PAR-6 is required for junction formation but not apicobasal polarization in C. elegans embryonic epithelial cells

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Epithelial cells perform important roles in the formation and function of organs and the genesis of many solid tumors. A distinguishing feature of epithelial cells is their apicobasal polarity and the presence of apical junctions that link cells together. The interacting proteins Par-6 (a PDZ and CRIB domain protein) and aPKC (an atypical protein kinase C) localize apically in fly and mammalian epithelial cells and are important for apicobasal polarity and junction formation. Caenorhabditis elegans PAR-6 and PKC-3/aPKC also localize apically in epithelial cells, but a role for these proteins in polarizing epithelial cells or forming junctions has not been described. Here, we use a targeted protein degradation strategy to remove both maternal and zygotic PAR-6 from C. elegans embryos before epithelial cells are born. We find that PKC-3 does not localize asymmetrically in epithelial cells lacking PAR-6, apical junctions are fragmented, and epithelial cells lose adhesion with one another. Surprisingly, junction proteins still localize apically, indicating that PAR-6 and asymmetric PKC-3 are not needed for epithelial cells to polarize. Thus, whereas the role of PAR-6 in junction formation appears to be widely conserved, PAR-6-independent mechanisms can be used to polarize epithelial cells.

KEY WORDS: Polarity, Organogenesis, Epithelium, Cell junctions, PAR-6, C. elegans

INTRODUCTION

Epithelial cells are integral components of organs and perform crucial roles in organ morphogenesis and function. As epithelial cells form, they acquire an apicobasal polarity that distinguishes their different membrane surfaces. Apical and basolateral surfaces differ in part because of targeted protein secretion (Nelson and Yeaman, 2001; Rodriguez-Boulan et al., 2004); junctions that form near the apical-basolateral interface also limit mixing between the two domains and promote adhesion by linking epithelial cells together (Anderson et al., 2004). Epithelial polarity allows different regions of the cell to develop specialized structures and functions, and, based on studies of Drosophila tumor suppressor proteins, is thought to inhibit cell proliferation and tumor formation (Humbert et al., 2003). However, how epithelial cells first polarize and assemble apical junctions is not well understood.

Many different cell types require the conserved scaffolding protein PAR-6 to polarize (Macara, 2004). PAR-6, which contains PB1, CRIB and PDZ domains that bind other polarity proteins such as the atypical protein kinase C (aPKC) PKC-3 (Hung and Kemphues, 1999; Joberty et al., 2000; Lin et al., 2000; Suzuki et al., 2001), was first identified for its role in polarizing the C. elegans zygote (Watts et al., 1996). The zygote is a highly polarized cell that cleaves asymmetrically to produce anterior and posterior daughter cells, which differ in size and developmental potential. PAR-6 and PKC-3 become restricted to an anterior cortical domain of the zygote and regulate the localization of developmental determinants and proteins required for asymmetric cleavage (Colombo et al., 2003; Gotta et al., 2003; Pellettieri and Seydoux, 2002). PAR-6 also functions later to polarize early embryonic cells: in response to the pattern of cell-cell contacts, PAR-6 becomes restricted to the outer (contact-free) cortex of cells and regulates inner-outer asymmetries in cell adhesion and cytoskeletal organization important for gastrulation (Hung and Kemphues, 1999; Nance et al., 2003; Nance and Priess, 2002).

