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 polarization 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; Kemphues 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 unpolared 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 relocation 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...
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; Kemphues 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 larva 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(b222, m1425) (Tonog et al., 2007; Watts et al., 1996), hT2 [h[+](e937) let-7(q782) q548]; LGIII: par-3[71w] (Cheng et al., 1995), unc-32(e189), unc-119(ed3), c[1](dpv-1(e1259) gfp-l1(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 5570 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[71w] 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), xns62 [hmr-1::gfp, unc-119], xns123-127 [par-3::gfp], xns1199 [yfp::par-3::zfs1::gfp, unc-119] (Nance et al., 2003), zuls20 [par-3::zfs1::gfp, unc-119] (Totong et al., 2007), zuls37 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 gsk7 selection in bacterial strains SW105 or SW106 (Tursun et al., 2001; Warming et al., 2005; Zhang et al., 2008). For analysis of par-3 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-3-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 PsiI site engineered at the 3’ end of the coding sequences; unc-119 was inserted into the vector NorI site (Nance et al., 2003). par-3::gfp was constructed by modifying par-3::gfp to remove sequence upstream of the Start site within the third intron, replacing par-3 exons and introns with an AvrII site and inserting par-3 cDNA. yfp::par-3 was created by recombineering fosmid WRM0616G01; yfp from plasmid pBALU2 (Tursun et al., 2009) was inserted at the par-3 start codon and unc-119 was recombined into the fosmid backbone using pLop unc-119 (Zhang et al., 2008). hmr-1::gfp was constructed by replacing the hmr-1 genomic sequence in plasmid pW02-21 (Broadbent and Petit, 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 xns196 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) 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::zfs1::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::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); 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; xns196 [hmr-1::gfp]] males. In experiments with par-3(MZ) embryos, controls were siblings of par-3(MZ) embryos that expressed PAR-3::GFP zygotically.

par-6(MZ) and par-6(MZ) embryos

par-6(MZ) embryos were obtained by crossing par-6(tm1425 or tm201) unc-32[qc1; him-8; nals8 [Plim-7::mCherry]] males with par-6(tm1425 or tm201) unc-32; zuls20 [pPie-1::gfp::par-6::zfs1] 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::GFP should be par-6(MZ); par-6(MZ) double mutants, i.e. par-6(tm1425); par-3(tm2716).
PAR-3 in C. elegans epithelia

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., 2003).

Live imaging

differential interference contrast (DIC) movies were acquired using a Zeiss AxioImager, 63× 1.4 NA or 40× 1.3 NA objective, Nomarski optics, an Axiocam MRM camera and AxioVision software. z-stacks of sections at 1-μm intervals were captured every 1-2 minutes. Fluorescence movies were acquired using a Leica SP5 confocal microscope, 61× 1.3 NA water-immersion objective, 488 or 514 nm laser and a 3× 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.

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-PSD-95 (recognizes DLG-1), 1:200 (Affinity BioReagents) (Firestein et al., 2001; Leung et al., 1999; McMahon et al., 2001; Totong et al., 2003) 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(RNAi) embryos (P1A5 stained nuclei, and P4A1 stained the epidermis or cuticle of late-elongation stage embryos).

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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-3YFP) from the par-3 promoter (see below) and captured time-lapse movies. In polarizing IPCs, PAR-3YFP formed foci similar to those observed in immunostained embryos (7 of 7 embryos) (Fig. 1F; see Movie 2 in the supplementary material). Individual PAR-3YFP 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 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 isofrom 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
Table 1. Phenyotypes of par-3(tm2716) and par-3(MZ) mutants

<table>
<thead>
<tr>
<th>Maternal genotype</th>
<th>Self-progeny phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
</tr>
<tr>
<td>Wild type</td>
<td>356 (99.5)</td>
</tr>
<tr>
<td>par-3(tm2716)/+</td>
<td>309 (78)</td>
</tr>
<tr>
<td>par-3(tm2716); par-3::zf1::gfp</td>
<td>697 (97.5)</td>
</tr>
<tr>
<td>par-3(tm2716); par-3::zf1::gfp/+</td>
<td>490 (61)</td>
</tr>
</tbody>
</table>

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

Table 2. Function of par-3 isoforms

<table>
<thead>
<tr>
<th>Genotype and treatment</th>
<th>L4 feeding</th>
<th>L1 feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hatched</td>
<td>Dead</td>
</tr>
<tr>
<td>par-3(tm2716); par-3::gfp, vector RNAi</td>
<td>385 (94)</td>
<td>27 (6)</td>
</tr>
<tr>
<td>par-3(tm2716); par-3::gfp, gfp RNAi</td>
<td>0</td>
<td>450</td>
</tr>
<tr>
<td>par-3(tm2716); yfp::par-3, vector RNAi</td>
<td>385 (99)</td>
<td>2 (1)</td>
</tr>
<tr>
<td>par-3(tm2716); yfp::par-3, gfp RNAi</td>
<td>341 (98)</td>
<td>9 (2)</td>
</tr>
</tbody>
</table>

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

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).
par-3::zf1::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::zf1::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::zf1::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 selected par-3(tm2716); par-3::zf1::gfp/+ worms and analyzed progeny lacking the par-3::zf1::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::zf1::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, epithelial 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 contract circumferentially to squeeze the embryo and elongate the body axis; simultaneously, the pharynx and intestine form a linked contract circumferentially to squeeze the embryo and elongate

**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-I; 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 μm.](image)
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. 4I). 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 epithelial 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

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**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 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-1GFP 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; Schmoranzer 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...
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 epithelial 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 apical basal polarization of MDCK cells (Nejsum and Nelson, 2007). Regardless of the mechanism that epithelial 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.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

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**References**


