, unc-119]*. Unless otherwise indicated, endogenous promoters were used.

Molecular biology

DNA was manipulated by standard techniques or by fosmid recombineering using *galK* selection in bacterial strains SW105 or SW106 (Tursun et al., 2009; Warming et al., 2005; Zhang et al., 2008). For analysis of *par-3s* transcripts, embryos were collected by alkaline hypochlorite treatment of adults and aged 4 hours before RNA was extracted with Trizol (Krause, 1995); poly(dT)-primed cDNA was amplified using *par-3s*-specific primers.

Transgenes and worm transformation

par-3::gfp was created by subcloning the 16,526 bp *SalI* fragment of cosmid F54E7 into pBluescript KS+ and inserting *gfp* (amplified from pPD95.75) into a *PstI* site engineered at the 3' end of the coding sequences; *unc-119* was inserted into the vector *NotI* site (Nance et al., 2003). *par-3s::gfp* was constructed by modifying *par-3::gfp* to remove sequence upstream of the *StuI* site within the third intron, replacing *par-3* exons and introns with an *AvrII* site and inserting *par-3s* cDNA. *yfp::par-3s* was created by recombineering fosmid WRM0616cG01; *yfp* from plasmid pBALU2 (Tursun et al., 2009) was inserted at the *par-3s* start codon and *unc-119* was recombined into the fosmid backbone using *pLoxp unc-119* (Zhang et al., 2008). *hmr-1::gfp* was constructed by replacing the *hmr-1* genomic sequence in plasmid pW02-21 (Broadbent and Pettitt, 2002) with *hmr-1* cDNA containing introns 2-4, inserting an *ApaI* site before the stop codon, and cloning *gfp* plus the *unc-54* 3' UTR into this site. HMR-1^{GFP} produced from the *hmr-1::gfp* transgene *xnIs96* is localized similarly to endogenous HMR-1 as detected by immunostaining, and rescues the strict embryonic lethality of *hmr-1(zu389)* mutants [1064 of 1485 (72%) were viable]. Transgene insertions were created by biolistic transformation of *unc-119* mutants (Praitis et al., 2001).

RNAi

RNAi was performed by the feeding method using HT115 bacteria containing empty vector (pPD129.36) or *gfp* plasmids (Timmons and Fire, 1998). Feeding was performed at room temperature as described (Kamath et al., 2001), but substituting β -lactose (0.2%) for IPTG.

par-3(MZ) and *par-6(MZ)*; *par-3(MZ)* embryos

par-3(MZ) embryos were obtained by crossing *par-3(tm2716 or tm2010) unc-32/qC1; him-8; nals8 [Plim-7::mCherry]* males with *par-3(tm2716 or tm2010) unc-32; zuls20 [par-3::zfl::gfp]* hermaphrodites and selfing the Unc outcross progeny. One quarter of the F₂ embryos were '*par-3(MZ)*', i.e. *par-3(tm2716) unc-32* homozygotes that lack *zuls20* and inherit maternal PAR-3^{ZF1-GFP} protein, which is degraded rapidly in early embryos. *par-3(MZ)* embryos were identified by a lack of GFP and arose at the expected frequency [40 of 173 (23%)]. *par-3(MZ)* embryos had normal anterior-posterior polarity (13 of 13 embryos localized PGL-1 to the germline precursor). In some experiments, *par-3(MZ)* embryos were obtained by crossing *par-3(tm2716)/hT2; zuls20* hermaphrodites with *par-3(tm2716)/hT2; him-8* males. *par-3(MZ)* embryos expressing HMR-1^{GFP} were obtained by using *par-3(tm2716) unc-32/qC1; him-8; xnIs96 [hmr-1::gfp]* males. In experiments with *par-3(MZ)* embryos, controls were siblings of *par-3(MZ)* embryos that expressed PAR-3^{ZF1-GFP} zygotically.

par-6(MZ); *par-3(MZ)* embryos were obtained by crossing *par-6(tm1425)/hT2; par-3(tm2716)/hT2; him-8* males with *unc-101 par-6(zu222); zuls20; par-3(tm2716) unc-32; zuls43 [Ppie-1::gfp::par-6::zfl]* hermaphrodites and allowing non-Unc F₁ lacking *hT2* to self. Because the *pie-1* promoter in *zuls43* is active only maternally (Totong et al., 2007), one quarter of embryos lacking zygotic PAR-3^{ZF1-GFP} should be '*par-6(MZ)*; *par-3(MZ)*' double mutants, i.e. *par-6(tm1425); par-3(tm2716)*

homozygotes that express maternal PAR-3^{ZF1-GFP} and PAR-6^{ZF1-GFP} protein only during early embryonic stages. Double-mutant embryos were triple stained for GFP, PAR-6 and DLG-1 and arose at the expected frequency [21/68 (32%) of the embryos lacking zygotic PAR-3^{ZF1-GFP}]. Control crosses were performed using *par-3(tm2716)/hT2*; *him-8* males; 34/35 control *par-3(MZ)* embryos showed normal junction maturation. *par-6(MZ)*; *par-3(MZ)* embryos and their siblings had normal anterior-posterior polarity (29/29 embryos had asymmetric first and second embryonic cleavages).

Immunostaining

Embryos were fixed in methanol and paraformaldehyde and stained as described (Anderson et al., 2008). The following antibodies and dilutions were used: mouse anti-AJM-1 ‘MH27’, 1:10 (Francis and Waterston, 1991); guinea pig anti-EAT-20, 1:100 (Shibata et al., 2000); rabbit anti-ERM-1, 1:200 (van Furden et al., 2004); rabbit anti-GFP, 1:2000 (Abcam); rat anti-GFP, 1:250 (Nacalai Tesque); rabbit anti-HMR-1, 1:50 (Costa et al., 1998); mouse anti-HMP-1, 1:10 (Costa et al., 1998); rabbit anti-HMP-2, 1:5 (Costa et al., 1998); mouse anti-IFB-2 ‘MH33’, 1:150 (Francis and Waterston, 1991); mouse anti-PAR-3 P4A1 (IgG₁) and P1A5 (IgG_{2a}), both 1:10 (Nance et al., 2003); rabbit anti-PAR-6, 1:40,000 (Schonegg et al., 2007) and 1:30 (Hung and Kemphues, 1999); rabbit anti-PGL-1, 1:10,000 (Kawasaki et al., 1998); rat anti-PKC-3, 1:30 (Tabuse et al., 1998); mouse anti-PSD-95 (recognizes DLG-1), 1:200 (Affinity BioReagents) (Firestein and Rongo, 2001); and rat anti- α -tubulin, 1:2000 (Harlan).

Mouse anti-PAR-3 monoclonal antibody P1A5 was isolated as described previously (Nance et al., 2003). In co-staining experiments, anti-PAR-3 monoclonal antibodies P1A5 and P4A1 showed a largely overlapping pattern that was absent in *par-3(MZ)* embryos; each antibody also showed non-specific staining that persisted in *par-3(MZ)* embryos and *par-3(RNAi)* embryos (P1A5 stained nuclei, and P4A1 stained the epidermis or cuticle of late-elongation stage embryos).

z-stacks of embryos were acquired and processed as described (Anderson et al., 2008). Unless stated otherwise, a minimum of 50 control and 40 *par-3(MZ)* embryos at the indicated stages were examined in each staining.

Live imaging

Differential interference contrast (DIC) movies were acquired using a Zeiss AxioImager, 63 \times 1.4 NA or 40 \times 1.3 NA objective, Nomarski optics, an AxioCam MRM camera and AxioVision software. *z*-stacks of sections at

1- μ m intervals were captured every 1-2 minutes. Fluorescence movies were acquired using a Leica SP5 confocal microscope, 63 \times 1.3 NA water-immersion objective, 488 or 514 nm laser and a 3 \times zoom. Laser intensity and scan speed were adjusted so that embryos hatched after imaging. Maximum intensity projections of several planes at 800-nm intervals were compiled in ImageJ (NIH) and processed using Photoshop (Adobe). For movies of *par-3(MZ)* embryos, mutant and control sibling embryos were imaged together. Mutant embryos were distinguished as those that arrested at the 2-fold stage and developed cell adhesion defects.