PAR-6 homologues are required to polarize epithelial cells in other organisms. Epithelial cells polarize either when clusters of precursor cells receive polarity cues provided by their external environment or when the membrane of the egg is divided into apical and basolateral domains by cleavage or cellularization (Drubin and Nelson, 1996; Muller, 2001). In Drosophila blastoderm epithelial cells, which form by cellularization of syncytial embryonic nuclei, Par-6 and aPKC localize to an apical domain adjacent to adherens junctions (Petronczki and Knoblich, 2001; Wodarz et al., 2000). aPKC requires Par-6 for its apical localization and regulates polarity effectors via phosphorylation, suggesting that aPKC asymmetry is critical for establishing polarity (Betschinger et al., 2003; Hutterer et al., 2004; Plant et al., 2003; Yamanaka et al., 2003). When both maternal and zygotic pools of Par-6 are removed, adherens junction proteins and other apical proteins fail to localize, and epithelial cells lose their laminar organization (Hutterer et al., 2004; Petronczki and Knoblich, 2001). Par-6 also regulates junction formation in cultured mammalian epithelial cells, which polarize upon contact with neighboring cells. Mammals contain four genes that encode different Par6 isoforms (A-D) (Gao and Macara, 2004; Joberty et al., 2000). Overexpressing full-length Par6B or truncated Par6A in canine kidney epithelial cells inhibits tight junction disassembly (Gao et al., 2002; Gao and Macara, 2004; Joberty et al., 2000; Yamanaka et al., 2001). In addition, Par6C in human kidney epithelial cells interacts with TGFβ receptors to mediate tight junction disassembly during TGFβ-induced epithelial-to-mesenchymal transitions (Ozdamar et al., 2005). However, because cells lacking all Par6 isoforms have not been described, it is unclear whether mammalian Par6 regulates aspects of epithelial polarization other than tight junction biogenesis.

C. elegans PAR-6 and PKC-3 localize asymmetrically within several epithelial cell types, but a role for these proteins in epithelial polarity or junction formation has not been described. In part, this may be due to difficulties in removing both maternal and zygotic PAR proteins from epithelial cells without also preventing polarization of the zygote (Hung and Kemphues, 1999; Watts et al., 1996). C.
**C. elegans** epithelial cells differentiate from clusters of precursor cells that polarize during organogenesis. The first epithelial cells form during the middle stages of embryogenesis and consist primarily of epidermal cells, pharyngeal cells, and intestinal cells (see Fig. 1). PAR-6 and PKC-3 localize to apical regions of pharyngeal and intestinal epithelial cells (Bossinger et al., 2001; Leung et al., 1999; McMahon et al., 2001). PAR-6 and PKC-3 are also found at apical surfaces of reproductive tract epithelial cells, such as distal spermathecal cells, that form during larval development (Aono et al., 2004; Hurd and Kemphues, 2003). Reducing larval levels of the PDZ domain protein PAR-3, which is required to localize PAR-6 and PKC-3, disrupts the polarity of distal spermathecal cells (Aono et al., 2004). However, because PAR-3 might have functions other than regulating PAR-6 and PKC-3 localization, it is not known whether PAR-6 and PKC-3 are needed to polarize spermathecal cells or any other type of *C. elegans* epithelial cell.

Here we use a targeted protein degradation strategy to remove both maternal and zygotic PAR-6 from *C. elegans* embryos before epithelial cells form. We find that PKC-3 can no longer localize asymmetrically in epithelial cells, and embryos arrest during morphogenesis with severe defects in epithelial cell adhesion and apical junction formation. Surprisingly however, epithelial cells are still able to polarize. Thus PAR-6 has a widely conserved role in regulating epithelial cell junctions but epithelial cells can utilize PAR-6-independent mechanisms to polarize.

**MATERIALS AND METHODS**

**Strains**

The following mutations and chromosomal rearrangements were utilized: LGI: *hmr-1(zu248)* (Costa et al., 1998), *unc-101(tm1), par-6(zu170), zu222, tm1425* (Watts et al., 1996); LGIII: *unc-119(ed3); LGIV: *him-8(e1489). zuEx3* is an extrachromosomal array containing *hmr-1* (+) and *rol-6* (4) (Costa et al., 1998). *xenEx12* (this study) is an extrachromosomal array containing par-6:gfp and *unc-119(+)* (Nance et al., 2003). The *par-6(tm1425)* deletion was provided by the National Bioresource Project (S. Mitani, Tokyo Women’s Medical University), outcrossed six times, balanced, and sequenced. *tm1425* removes bp 117 to 969 of the par-6 genomic sequence (start codon=1), *par-6(tm1425)* failed to complement par-6(zu222) for the maternal-effect Par phenotype (12/12) and caused a recessive larval lethal phenotype (2/135 eggs from *par-6(tm1425)/+* homohydrops did not hatch, 40/135 (30%) progeny died as early larvae, 93/135 (69%) grew to fertile adults). The larval lethal phenotype of *par-6(tm1425)* homozygotes is rescued by the *xenEx12 par-6:gfp* transgenic array: *par-6(tm1425)/par-6(tm1425); xenEx12* epithelial cells are viable, fertile, and produce viable progeny (95/101 eggs developed to fertile adults).

**Transformation**

*xds17 [dlg-1::gfp]* was created by integrating a *dlg-1::gfp* extrachromosomal array (Firestein and Rongo, 2001) using trimethylpsoralen mutagenesis and UV irradiation (Clark and Chiu, 2003). All other extrachromosomal and integrated transgenic lines were created by biolistic transformation of *unc-119* worms (Prattis et al., 2001).

**Transgene construction**

A transgene expressing *PAR-6*::GFP driven by the maternal *pie-1* promoter was created by digesting the *pie-1::gfp:actin* plasmid pH4.64 (Reese et al., 2000) with SpeI to remove *actin* coding sequences, then replacing with *par-6* and *gfp* coding sequences. pH4.64 was also modified to eliminate an internal *NolI* site. The complete *par-6* cDNA (Hung and Kemphues, 1999) was amplified by RT-PCR using an *Nhel*-tagged forward primer and a SpeI-tagged reverse primer; digested RT-PCR product was inserted into the SpeI site 3’ of *gfp*. The *unc-119* (+) gene from plasmid pH254 (Nance et al., 2003) was inserted into the *NolI* site. The *gfp* coding sequence (codons 97-132 from the *pie-1* gene) (Reese et al., 2000) was amplified using SpeI-tagged primers and inserted into the SpeI site 3’ of *par-6*. Two transgenic lines were obtained (zds43 and zds44).

**par-6(M2/Z) embryos**

*par-6(M2/Z) embryos* were obtained by crossing *unc-101+/+ par-6(tm1425); him-8 males* with *unc-101 par-6(zu170); zds43* [pie-1::gfp:par-6::gfp: *hermaphrodites* (P0 cross) and allowing the non-Unc F1 progeny to self-fertilize; 25% of F2 embryos are expected to be *par-6(M2/Z); par-6(tm1425)/par-6(tm1425) homozygotes* that express maternal *PAR-6*::GFP protein only during early embryonic stages. *par-6(M2/Z) embryos* were distinguished from their wild-type siblings in immunostaining experiments by including PAR-6 or PKC-3 antibodies. Where indicated, the *par-6(zu222) mutation* was used in place of *par-6(zu170)* or the *zds43* transgene was used in place of *zds43*; all gave similar results.

*par-6(M2/Z) embryos* expressing DLG-1::GFP were obtained by replacing *unc-101 par-6(zu170); zds43* *P0* hermaphrodites with *unc-101 par-6(zu170); zds43; xen17 [dlg-1::gfp]. *par-6(M2/Z) embryos* depleted of DLG-1 or LET-413 were obtained by injecting non-Unc F1 hermaphrodites from the P0 cross described above with dsRNA from the *dlg-1* or *let-413* gene (see below). *hmnr-1(zu248), RNAi par-6(M2/Z) embryos* were obtained by replacing the *P0* male strain with *hmnr-1(zu248) + par-6(tm1425)+ unc-101* (+); *him-8* males and placing non-Unc F1 hermaphrodites on *hmnr-1 RNAi* feeding plates (see below) to remove maternal HMR-1. *hmnr-1(zu248), RNAi par-6(M2/Z) embryos* were distinguished from siblings by co-staining with HMR-1 and PKC-3 antibodies; only embryos with no detectable HMR-1 and uniformly cytoplasmic PKC-3 in intestinal epithelial cells were scored. For control *hmnr-1 embryos*, *hmnr-1(zu248) hermaphrodites* expressing an extrachromosomal array containing *hmnr-1 (zuEx3)* were placed on *hmnr-1 RNAi* plates; only embryos lacking HMR-1 immunostaining in intestinal epithelial cells were scored.