RESULTS

PAR-3 colocalizes with junction and polarity proteins in apically targeted foci

PAR-3 localizes to apical regions of polarized embryonic epithelial cells, including those of the intestine, pharynx and epidermis (Fig. 1A,G) (Bossinger et al., 2001; McMahon et al., 2001; Nance et al., 2003). We used two different anti-PAR-3 monoclonal antibodies to determine whether PAR-3 is present within embryonic epithelial cells as apicobasal polarity first appears, and where within the cell it localizes. Initially, we focused our analysis on intestinal epithelial cells, which are among the first epithelial cells to form during embryogenesis and have a simple organization. The intestinal epithelium differentiates from intestinal precursor cells (IPCs) that are arranged in contacting left and right rows aligned with the anterior-posterior axis (Fig. 1C). Each IPC polarizes such that its nascent apical surface forms at the midline where the left and right rows of IPCs meet; apical surfaces of cells on opposite sides of the midline eventually separate to form the intestinal lumen (Leung et al., 1999). Before the IPCs showed other signs of polarity, we observed PAR-3 immunostaining in small foci that formed at regions of contact between these cells (Fig. 1B; see Fig. S1A and Movie 1 in the supplementary material); PAR-3 foci gradually accumulated at the nascent apical surface (Fig. 1C; see Fig. S1B in the supplementary material). Similar spot-like formations within polarizing epithelial cells have been described for other PAR polarity proteins and adherens junction proteins (Bossinger et al., 2001; Leung et al., 1999; McMahon et al., 2001; Totong et al.,

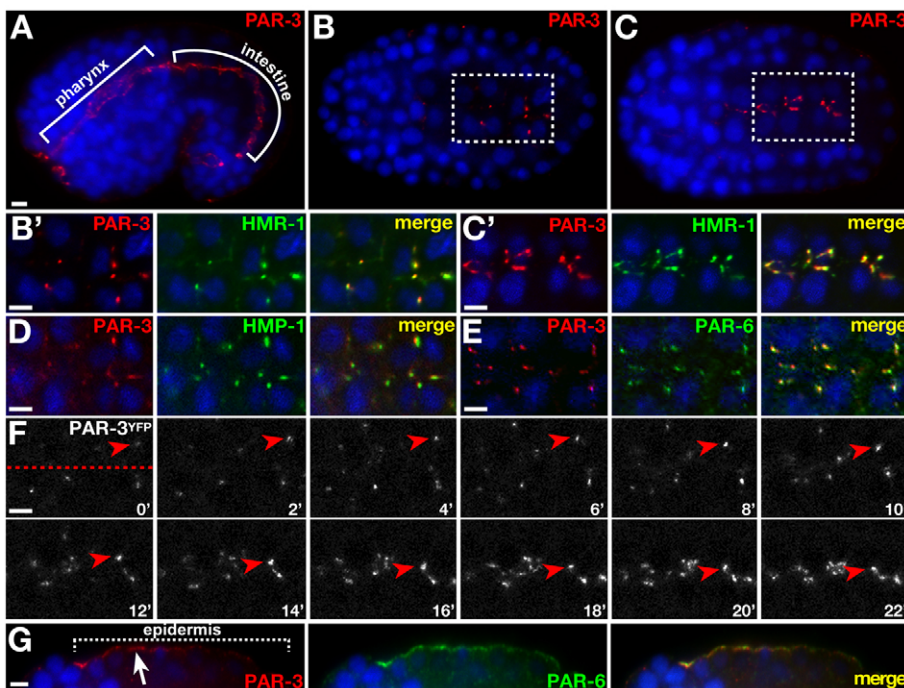


Fig. 1. PAR-3 localization within polarizing epithelial cells. DNA is blue (in this and all subsequent figures). Anterior is left. *C. elegans* embryos are ~50 μ m. (A-C') PAR-3 in polarized intestinal and pharyngeal cells (A), in intestinal precursor cells (IPCs) that are beginning to polarize (B), or during polarization (C). The boxed regions in B,C are shown in B',C' and show colocalization of PAR-3 with HMR-1. (D,E) PAR-3 colocalization with HMP-1 (D) and PAR-6 (E) in IPCs at the onset of polarization. (F) Stills from Movie 2 of IPCs expressing PAR-3^{YFP} (see Movie 2 in the supplementary material). Dashed line indicates the future apical surface. A focus of PAR-3^{YFP} (arrowhead) is shown over time (minutes). (G) Lateral view of polarizing epidermis (bracketed region) showing apical PAR-3 (arrow) and PAR-6. Scale bars: 2.5 μ m.

2007). In co-staining experiments, we observed that PAR-3 foci contained the adherens junction proteins HMR-1, HMP-1 (α -catenin), HMP-2 (β -catenin) (Fig. 1B',D; data not shown) and the PAR proteins PAR-6 and PKC-3 (Fig. 1E; data not shown). By contrast, we could not detect the junction proteins DLG-1 and AJM-1 within PAR-3 foci. DLG-1 and AJM-1, which localize to a distinct basal region of mature junctions (Koppen et al., 2001; McMahon et al., 2001), first appeared within intestinal epithelial cells after the apical accumulation of PAR and adherens junction proteins was already evident.

To determine whether the asymmetric localization of PAR-3 within mature epithelial cells arises from apical movement of PAR-3 foci, we created a transgene expressing functional YFP-tagged PAR-3 (PAR-3^{YFP}) from the *par-3* promoter (see below) and captured time-lapse movies. In polarizing IPCs, PAR-3^{YFP} formed foci similar to those observed in immunostained embryos (7 of 7 embryos) (Fig. 1F; see Movie 2 in the supplementary material). Individual PAR-3^{YFP} foci were dynamic and moved erratically along the surfaces of IPCs until they adopted a more directed motion, concentrated apically and aggregated. These results, taken together with the co-staining experiments described above, suggest that foci containing PAR-3, adherens junction proteins and the polarity proteins PAR-6 and PKC-3 travel apically and condense as IPCs begin to polarize.

Within fully polarized intestinal epithelial cells, PAR-3 segregated away from apical PAR-6 and PKC-3 and colocalized with HMR-1, HMP-1 and HMP-2 at adherens junctions, which form where apical and lateral surfaces meet (data not shown) (Totong et al., 2007). In contrast to adherens junction proteins, which maintained high levels of expression within epithelial cells throughout embryogenesis, PAR-3 immunostaining peaked during and after epithelial polarity establishment and diminished during subsequent stages. PAR-3 showed a similar localization within polarizing pharyngeal epithelial cells (Fig. 1A). In polarizing epidermal epithelial cells, we also detected foci of apically directed PAR-3^{YFP} (see Movie 2 in the supplementary material). However, foci did not persist upon reaching the apical surface. Rather, PAR-

3 developed a smooth cortical enrichment at the contact-free apical surface, where it colocalized transiently with PAR-6 and PKC-3 before becoming enriched at apical junctions (Fig. 1G; see Fig. S6 in the supplementary material; data not shown). Combined, these observations show that PAR-3 is present within epithelial precursor cells at the initial stages of polarity establishment, is among the earliest proteins to develop apicobasal asymmetry, colocalizes with other polarity proteins and junction proteins within apically targeted foci, and appears to peak in intensity as epithelial polarization is established and elaborated.

Zygotic *par-3* expression is required for viability

To determine whether *par-3* is required to establish polarity in epithelial cells, we first searched for *par-3* null mutants. We obtained two uncharacterized *par-3* deletion mutants from the National BioResource Project, Japan (Fig. 2A). *tm2010* is a 408 bp deletion that removes sequences just prior to the first PDZ domain and is predicted to alter splicing. The 601 bp *tm2716* deletion removes most of the first PDZ domain and causes a frameshift that is predicted to terminate translation just after the deletion; this allele is likely to be a genetic null, as the expected mutant gene product is severely truncated and lacks most of the functional domains of PAR-3. *tm2010* and *tm2716* failed to complement the maternal-effect lethal allele *par-3(it71)*, but each mutation on its own caused a more severe L1 larval lethal phenotype (Table 1; see Materials and methods). We reasoned that the deletions disrupt an essential *par-3* isoform that is unaffected by existing *par-3* mutations, all of which cause maternal-effect lethal phenotypes.

We searched for alternative *par-3* transcripts by sequence comparison and RT-PCR. By aligning genomic sequences from *C. elegans* and *C. briggsae*, we identified a highly conserved region near the end of the large third intron. The conserved region contains an SL1 trans-splice acceptor, which indicates the 5' end of a transcript (Hwang et al., 2004), and forms an open reading frame that extends in-frame into the fourth exon. Using RT-PCR, we verified that the conserved region is the beginning of an alternative *par-3* transcript (which we named *par-3s*) that contains

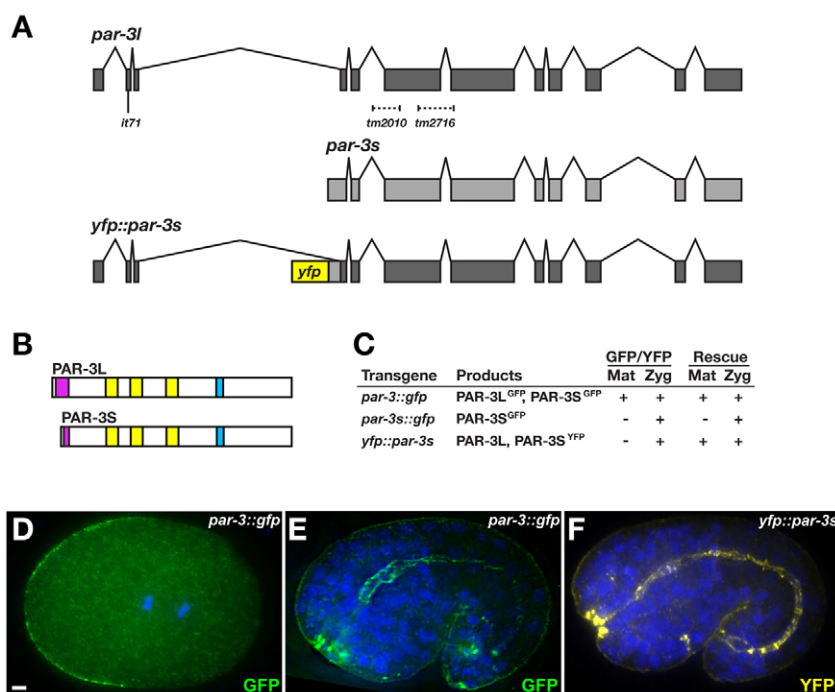


Fig. 2. Isoforms of *C. elegans par-3*. (A) *par-3l*, *par-3s* and *yfp::par-3s*. Exons (rectangles), introns (chevrons) and mutations are indicated. (B) Predicted PAR-3L and PAR-3S products showing the oligomerization domain (magenta), PDZ domains (yellow) and aPKC-binding domain (cyan). (C) Expression and rescuing activity of *par-3* transgenes. (D,E) Expression of *par-3::gfp* in one-cell (D) and 1.5-fold stage (E) embryos. (F) Expression of *yfp::par-3s* in 1.5-fold stage embryo. Scale bar: 2.5 μ m.

Table 1. Phenotypes of *par-3(tm2716)* and *par-3(MZ)* mutants

Maternal genotype	Self-progeny phenotype		
	Wild type	Embryonic lethal	Larval lethal
Wild type	356 (99.5)	2 (0.5)	0
<i>par-3(tm2716)/+</i>	309 (78)	7 (1.7)	82 (20.3)
<i>par-3(tm2716); par-3::zf1::gfp</i>	697 (97.5)	18 (2.5)	0
<i>par-3(tm2716); par-3::zf1::gfp/+</i>	490 (61)	273 (34)	45 (5)

The number of embryos or larvae is shown, with the percentage in parentheses.

each of the previously annotated downstream *par-3* exons (Fig. 2A; see Fig. S2 in the supplementary material). The predicted PAR-3S protein includes all of the known functional domains of full-length PAR-3 (hereafter PAR-3L), although the putative N-terminal oligomerization domain is truncated (Fig. 2B). *par-3s* mRNA is disrupted by the *tm2010* and *tm2716* deletions, but is unlikely to be affected by the two molecularly characterized *par-3* maternal-effect lethal mutations; these nonsense mutations occur in upstream exons that are not included in the *par-3s* transcript (Aono et al., 2004) (Fig. 2A).

To determine where the different PAR-3 isoforms are expressed, we created translational reporters by fusing *gfp* to *par-3* within large genomic clones. First, we created a C-terminal GFP fusion (*par-3::gfp*) that reports on both PAR-3L and PAR-3S expression. *par-3::gfp* expressed PAR-3^{GFP} in the early embryo and in epithelial cells (Fig. 2C-E), in a pattern similar to that of endogenous PAR-3 (2/2 lines). Reasoning that the large third intron of *par-3* contains an internal promoter driving *par-3s* transcription, we created a *par-3::gfp* derivative lacking all sequences upstream of the third intron (*par-3s::gfp*). Embryos expressing *par-3s::gfp* showed zygotic PAR-3S^{GFP} expression in epithelial cells (6/6 lines) but no maternal expression (Fig. 2C; data not shown). Our inability to detect maternal PAR-3S^{GFP} suggests that sequences upstream of the third intron might be required for germline PAR-3 expression; alternatively, *par-3s::gfp* transgenic lines could be silenced within the germ line (Kelly et al., 1997). To distinguish between these possibilities, we fused *yfp* to the beginning of *par-3s* within a genomic clone containing the entire *par-3* gene (Fig. 2A); since the *yfp* insertion site is within coding sequences of *par-3s* but within intronic sequences of *par-3l*, the *yfp::par-3s* transgene should express PAR-3S^{YFP} as well as untagged PAR-3L. To detect both transgenic PAR-3L and PAR-3S^{YFP} we crossed the *yfp::par-3s* transgene into *par-3(tm2716)* mutants, which lack endogenous PAR-3 immunostaining (see below), and co-stained embryos with PAR-3 and GFP antibodies. In a line of *yfp::par-3s* that did not show germline silencing, we detected PAR-3S^{YFP} only in epithelial cells, and PAR-3L was present weakly in early embryos (Fig. 2C,F; data not shown). These results indicate that PAR-3L is expressed maternally, and that PAR-3S is expressed zygotically but not maternally. Consistent with these expression patterns, transgenes

expressing both PAR-3L and PAR-3S (*par-3::gfp* and *yfp::par-3s*) rescued larval and maternal-effect lethality of *par-3(tm2716)* mutants (*par-3::gfp*, 2/2 lines; *yfp::par-3s*, 1/1 line) (Table 2); a transgene expressing only PAR-3S (*par-3s::gfp*) rescued larval lethality but not maternal-effect lethality [1 (the highest-expressing) of 4 lines rescued] (Fig. 2C).

To establish whether PAR-3S has an important role during development, we treated *par-3(tm2716); yfp::par-3s* worms with *gfp* dsRNA, which targets transgenic *par-3* tagged with *gfp* or *yfp* (Table 2). When fed to *par-3(tm2716); yfp::par-3s* worms, *gfp* dsRNA targets expression of zygotic PAR-3S^{YFP} but not maternal PAR-3L, which is untagged. *par-3(tm2716); yfp::par-3s* worms fed at the L4 stage produced embryos that hatched but frequently developed into sterile adults. This phenotype is observed in wild-type larvae treated with *par-3* dsRNA and is due to a reduction in zygotic PAR-3 protein (Aono et al., 2004), indicating that PAR-3S has an essential zygotic function. *gfp* dsRNA fed to control *par-3(tm2716); par-3::gfp* worms targets expression of both PAR-3L^{GFP} and PAR-3S^{GFP}. As expected, *par-3(tm2716); par-3::gfp* worms fed *gfp* dsRNA at the L4 stage produced dead eggs owing to the loss of maternal PAR-3. When fed at the L1 stage to bypass the requirement for maternal PAR-3, some *par-3(tm2716); par-3::gfp* worms became sterile, as we observed for *par-3(tm2716); yfp::par-3s* worms. Taken together, these findings indicate that maternal PAR-3L has an essential function in the early embryo, whereas zygotic PAR-3S, which is found in epithelial cells, has an essential function during embryonic or larval stages.

***par-3* is required for morphogenesis of epithelial organs**

To determine whether PAR-3 is required for the initial polarization of epithelial cells, we designed a strategy to eliminate PAR-3 protein from the embryo after its essential role in polarizing the one-cell embryo has been completed but before epithelial cells are formed. We previously described the *par-3::zf1::gfp* transgene, which transiently expresses maternal PAR-3^{ZF1-GFP} (Nance et al., 2003). *par-3::zf1::gfp* is a derivative of *par-3::gfp* that includes sequences encoding the PIE-1 protein ZF1 (zinc-finger) domain fused to *par-3*; proteins tagged with the ZF1 domain degrade rapidly in all early embryonic somatic cells (Reese et al., 2000).