**RNAi**

*par-6* 3’ UTR RNAi and *hmnr-1 RNAi* was performed by the feeding method. Base pairs 1-386 of the *par-6* 3’ UTR or hp 13,345-13,988 of the *hmnr-1* gene (start codon=1) were amplified and cloned into RNAi feeding vector pPD129.36 (Timmons and Fire, 1998). Feeding RNAi was performed as described (Kamath et al., 2001), substituting β-lactose for IPTG (Gobel et al., 2004). L4 hermaphrodites placed on feeding plates were incubated at 25°C and embryos laid after 36-48 hours were analyzed. In control experiments using wild-type hermaphrodites, 241/245 *par-6(UTR)RNAi* embryos died and 15/16 embryos showed a Par first cleavage. All *hmnr-1(RNAi) embryos* died (103/103).

*let-413 and dlg-1 RNAi* was performed by the injection method. Coding sequences from each gene were amplified from genomic DNA using T7 promoter-tagged primers [*let-413: bp 1014-1613; dlg-1: bp 3185-3675*] and dsRNA was produced by in vitro transcription (Nance and Priess, 2002). Young adult hermaphrodites were injected with 1-3 µg/µL dsRNA and allowed to recover at 20°C; eggs laid 20-32 hours post-injection were collected and immunostained. To monitor the extent of protein depletion following RNAi, a subset of embryos in each experiment was stained for LET-413 or DLG-1. Of the 30 *let-413(RNAi) embryos* 29 lacked intestinal LET-413 staining. A total of 89/101 *dlg-1(RNAi) embryos* lacked intestinal DLG-1 staining, 11/101 showed trace amounts of staining and 1/101 showed normal levels of staining.

**Videomicroscopy**

Three-dimensional timelapse Nomarski movies were acquired using a Zeiss Imager equipped with a 63× 1.4 NA or 40× 1.3 NA objective, Nomarski optics, an Axioptic MRM digital camera, and Axiovision software. Z-stacks of sections spaced at 1 µm intervals were captured every 2-5 minutes. Fluorescence timelapse movies of DLG-1::GFP in hypodermal cells were acquired using a Zeiss 510 LSM confocal microscope, 63× 1.3 NA objective, 488 nm laser at 9% strength and 2× zoom. Laser intensity and scan speed were adjusted so that imaged control embryos hatched. Maximum intensity projections of several planes at 1 µm intervals were compiled and optimized using ImageReady. For movies of *par-6(M2/Z) embryos*, mutant embryos and control sibling embryos were mounted together. After acquiring movies, mutant embryos were distinguished as those that arrested before the twofold stage and developed cell adhesion defects; in all cases, arrested embryos expressing DLG-1::GFP had fragmented junctions.
**Immunostaining**

Embryos were fixed using the freeze-crack methanol procedure and stained as described (Leung et al., 1999). The following primary antibodies and dilutions were used: rabbit (Rb) α-ACT-5 1:50 (MacQueen et al., 2005), mouse (Ms) α-AJM-1 ‘MH27’ 1:10 (Francis and Waterston, 1991; Koppen et al., 2001), Rb α-EBP-2 1:3000 (Srayko et al., 2005), Ms ‘F2-P3E3’ 1:5 (Eisenhut et al., 2005), chicken α-GFP 1:200 (Chemicon), Rb α-GFP 1:2000 (Abcam), Rb α-HMR-1 1:1100 (Costa et al., 1998), Ms α-HMP-1 1:110 (Costa et al., 1998), Ms α-IFB-2 ‘MH33’ 1:150 (Bossinger et al., 2004; Francis and Waterston, 1991), Rb α-LET-413 1:5000 (Aono et al., 2004), Ms α-PAR-3 1:25 (Nance et al., 2003), Rb α-PAR-6 1:20 (Hung and Kemphues, 1999), rat (Rt) α-PK-3 1:30 (Tabuse et al., 1998), Ms α-PSD-95 1:200 (Affinity Bioreagents; recognizes DLG-1) (Firestein and Rongo, 2001), Rt α-alpha-tubulin 1:2000 (Harlan). Primary antibodies were detected using dye-coupled secondary antibodies (Molecular Probes, Jackson ImmunoResearch). For tubulin plus PAR-6 and EBP-2 plus PKC-3 co-stainings, secondary antibody incubations and washes were performed sequentially. Z-stacks of embryos were acquired with a Zeiss Imager, 63×1.4 NA objective and Axiocam MRM camera and were deconvolved using Axioscan software. Images shown are maximum intensity projections of several adjacent planes spaced at 300 nm intervals. For embryos stained with α-EBP-2 or α-tubulin antibodies, confocal images were acquired with a Zeiss 510 LSM and a 63×1.3 NA objective. Unless stated otherwise, a minimum of 50 control and 20 par-6(M/Z) embryos at the indicated stages were examined in each staining experiment.