Table 2. Function of *par-3* isoforms

Genotype and treatment [†]	L4 feeding Embryo phenotype		L1 feeding Adult phenotype*		
	Hatched	Dead	Fertile	Sub-fertile	Sterile
<i>par-3(tm2716); par-3::gfp</i> , vector RNAi	389 (94)	27 (6)	68	0	0
<i>par-3(tm2716); par-3::gfp</i> , <i>gfp</i> RNAi	0	450	63 (88)	0	9 (12)
<i>par-3(tm2716); yfp::par-3s</i> , vector RNAi	385 (99)	2 (1)	62 (91)	5 (7)	1 (1)
<i>par-3(tm2716); yfp::par-3s</i> , <i>gfp</i> RNAi	341 (98)	9 (2)	3 (4)	42 (61)	24 (35)

The number of embryos or adults is shown, with the percentage in parentheses.

*Sterile worms laid no eggs; sub-fertile worms laid 1-20 eggs; fertile worms laid more than 100 eggs.

[†]Worms were also homozygous for *unc-32*. Transgenes used were *zuls73 [par-3::gfp]* and *xnls199 [yfp::par-3s]*.

par-3::zfl::gfp rescues the polarity defects of *par-3* mutant one-cell embryos before the fusion protein starts to rapidly degrade at the four-cell stage (Nance et al., 2003). Zygotic *par-3::zfl::gfp* expression begins later in epithelial cell precursors and is stable because ZF1-mediated degradation appears to be limited to early embryos. Therefore, *par-3(tm2716)* mutants expressing *par-3::zfl::gfp* were viable and produced embryos that had normal anterior-posterior polarity (Table 1; see Materials and methods).

To obtain embryos with epithelial cells lacking both maternal and zygotic PAR-3, we selfed *par-3(tm2716); par-3::zfl::gfp/+* worms and analyzed progeny lacking the *par-3::zfl::gfp* transgene. Hereafter, we refer to these embryos as *par-3(MZ)* embryos [*par-3(MZ)* embryos differ from the previously described *par-3(ZF1)* embryos (Nance et al., 2003), which still express PAR-3^{ZF1-GFP} zygotically]. Using antibodies that recognize sequences downstream of the *tm2716* deletion, we could not detect PAR-3 in *par-3(MZ)* embryos after PAR-3^{ZF1-GFP} had degraded from early blastomeres (Fig. 4B; see Fig. S6B in the supplementary material). Therefore, PAR-3 is depleted from *par-3(MZ)* embryos well before epithelial cells form.

One quarter of the progeny from *par-3(tm2716); par-3::zfl::gfp/+* mothers died during embryogenesis, suggesting that *par-3(MZ)* embryos arrest at an earlier stage than *par-3* deletion mutants, which inherit maternal PAR-3 (Table 1). We acquired time-lapse DIC movies of developing embryos to determine why *par-3(MZ)* embryos arrested. In wild-type embryos, epidermal epithelial cells migrate ventrally to encase the embryo in skin then contract circumferentially to squeeze the embryo and elongate the body axis; simultaneously, the pharynx and intestine form a linked tube that connects to the mouth and rectum. *par-3(MZ)* embryos elongated much more slowly than wild-type embryos and arrested before reaching 2-fold elongation, as compared with the 4-fold elongation of wild-type embryos at this stage (Fig. 3A-C; see Movies 3, 4 in the supplementary material). We observed defects in morphogenesis of the pharynx and intestine, such as mispositioned intestinal cells and an improperly elongated pharynx (Fig. 3A,B, outlined). Mutant embryos also frequently formed epidermal lesions, although these were restricted to the mouth and rectum, where internal and external epithelial cells connect (Fig. 3B, arrowheads). This phenotype contrasts with that of *par-6(MZ)* embryos, which form only immature junctions and develop lesions throughout the epidermis (Totong et al., 2007). The phenotype of *par-3(MZ)* mutants is consistent with a defect in epithelial cell polarization or junction formation that interferes with the morphogenesis of internal epithelial organs. Additionally, the different phenotypes of *par-3(MZ)* and *par-6(MZ)* embryos suggest that PAR-3 and PAR-6 have distinct roles in epithelial cells.

PAR-3 mediates intestinal epithelial cell polarization

To investigate the cellular basis of the morphogenesis defects in *par-3(MZ)* embryos, we immunostained for proteins that develop asymmetric localizations within polarized epithelial cells. PAR-6 and PKC-3 localize to the apical cortex of wild-type intestinal epithelial cells (Totong et al., 2007), but in *par-3(MZ)* embryos both proteins were present diffusely within the cytoplasm and failed to enrich apically (Fig. 4C,D; data not shown). The intermediate filament protein IFB-2 is found just beneath apical microvilli in wild-type intestinal cells (Bossinger et al., 2004). In *par-3(MZ)* embryos, IFB-2 formed large discontinuous aggregates, rather than the continuous apical band observed in wild-type embryos (see Fig. S4C,D in the supplementary material). HMP-1 localizes to adherens junctions, which form at the interface of apical and lateral surfaces. In *par-3(MZ)* embryos, HMP-1 accumulated within irregular aggregates, similar to those seen in embryos stained for IFB-2 (see Fig. S4A,B in the supplementary material). The junction proteins DLG-1 and AJM-1 also formed irregular aggregates in *par-3(MZ)* embryos (Fig. 4E,F; data not shown). We co-stained embryos for DLG-1 and either HMP-1 or IFB-2 to determine the composition of aggregates (see Fig. S4 in the supplementary material). In most aggregates, DLG-1 showed considerable colocalization with HMP-1 or IFB-2. Therefore, although PAR-3 is required to properly position these apically enriched proteins, they retain the ability to aggregate when PAR-3 is absent.

In addition to these cortical proteins, we examined two apically enriched transmembrane proteins: the apical Crumbs-like protein EAT-20 and the adherens junction protein HMR-1 (Shibata et al., 2000). Both EAT-20 and HMR-1 were mislocalized in *par-3(MZ)* embryos but were also more dispersed, unlike DLG-1 and HMP-1, and did not form large aggregates (Fig. 4G-J; see Fig. S4E,F in the supplementary material). The irregular organization of apical surfaces in *par-3(MZ)* embryos made it difficult to assess the localization of two other markers: LET-413 (the homolog of *Drosophila* Scribble) and microtubules. LET-413 normally localizes to basolateral surfaces and is excluded from apical surfaces (Legouis et al., 2000). *par-3(MZ)* embryos showed normal basolateral LET-413 localization but apical exclusion was difficult to ascertain given the lack of a defined apical surface in mutant embryos (Fig. 4K,L, arrowheads). Microtubules are normally enriched just below the apical cortex as the IPCs polarize (see Fig. S3A in the supplementary material). In *par-3(MZ)* embryos, microtubules in the IPCs were concentrated in patches, rather than uniformly, beneath the apical surface (see Fig. S3B in the supplementary material). In summary, PAR-3 is required to

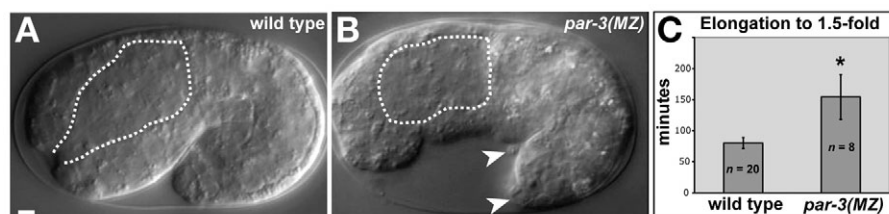


Fig. 3. Morphogenesis in *par-3(MZ)* embryos. (A,B) DIC time-lapse stills of wild-type and *par-3(MZ)* mutant *C. elegans* embryos at 1.5-fold stage. The pharynx (outlined) does not extend anteriorly in the *par-3(MZ)* embryo and extruded cells (arrowheads) are visible. (C) Elongation rate to 1.5-fold stage. Time is minutes from completion of ventral enclosure to 1.5-fold stage. Error bars indicate s.d.; *, $P < 0.003$ (Student's *t*-test). Scale bar: 2.5 μ m.

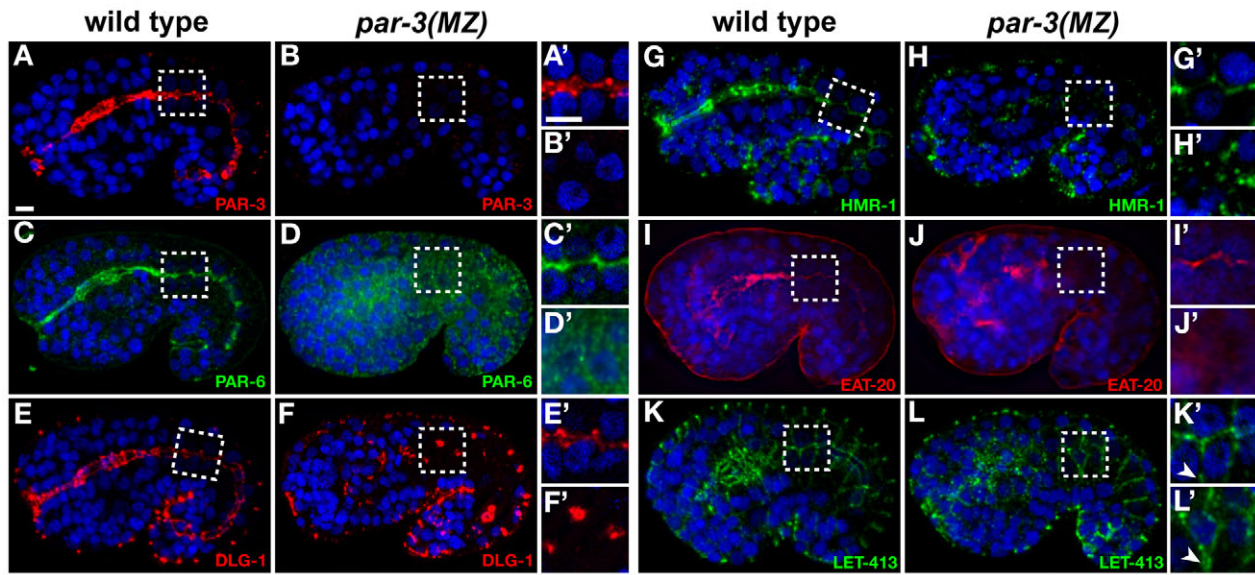


Fig. 4. Junction and polarity proteins in *par-3(MZ)* embryos. (A-L') Wild-type and *par-3(MZ)* *C. elegans* embryos at 1.25- to 1.5-fold stage, immunostained as indicated. The boxed regions are shown at higher magnification to the right; the contrast in some of these has been increased to highlight local staining. Arrowheads indicate LET-413 at lateral surfaces. Scale bars: 2.5 μ m.

properly position both cortical and transmembrane apical proteins within intestinal epithelial cells, and in mutant embryos many apical proteins eventually form irregular clusters.

PAR-3 is required for the clustering and apical accumulation of junction and polarity proteins

We hypothesized that PAR-3 polarizes IPCs by assembling clusters of adherens junction proteins and polarity proteins, directly or indirectly helping in their delivery to the nascent apical surface. To examine the movement of an apical protein that colocalizes with PAR-3 in foci as IPCs polarize, we created a functional *hmr-1::gfp* transgene. Zygotic HMR-1^{GFP} expression began in somatic cells of early embryos well before epithelial cells differentiated. At these stages, HMR-1^{GFP} in living embryos showed a similar localization to endogenous HMR-1 as detected by immunostaining. Foci of HMR-1^{GFP} formed at surfaces of polarizing IPCs (19/19 embryos) (Fig. 5A,A'; see Movie 5 in the supplementary material), but these foci were not observed in *par-3(MZ)* embryos (12/12 embryos) (Fig. 5B,B'; see Movie 6 in the supplementary material). HMR-1^{GFP} eventually accumulated at the apical surfaces of IPCs in wild-type embryos, but this apical accumulation did not occur in *par-3(MZ)* embryos. At later stages, when belt-like junctions of HMR-1^{GFP} were evident in wild-type embryos, we observed small irregular clusters of HMR-1^{GFP} in *par-3(MZ)* embryos (see Fig. S5 in the supplementary material), similar to those of HMR-1 detected in immunostained *par-3(MZ)* embryos. We conclude that PAR-3 polarizes intestinal epithelial cells by helping to organize HMR-1, and probably other polarity and junction proteins, into foci that accumulate at the apical surface during polarization.

Epidermal epithelial cells do not require PAR-3 to form apical junctions

To determine whether PAR-3 has a similar role in polarizing other types of epithelial cells, we examined pharyngeal and epidermal cells. Pharyngeal epithelial cells, like intestinal epithelial cells, form from clusters of internal precursor cells. Apical proteins were also mislocalized in the pharynx of *par-3(MZ)* embryos, suggesting

that PAR-3 performs a similar function in polarizing pharyngeal epithelial cells (Fig. 4; see Fig. S5 in the supplementary material). In contrast to intestinal and pharyngeal epithelial cells, epidermal epithelial cells form on the embryo surface from a single layer of superficial precursor cells. In epidermal cells of *par-3(MZ)* embryos, PAR-6 and PKC-3 were distributed uniformly within the cytoplasm, rather than concentrated apically as in wild type (Fig. 6A-D). EAT-20 apical localization was variably disrupted in the epidermis, with cells in dorsal regions of the embryo showing the most severe mislocalization (see Fig. 4J). Surprisingly, however, the junction proteins HMR-1, HMP-1, DLG-1 and AJM-1 were properly positioned in *par-3(MZ)* embryos (Fig. 6E,F; data not shown) and formed mature belt-like structures as in wild-type epidermal epithelial cells (see Fig. S6C,D in the supplementary material). A similar phenotype was observed when *par-3(MZ)* embryos were constructed using the *par-3(tm2010)* deletion allele (see Fig. S7 in the supplementary material). Altogether, these observations indicate that internal epithelial cells of the intestine and pharynx require PAR-3 to polarize, but external epidermal cells can utilize PAR-3-independent mechanisms to form apical junctions. Functional apical junctions within the epidermis of *par-3(MZ)* embryos are likely to explain why these cells do not rupture in random positions, as occurs in *par-6(MZ)* embryos in which junctions fail to mature (Totong et al., 2007).

PAR-6 functions independently of PAR-3 to promote apical junction maturation

Given that PAR-6 was not enriched apically within *par-3(MZ)* mutant epidermal cells, the observation that *par-3(MZ)* embryos did not have a defect in junction maturation within the epidermis was surprising (see Discussion). The different junction phenotypes of *par-3(MZ)* and *par-6(MZ)* embryos suggest several possible relationships between PAR-3 and PAR-6 within the epidermis. One possibility is that PAR-6 has an essential, PAR-3-independent role in promoting apical junction maturation. Alternatively, the normal role of PAR-6 could be to inhibit apical PAR-3 during junction maturation; an excess of PAR-3 activity in *par-6(MZ)* mutants

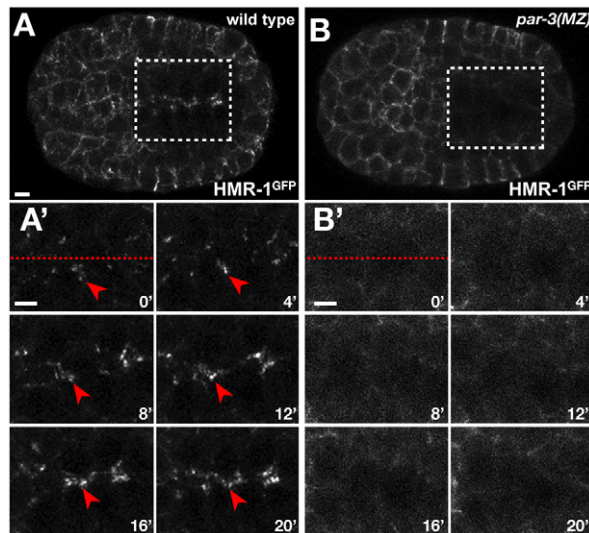


Fig. 5. HMR-1^{GFP} localization during epithelial cell polarization. (A–B') Stills from movies of wild-type (A, from Movie 5) and *par-3(MZ)* (B, from Movie 6) *C. elegans* embryos expressing HMR-1^{GFP} (see Movies 5, 6 in the supplementary material). The boxed regions in A,B are shown in A',B' in a time-lapse sequence covering the preceding 20 minutes. 0' represents the time at which HMR-1^{GFP} is first detected in foci in wild-type IPCs. Dashed lines in A',B' denote the future apical surface. Scale bars: 2.5 μm.

could prevent apical junctions from maturing. To test these hypotheses, we created *par-6(MZ); par-3(MZ)* double-mutant embryos using a strategy analogous to that used for *par-3(MZ)* single mutants (see Materials and methods). To observe junctions, we stained embryos for DLG-1 and examined the epidermis from a superficial view, from which defects in junction maturation are most apparent (Fig. 7A). In *par-6(MZ); par-3(MZ)* double-mutant embryos, DLG-1-containing epidermal junctions formed but failed to mature; instead, DLG-1 accumulated in spot-like clusters identical to those observed in *par-6(MZ)* single-mutant embryos

(Fig. 7C,D). Control embryos, which lacked PAR-3 but still expressed PAR-6, formed mature junctions within the epidermis (Fig. 7B). These results indicate that within epidermal cells, and perhaps other types of epithelial cells, PAR-6 has a PAR-3-independent role in promoting apical junction maturation.