**RESULTS**

**PAR-6 expression and localization in embryonic epithelial cells**

We used PAR-6 antibodies (Hung and Kemphues, 1999) and GFP-tagged PAR-6 (hereafter PAR-6GFP) to examine the localization of PAR-6 in embryonic epithelial cells. Early embryos contain maternally supplied PAR-6, which diminishes in level after the onset of gastrulation (26-cell stage) (Hung and Kemphues, 1999; Nance and Priess, 2002). Levels of PAR-6 immunostaining increase in epithelial cells that form during the middle stages of embryogenesis (~300- to 400-cell stage) (Bossinger et al., 2001; Leung et al., 1999; McMahon et al., 2001). Epidermal epithelial cells are superficial and have contact-free apical surfaces that face the eggshell, whereas pharyngeal and intestinal epithelial cells are internal and have apical surfaces that contact other epithelial cells and the digestive tract lumen (Fig. 1A,B). We noted a previously undescribed PAR-6 immunostaining at the apical surfaces of epidermal cells (arrow, Fig. 1A); staining was most intense when epidermal cells first formed and diminished as embryos aged (compare Fig. 1A,B). PAR-6 antibodies also labeled the apical surfaces of pharyngeal and intestinal epithelial cells, as has been described by others (Bossinger et al., 2001; Leung et al., 1999; McMahon et al., 2001), as well as the excretory cell (Fig. 1B). Because specificity of PAR-6 immunostaining in epithelial cells has not been established, we analyzed the localization of a functional PAR-6GFP fusion protein expressed from the par-6 promoter (Nance et al., 2003) (see Materials and methods). PAR-6GFP was expressed in epidermal, pharyngeal, intestinal, and excretory epithelial cells, colocalized with PAR-6 at apical surfaces, and was present even when we introduced the transgene at fertilization (Fig. 1C,F and data not shown). In summary, PAR-6 localizes to the apical cortex of most or all embryonic epithelial cells and at least some PAR-6 in epithelial cells arises from zygotic par-6 expression.

We performed triple immunostaining experiments to determine whether PAR-6 colocalized with PKC-3 and PAR-3. In polarizing epithelial cells, PAR-6 colocalized broadly with both PKC-3 and PAR-3 (Fig. 1A′ and data not shown). In fully polarized epithelial cells PAR-6 continued to colocalize with PKC-3 at the apical cortex (see Fig. 3B′), but PAR-3 developed a more lateral localization (Fig. 1B′). Thus, whereas PAR-6 and PKC-3 always appear to colocalize, PAR-6 and PAR-3 overlap only transiently as epithelial cells polarize.