DISCUSSION

PAR-3 as an apical organizer during epithelial polarization

Our analysis of polarizing IPCs provides the first in vivo cellular model for PAR-3 function as precursor cells polarize to become epithelial cells. PAR-3 foci containing adherens junction proteins and apical polarity proteins assembled at the cortex of polarizing IPCs, moved apically and clustered at the nascent apical surface. Apical and junction proteins were mislocalized in *par-3(MZ)* embryos, and foci of HMR-1^{GFP} that normally appear at the onset of polarization failed to form at this stage. We propose that PAR-3 helps to polarize epithelial cells by functioning as a local organizer of apical proteins at the cell surface. In this model, PAR-3 directly or indirectly concentrates and clusters adherens junction proteins and PAR polarity proteins, facilitating their subsequent apical transport. It will be important to determine whether PAR-3 has a similar role in vertebrate cells that form through mesenchymal-to-epithelial transition, a conceptually analogous process.

Based on studies in other types of polarized cells that have demonstrated that PAR-3 can interact with microtubule motor proteins (Fan et al., 2004; Schmoranzner et al., 2009), it is reasonable to speculate that foci travel apically along microtubules, as has been proposed in the *Drosophila* blastoderm (Harris and Peifer, 2005). Microtubules concentrate apically in polarizing intestinal and pharyngeal epithelial cells (Leung et al., 1999; Portereiko et al., 2004), potentially providing a mechanism for enriching PAR-3 foci within this region of the cell.

Our model for PAR-3 function contrasts with that of Baz in the *Drosophila* blastoderm, the only other epithelial cell type in which PAR-3 function has been analyzed through live imaging of mutant embryos (Harris and Peifer, 2005; McGill et al., 2009). As the blastoderm cellularizes, Baz accumulates in apical regions of the

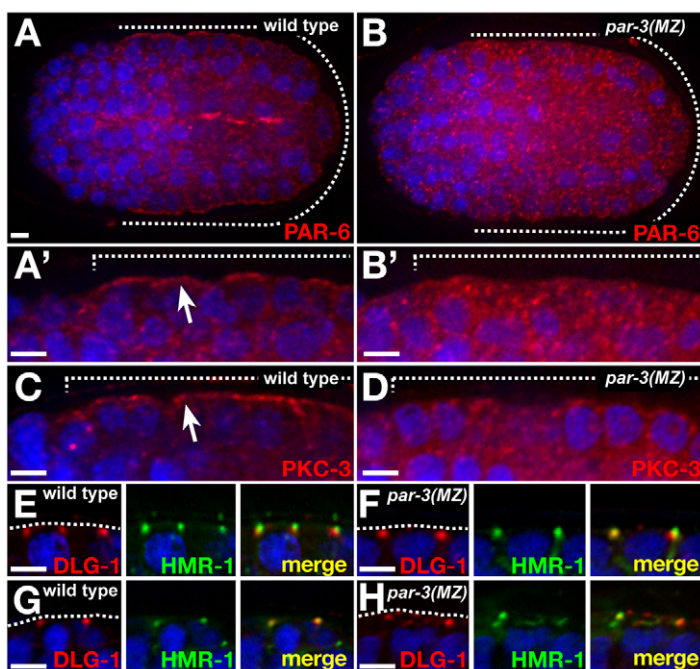


Fig. 6. Epidermal epithelial cells in *par-3(MZ)* embryos. (A–B') PAR-6 staining in the epidermis (underlying the dashed lines). The epidermis is shown at higher magnification in A',B'. (C,D) PKC-3 staining in the epidermis. (E–H) Epidermal cells showing DLG-1 and HMR-1 in apical junctions at comma stage (E,F) or 1.5-fold stage (G,H), when junction proteins become more dispersed in *par-3(MZ)* than in wild-type *C. elegans* embryos. Scale bars: 2.5 μm.

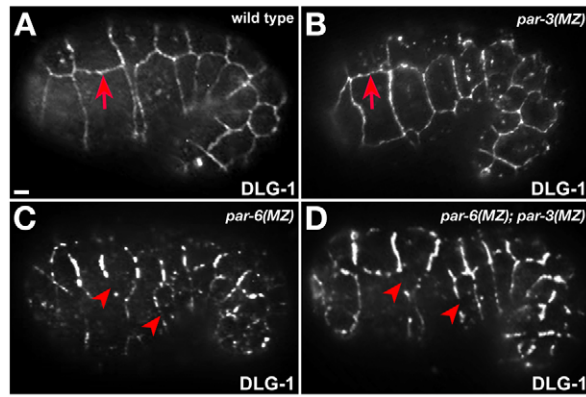


Fig. 7. Junction maturation in *par-6(MZ); par-3(MZ)* double-mutant embryos. (A–D) Superficial views of 1.25- to 1.5-fold stage embryos stained for DLG-1. Junctions are continuous (arrows) in wild-type and *par-3(MZ)* *C. elegans* embryos, but thick and fragmented (arrowheads) in *par-6(MZ)* and *par-6(MZ); par-3(MZ)* embryos. Scale bar: 2.5 μm .

invaginating lateral surface (Harris and Peifer, 2004; Harris and Peifer, 2005). Clusters of E-cadherin form independently at the apical surface, move laterally and basally, and are trapped at the apicolateral region by Baz (McGill et al., 2009). Thus, whereas PAR-3 promotes clustering and apical accumulation of junction proteins in *C. elegans* intestinal epithelial cells, in the *Drosophila* blastoderm Baz functions as a net that traps clusters of junction proteins that form independently at the apical surface and travel laterally. These differences might reflect the contrasting ways in which these two cell types form. For instance, the insertion of new membrane that occurs as the lateral surfaces of blastoderm cells invaginate might make this environment too dynamic to support local clustering of Baz and E-cadherin.

Distinct roles for PAR-3 and PAR-6 in junction formation

Although PAR-3 and PAR-6 can function in a complex and interact directly (Joberty et al., 2000; Lin et al., 2000), the sorting of these proteins to distinct apical domains within differentiated epithelial cells suggests that they might have different functions. We showed previously that PAR-6 is not required for *C. elegans* embryonic epithelial cells to polarize, but instead promotes the maturation of apical spot junctions into belt-like junctions (Totong et al., 2007). Here, we demonstrated that within internally forming epithelial cells, PAR-3 functions at an earlier stage to cluster and position junction proteins at the apical surface. Thus, PAR-3 and PAR-6 have different roles in the positioning and maturation of apical junctions. These results are consistent with findings in the *Drosophila* notum, in which Par-6, but not Baz, is required for maintenance of adherens junction morphology (Georgiou et al., 2008; Leibfried et al., 2008).

Until now it has not been possible to determine whether PAR-6 regulates junction formation independently of PAR-3, or if PAR-3 and PAR-6 work together during this step. The ability of epidermal epithelial cells to form apical junctions in the absence of PAR-3 allowed us to test these hypotheses with double mutants. *par-6(MZ); par-3(MZ)* double-mutant embryos showed the *Par-6* phenotype (fragmented apical junctions) in epidermal cells, indicating that PAR-6 regulates junction maturation independently of PAR-3 in these cells. This finding is somewhat surprising because PAR-6 and its binding partner PKC-3 do not become

apically enriched in *par-3(MZ)* mutant epidermal cells. It is possible that PAR-6 retains an asymmetric activity in *par-3(MZ)* mutant epidermal cells, or that the asymmetric apical positioning of its substrates is sufficient for PAR-6 function.