To determine when PAR-6 first localizes asymmetrically, we examined the distribution of PAR-6GFP in intestinal precursor cells that are just beginning to polarize. Intestinal precursor cells are arranged in left and right columns; as apicobasal polarity develops, nuclei migrate to the midline between the columns where the apical surface forms (compare Fig. 1C,F) (Leung et al., 1999; Sulston et al., 1983). Prior to apical nuclear movements, we observed spot-like accumulations of cortical PAR-6GFP at lateral and apical interfaces of intestinal precursor cells (Fig. 1C). PAR-6GFP puncta congregated at apical surfaces (Fig. 1F) before organizing into a continuous band (see Fig. 1B). We co-stained embryos with GFP antibodies and junction protein antibodies to determine if apical PAR-6GFP accumulation preceded the formation of apical junctions. The adherens junction protein HMP-1α-catenin and the junction protein DLG-1/Discs large accumulate in puncta that congregate near the apical-lateral interface before forming continuous belt-like junctions (Bossinger et al., 2001; Costa et al., 1998; Firestein and Rongo, 2001).
pie-1 promoter expressing PAR-6 fused to ZF1 and GFP (hereafter PAR-6ZF1GFP), then crossed the transgene into a par-6 mutant background where maternal PAR-6 is not detectably expressed (Hung and Kemphues, 1999). As we observed with PAR-6ZF1GFP expressed from the par-6 promoter (Nance et al., 2003), PAR-6ZF1GFP expressed from the pie-1 promoter localized to the anterior cortex of the zygote and to the outer cortex of early embryonic cells (Fig. 2A–C and data not shown). Unlike par-6 mutant embryos, par-6 mutant embryos expressing maternal PAR-6ZF1GFP appeared to have normal anteroposterior polarity, including an asymmetric first division and asymmetric localization of germline P granules (‘P’ in Fig. 2A). PAR-6ZF1GFP levels dropped in somatic cells beginning at the six- to eight-cell stage (Fig. 2C and data not shown), and after the 50-cell stage we were unable to detect PAR-6ZF1GFP in any somatic cell (Fig. 2D and data not shown). In summary, maternal PAR-6ZF1GFP restores anteroposterior polarity to par-6 mutant embryos then degrades well before epithelial cells are born.

Surprisingly, most par-6 embryos expressing PAR-6ZF1GFP appeared to develop normally (803/811 hatched) and grew to fertile adults. We stained embryos to determine if PAR-6 and PAR-6ZF1GFP were indeed removed from epithelial cells. As expected, GFP antibodies did not stain epithelial cells, indicating that maternal PAR-6ZF1GFP was not present (Fig. 2F). However, epithelial cells in par-6 embryos expressing maternal PAR-6ZF1GFP showed high levels of PAR-6 immunostaining; we obtained similar results using two different par-6 alleles (zu170, zu222) (Fig. 2E and data not shown). These findings suggest either that PAR-6 immunostaining in epithelial cells is nonspecific or that par-6 mutations do not prevent zygotic PAR-6 expression. Because we demonstrate below that PAR-6 antibody staining is specific, we conclude that par-6 mutations do not prevent maternal PAR-6 expression. 

### par-6 deletion mutation causes larval lethality

par-6 alleles defective for maternal but not zygotic PAR-6 expression contain a transposable element insertion in the par-6 3’ untranslated region (UTR) (Hung and Kemphues, 1999). We obtained a par-6 deletion allele (tm1425; provided by the National Bioresource Project, Tokyo) lacking sequences encoding much of the N-terminal PB1 domain (green in Fig. 3A), which in mammalian Par6 is required for binding to aPKC (Lin et al., 2000; Suzuki et al., 2001). The deletion also causes a frameshift six codons beyond the deleted region, which is predicted to truncate PAR-6 before the CRIB and PDZ domains (Fig. 3A and Materials and methods). par-6(tm1425) failed to complement existing par-6 alleles and had a more severe larval lethal phenotype that was rescued by a par-6:gfpc transgene (see Materials and methods). These observations strongly suggest that par-6(tm1425) inactivates full-length PAR-6 and that zygotic expression of PAR-6 is required for viability.