Alternative junction formation mechanisms for internal and external epithelial cells

An unexpected finding was that *par-3(MZ)* mutant epidermal epithelial cells were able to assemble functional apical junctions. Several observations, including the lack of PAR-3 immunostaining and the mislocalization of PAR-6 and PKC-3, strongly suggest that this is not simply due to residual PAR-3 protein. Rather, we propose that epidermal cells can utilize a PAR-3-independent mechanism to form apical junctions. However, PAR-3 does appear to have a role within the epidermis and could function redundantly with alternative polarization mechanisms. For example, we detected foci of PAR-3 in polarizing wild-type epidermal cells, similar to PAR-3 foci that form as IPCs polarize, and PAR-3 immunostaining was evident at junctions during elongation (see Fig. S6A–B' in the supplementary material). Moreover, *par-3(MZ)* embryos elongated more slowly than the wild type, and mutant embryos arrested before elongation was complete. Apical junction proteins within the epidermis of terminal stage *par-3(MZ)* embryos often showed a more disperse localization than in wild type (see Fig. 6G,H). One possibility is that subtle defects in the assembly of epidermal apical junctions in *par-3(MZ)* embryos are exacerbated during elongation, when the epidermis is under mechanical stress and junctions must remodel as cells change shape.

An important difference between epidermal cells and internal epithelial cells is asymmetry in cell contact patterns. Intestinal and pharyngeal cells are contacted on all surfaces by neighboring cells; as these cells polarize, they must define an apical surface and subsequently form a lumen at this surface. By contrast, epidermal epithelial cells have a surface that is not contacted by other cells and do not form a lumen. Asymmetries in cell contact could provide polarity landmarks to epidermal cells, obviating the need for PAR-3 in junction formation. Asymmetric patterns of cell adhesion, mediated by E-cadherin interactions, are sufficient to initiate the apicobasal polarization of MDCK cells (Nejsum and Nelson, 2007). Regardless of the mechanism that epidermal cells use to polarize and form junctions, the distinct requirements for PAR-3 in internal versus external epithelial cells stresses the variability in ways that epithelial polarity can develop, and highlights the importance of examining the function of polarity regulators in multiple epithelial cell types.

Acknowledgements

We thank Jonathan Pettitt, Shin Takagi, Jim Priess, Olaf Bossinger, Ken Kemphues and Tony Hyman for reagents; Shohei Mitani for *par-3* deletions; and Jane Hubbard and J.N. laboratory members for comments. Some antibodies and strains were obtained from the NIH-funded Developmental Studies Hybridoma Bank and *Caenorhabditis* Genetics Center. Funding was provided by an ACS Postdoctoral Fellowship (A.M.W.), AHA Grant-in-Aid 0655893T (J.N.) and NIH grant R01GM078341 (J.N.). Deposited in PMC for release after 12 months.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.047647/-/DC1>

References

Anderson, D. C., Gill, J. S., Cinalli, R. M. and Nance, J. (2008). Polarization of the *C. elegans* embryo by RhoGAP-mediated exclusion of PAR-6 from cell contacts. *Science* **320**, 1771–1774.

- Aono, S., Legouis, R., Hoose, W. A. and Kemphues, K. J. (2004). PAR-3 is required for epithelial cell polarity in the distal spermatheca of *C. elegans*. *Development* **131**, 2865-2874.
- Bilder, D. (2004). Epithelial polarity and proliferation control: links from the *Drosophila* neoplastic tumor suppressors. *Genes Dev.* **18**, 1909-1925.
- Bossinger, O., Klebes, A., Segbert, C., Theres, C. and Knust, E. (2001). Zonula adherens formation in *Caenorhabditis elegans* requires *dlg-1*, the homologue of the *Drosophila* gene *discs large*. *Dev. Biol.* **230**, 29-42.
- Bossinger, O., Fukushige, T., Claeys, M., Borgonie, G. and McGhee, J. D. (2004). The apical disposition of the *Caenorhabditis elegans* intestinal terminal web is maintained by LET-413. *Dev. Biol.* **268**, 448-456.
- Broadbent, I. D. and Pettitt, J. (2002). The *C. elegans* *hmr-1* gene can encode a neuronal classic cadherin involved in the regulation of axon fasciculation. *Curr. Biol.* **12**, 59-63.
- Chen, X. and Macara, I. G. (2005). Par-3 controls tight junction assembly through the Rac exchange factor Tiam1. *Nat. Cell Biol.* **7**, 262-269.
- Cheng, N. N., Kirby, C. M. and Kemphues, K. J. (1995). Control of cleavage spindle orientation in *Caenorhabditis elegans*: the role of the genes *par-2* and *par-3*. *Genetics* **139**, 549-559.
- Costa, M., Raich, W., Agbunag, C., Leung, B., Hardin, J. and Priess, J. R. (1998). A putative catenin-cadherin system mediates morphogenesis of the *Caenorhabditis elegans* embryo. *J. Cell Biol.* **141**, 297-308.
- Dow, L. E. and Humbert, P. O. (2007). Polarity regulators and the control of epithelial architecture, cell migration, and tumorigenesis. *Int. Rev. Cytol.* **262**, 253-302.
- Etamad-Moghadam, B., Guo, S. and Kemphues, K. J. (1995). Asymmetrically distributed PAR-3 protein contributes to cell polarity and spindle alignment in early *C. elegans* embryos. *Cell* **83**, 743-752.
- Fan, S., Hurd, T. W., Liu, C. J., Straight, S. W., Weimbs, T., Hurd, E. A., Domino, S. E. and Margolis, B. (2004). Polarity proteins control ciliogenesis via kinesin motor interactions. *Curr. Biol.* **14**, 1451-1461.
- Firestein, B. L. and Rongo, C. (2001). DLG-1 is a MAGUK similar to SAP97 and is required for adherens junction formation. *Mol. Biol. Cell* **12**, 3465-3475.
- Francis, R. and Waterston, R. H. (1991). Muscle cell attachment in *Caenorhabditis elegans*. *J. Cell Biol.* **114**, 465-479.
- Georgiou, M., Marinari, E., Burden, J. and Baum, B. (2008). Cdc42, Par6, and aPKC regulate Arp2/3-mediated endocytosis to control local adherens junction stability. *Curr. Biol.* **18**, 1631-1638.
- Giepmans, B. N. and van Ijzendoorn, S. C. (2009). Epithelial cell-cell junctions and plasma membrane domains. *Biochim. Biophys. Acta* **1788**, 820-831.
- Goldstein, B. and Macara, I. G. (2007). The PAR proteins: fundamental players in animal cell polarization. *Dev. Cell* **13**, 609-622.
- Harris, T. J. and Peifer, M. (2004). Adherens junction-dependent and -independent steps in the establishment of epithelial cell polarity in *Drosophila*. *J. Cell Biol.* **167**, 135-147.
- Harris, T. J. and Peifer, M. (2005). The positioning and segregation of apical cues during epithelial polarity establishment in *Drosophila*. *J. Cell Biol.* **170**, 813-823.
- Hirose, T., Karasawa, M., Sugitani, Y., Fujisawa, M., Akimoto, K., Ohno, S. and Noda, T. (2006). PAR3 is essential for cyst-mediated epicardial development by establishing apical cortical domains. *Development* **133**, 1389-1398.
- Horikoshi, Y., Suzuki, A., Yamanaka, T., Sasaki, K., Mizuno, K., Sawada, H., Yonemura, S. and Ohno, S. (2009). Interaction between PAR-3 and the aPKC-PAR-6 complex is indispensable for apical domain development of epithelial cells. *J. Cell Sci.* **122**, 1595-1606.
- Hung, T. J. and Kemphues, K. J. (1999). PAR-6 is a conserved PDZ domain-containing protein that colocalizes with PAR-3 in *Caenorhabditis elegans* embryos. *Development* **126**, 127-135.
- Hwang, B. J., Muller, H. M. and Sternberg, P. W. (2004). Genome annotation by high-throughput 5' RNA end determination. *Proc. Natl. Acad. Sci. USA* **101**, 1650-1655.
- Izumi, Y., Hirose, T., Tamai, Y., Hirai, S., Nagashima, Y., Fujimoto, T., Tabuse, Y., Kemphues, K. J. and Ohno, S. (1998). An atypical PKC directly associates and colocalizes at the epithelial tight junction with ASIP, a mammalian homologue of *Caenorhabditis elegans* polarity protein PAR-3. *J. Cell Biol.* **143**, 95-106.
- Joberty, G., Petersen, C., Gao, L. and Macara, I. G. (2000). The cell-polarity protein Par6 links Par3 and atypical protein kinase C to Cdc42. *Nat. Cell Biol.* **2**, 531-539.
- Kamath, R. S., Martinez-Campos, M., Zipperlen, P., Fraser, A. G. and Ahringer, J. (2001). Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. *Genome Biol.* **2**, RESEARCH0002.
- Kawasaki, I., Shim, Y. H., Kirchner, J., Kaminker, J., Wood, W. B. and Strome, S. (1998). PGL-1, a predicted RNA-binding component of germ granules, is essential for fertility in *C. elegans*. *Cell* **94**, 635-645.
- Kelly, W. G., Xu, S., Montgomery, M. K. and Fire, A. (1997). Distinct requirements for somatic and germline expression of a generally expressed *Caenorhabditis elegans* gene. *Genetics* **146**, 227-238.
- Kemphues, K. J., Priess, J. R., Morton, D. G. and Cheng, N. S. (1988). Identification of genes required for cytoplasmic localization in early *C. elegans* embryos. *Cell* **52**, 311-320.
- Koppen, M., Sims, J. S., Sims, P. A., Firestein, B. L., Hall, D. H., Radice, A. D., Rongo, C. and Hardin, J. D. (2001). Cooperative regulation of AJM-1 controls junctional integrity in *Caenorhabditis elegans* epithelia. *Nat. Cell Biol.* **3**, 983-991.
- Krause, M. (1995). Techniques for analyzing transcription and translation. *Methods Cell Biol.* **48**, 513-529.
- Legouis, R., Gansmuller, A., Sookharee, S., Bosher, J. M., Baillie, D. L. and Labouesse, M. (2000). LET-413 is a basolateral protein required for the assembly of adherens junctions in *Caenorhabditis elegans*. *Nat. Cell Biol.* **2**, 415-422.
- Leibfried, A., Fricke, R., Morgan, M. J., Bogdan, S. and Bellaiche, Y. (2008). *Drosophila* Cip4 and WASp define a branch of the Cdc42-Par6-aPKC pathway regulating E-cadherin endocytosis. *Curr. Biol.* **18**, 1639-1648.
- Leung, B., Hermann, G. J. and Priess, J. R. (1999). Organogenesis of the *Caenorhabditis elegans* intestine. *Dev. Biol.* **216**, 114-134.
- Lin, D., Edwards, A. S., Fawcett, J. P., Mbamalu, G., Scott, J. D. and Pawson, T. (2000). A mammalian PAR-3-PAR-6 complex implicated in Cdc42/Rac1 and aPKC signalling and cell polarity. *Nat. Cell Biol.* **2**, 540-547.
- McGill, M. A., McKinley, R. F. and Harris, T. J. (2009). Independent cadherin-catenin and Bazooka clusters interact to assemble adherens junctions. *J. Cell Biol.* **185**, 787-796.
- McMahon, L., Legouis, R., Vonesch, J. L. and Labouesse, M. (2001). Assembly of *C. elegans* apical junctions involves positioning and compaction by LET-413 and protein aggregation by the MAGUK protein DLG-1. *J. Cell Sci.* **114**, 2265-2277.
- Nance, J., Munro, E. M. and Priess, J. R. (2003). *C. elegans* PAR-3 and PAR-6 are required for apicobasal asymmetries associated with cell adhesion and gastrulation. *Development* **130**, 5339-5350.
- Nejsum, L. N. and Nelson, W. J. (2007). A molecular mechanism directly linking E-cadherin adhesion to initiation of epithelial cell surface polarity. *J. Cell Biol.* **178**, 323-335.
- Oshio, T., Fujita, N., Yamada, A., Sato, T., Kitagawa, Y., Okamoto, R., Nakata, S., Miki, A., Irie, K. and Takai, Y. (2007). Cooperative roles of Par-3 and afadin in the formation of adherens and tight junctions. *J. Cell Sci.* **120**, 2352-2365.
- Portereiko, M. F., Saam, J. and Mango, S. E. (2004). ZEN-4/MKLP1 is required to polarize the foregut epithelium. *Curr. Biol.* **14**, 932-941.
- Praitis, V., Casey, E., Collar, D. and Austin, J. (2001). Creation of low-copy integrated transgenic lines in *Caenorhabditis elegans*. *Genetics* **157**, 1217-1226.
- Reese, K. J., Dunn, M. A., Waddle, J. A. and Seydoux, G. (2000). Asymmetric segregation of PIE-1 in *C. elegans* is mediated by two complementary mechanisms that act through separate PIE-1 protein domains. *Mol. Cell* **6**, 445-455.
- Schmoranzer, J., Fawcett, J. P., Segura, M., Tan, S., Vallee, R. B., Pawson, T. and Gunderson, G. G. (2009). Par3 and dynein associate to regulate local microtubule dynamics and centrosome orientation during migration. *Curr. Biol.* **19**, 1065-1074.
- Schonegg, S., Constantinescu, A. T., Hoege, C. and Hyman, A. A. (2007). The Rho GTPase-activating proteins RGA-3 and RGA-4 are required to set the initial size of PAR domains in *Caenorhabditis elegans* one-cell embryos. *Proc. Natl. Acad. Sci. USA* **104**, 14976-14981.
- Shibata, Y., Fujii, T., Dent, J. A., Fujisawa, H. and Takagi, S. (2000). EAT-20, a novel transmembrane protein with EGF motifs, is required for efficient feeding in *Caenorhabditis elegans*. *Genetics* **154**, 635-646.
- Shin, K., Fogg, V. C. and Margolis, B. (2006). Tight junctions and cell polarity. *Annu. Rev. Cell Dev. Biol.* **22**, 207-235.
- Tabuse, Y., Izumi, Y., Piano, F., Kemphues, K. J., Miwa, J. and Ohno, S. (1998). Atypical protein kinase C cooperates with PAR-3 to establish embryonic polarity in *Caenorhabditis elegans*. *Development* **125**, 3607-3614.
- Timmons, L. and Fire, A. (1998). Specific interference by ingested dsRNA. *Nature* **395**, 854.
- Totong, R., Achilleos, A. and Nance, J. (2007). PAR-6 is required for junction formation but not apicobasal polarization in *C. elegans* embryonic epithelial cells. *Development* **134**, 1259-1268.
- Tursun, B., Cochella, L., Carrera, I. and Hobert, O. (2009). A toolkit and robust pipeline for the generation of fosmid-based reporter genes in *C. elegans*. *PLoS One* **4**, e4625.
- van Furden, D., Johnson, K., Segbert, C. and Bossinger, O. (2004). The *C. elegans* ezrin-radixin-moesin protein ERM-1 is necessary for apical junction remodelling and tubulogenesis in the intestine. *Dev. Biol.* **272**, 262-276.
- Voutev, R., Keating, R., Hubbard, E. J. and Vallier, L. G. (2009). Characterization of the *Caenorhabditis elegans* Islet LIM-homeodomain ortholog, *lim-7*. *FEBS Lett.* **583**, 456-464.
- Warming, S., Costantino, N., Court, D. L., Jenkins, N. A. and Copeland, N. G. (2005). Simple and highly efficient BAC recombineering using galK selection. *Nucleic Acids Res.* **33**, e36.
- Watts, J. L., Etamad-Moghadam, B., Guo, S., Boyd, L., Draper, B. W., Mello, C. C., Priess, J. R. and Kemphues, K. J. (1996). *par-6*, a gene involved in the establishment of asymmetry in early *C. elegans* embryos, mediates the asymmetric localization of PAR-3. *Development* **122**, 3133-3140.
- Wodarz, A. and Nathke, I. (2007). Cell polarity in development and cancer. *Nat. Cell Biol.* **9**, 1016-1024.
- Zhang, Y., Nash, L. and Fisher, A. L. (2008). A simplified, robust, and streamlined procedure for the production of *C. elegans* transgenes via recombineering. *BMC Dev. Biol.* **8**, 119.