### Removing maternal and zygotic PAR-6 causes embryonic lethality and disrupts epithelial cell adhesion

We performed crosses to obtain par-6(zu170)/par-6(tm1425) hermaphrodites expressing maternal PAR-6ZF1GFP. Of the self-progeny from these hermaphrodites, 25% should be par-6(tm1425) homozygotes lacking both maternal and zygotic PAR-6 and expressing maternal PAR-6ZF1GFP only at early embryonic stages. We found that 24.7±1.0% of the embryos arrested before hatching (n=1304 embryos). To confirm that arrested embryos lacked PAR-6...
in epithelial cells, we allowed synchronized embryos to develop through much of embryogenesis then co-stained with PAR-6 and PKC-3 antibodies. All embryos that developed beyond the twofold stage (141 of 204, 69%) contained apical PAR-6 and PKC-3 in epithelial cells (data not shown, see Fig. 3B). All embryos that arrested before the twofold stage (63 of 204, 31%) lacked PAR-6 immunostaining, and PKC-3 was distributed uniformly throughout the cytoplasm of epithelial cells (Fig. 3C). As PAR-6 is required to position PKC-3 asymmetrically in the one-cell embryo and in early embryonic cells (Nance et al., 2003; Tabuse et al., 1998), these observations indicate that arrested embryos lack PAR-6 activity in epithelial cells. We refer to these arrested embryos as ‘par-6(M/Z)’ embryos.

We used 3D timelapse videomicroscopy to determine when par-6(M/Z) embryos first developed defects. Using our crossing scheme, we expected 25% of the embryos analyzed to be par-6(M/Z). All of the embryos filmed (n=160) appeared to develop normally through the first half of embryogenesis, when most cell divisions occur. During the second phase of embryogenesis, which is characterized by morphogenesis, 24% arrested. Two prominent morphogenetic events occur during this phase of development. At the beginning of the morphogenesis phase, dorsal epidermal cells migrate ventrally to encase the embryo in skin (‘enclosure’); subsequently, epidermal cells undergo cell shape changes that squeeze and elongate the embryo fourfold in length (‘elongation’; see Fig. 4A) (Chin-Sang and Chisholm, 2000; Simske and Hardin, 2001). Arrested embryos completed enclosure, but did not progress beyond the initial stages of elongation (not beyond the twofold stage) (Fig. 4B,C). Epidermal cells of arrested embryos eventually began to separate from one another, allowing internal cells to extrude to the embryo’s surface (arrowheads in Fig. 4C). Separations were not restricted to the ventral epidermal cells, which form new contacts during enclosure, but occurred at many different positions. Because, as demonstrated above, embryos arresting before the twofold stage lack PAR-6 immunostaining, these timelapse experiments indicate that PAR-6 is required for embryonic elongation and epithelial cell adhesion.

par-6(M/Z) epithelial cells polarize but develop abnormal junctions

The cell adhesion defects of par-6(M/Z) embryos suggest that epithelial cells lacking PAR-6 fail to polarize and/or cannot form functional apical junctions. We examined epithelial polarity by staining embryos for cytoskeletal markers that develop apico-basal asymmetries in intestinal epithelial cells. Microtubules concentrate at the apical cortex of intestinal epithelial cells as they first polarize (Leung et al., 1999). Intestinal epithelial cells of wild-type and par-6(M/Z) embryos showed similar apical enrichments of both microtubules and EBP-2/EB1, a plus-end microtubule-binding protein (Srayko et al., 2005), although in par-6(M/Z) embryos both apical microtubules and EBP-2 appeared somewhat less compact than in wild type (Fig. 5A-B’ and see Fig. S1A-B’ in the supplementary material). The largely coincident localization of microtubules and EBP-2 was somewhat surprising, given that the plus ends of microtubules in mammalian epithelial cells concentrate basally (Musch, 2004); it is unclear whether these differences reflect an alternative organization of microtubules in C. elegans epithelial cells, or whether in C. elegans the microtubules are shorter and thus present an abundance of plus ends. We also examined the distribution of the intermediate filament protein IFB-2 and the microvillar actin ACT-5, the levels of which increase apically at slightly later stages, as microvilli begin to form at the apical surfaces of intestinal cells (Bosinger et al., 2004; MacQueen et al., 2005). When IFB-2 and ACT-5 were first expressed, both proteins showed apical concentrations in wild-type and par-6(M/Z) intestinal epithelial cells, although occasional gaps in IFB-2 apical localization were evident between cells in par-6(M/Z) embryos (see below; Fig. 5C,D and see Fig. S1C,D in the supplementary material). We
observed apical localization of IFB-2 in par-6(M/Z) embryos when using a different transgene insertion (zu144) to express maternal PAR-6F1GFP, either of two maternal alleles of par-6 (zu170, zu222) in our crossing scheme, or after treating worms to induce RNAi of any hypothetical remaining endogenous PAR-6 (n=11 par-6(M/Z) embryos; see Materials and methods). In summary, the asymmetric localization of microtubules, IFB-2 and ACT-5 in par-6(M/Z) embryos indicates that detectable levels of PAR-6 and asymmetric localization of PKC-3 are not needed for C. elegans embryonic epithelial cells to polarize.

In older arrested par-6(M/Z) embryos, apical IFB-2 and ACT-5 were often present in isolated apical patches (Fig. 5E and data not shown). These apical patches did not appear to result from apoptosis of epithelial cells as par-6(M/Z) embryos showed no increase in staining with the F2-P3E3 antibody (data not shown), which recognizes apoptotic bodies (Eisenhut et al., 2005). Rather, we interpret these isolated apical patches as the result of failed adhesion between adjacent epithelial cells. Together with our 3D timelapse analysis, these staining experiments suggest that epithelial cells in par-6(M/Z) embryos develop apicobasal polarity but eventually detach from one another.

To determine whether abnormal apical junctions might explain the adhesion defects we observed, we examined the localization of junction proteins HMR-1/E-cadherin, HMP-1/i-catenin, DLG-1/Discs large, and AJM-1 in par-6(M/Z) embryos. Each of these junction proteins was positioned apically within intestinal cells; however, in contrast to the continuous belt-like organization of HMP-1, HMR-1, DLG-1, and AJM-1 in wild-type embryos, each protein showed a fragmented distribution (Fig. 6 and data not shown). In some cell types, especially lateral epidermal cells, HMR-1 and HMR-1 fragmentation was often less severe than DLG-1 or AJM-1 fragmentation. PAR-3 showed a similar apical but fragmented localization in par-6(M/Z) embryos (Fig. 3C). These defects in apical junction protein and PAR-3 organization were not caused by mislocalization of the basolateral protein LET-413 (Fig. 6E,F), which is also required to form continuous apical junctions (Legouis et al., 2000). In summary, apical junction proteins, PAR-3, and the basolateral protein LET-413 can become positioned asymmetrically without PAR-6, but apical junction proteins and PAR-3 require PAR-6 for their organization into belt-like structures.

Our failure to observe epithelial polarity defects in par-6(M/Z) embryos could be explained if PAR-6 and another protein(s) function redundantly to polarize epithelial cells. Because E-cadherin and Discs large are required to polarize Drosophila epithelial cells (Humbert et al., 2003) but their C. elegans orthologs (HMR-1/E-cadherin and DLG-1/Discs large) are dispensable for epithelial polarity (Bossinger et al., 2001; Costa et al., 1998; Firestein and Rongo, 2001; Koppen et al., 2001; McMahon et al., 2001), we created ‘double mutants’ to ask whether PAR-6 functions redundantly with HMR-1 or DLG-1 to polarize epithelial cells. Using RNAi to deplete embryos of DLG-1 or a combination of RNAi and the hmr-1(zu248) mutation to deplete embryos of HMR-1, we were unable to detect either protein in intestinal epithelial cells (see Materials and methods). Consistent with reports by others (Bossinger et al., 2001; Costa et al., 1998; Firestein and Rongo, 2001; Koppen et al., 2001; McMahon et al., 2001), we observed that DLG-1 and PKC-3 localized apically in intestinal cells of embryos depleted of HMR-1 (n=27/28), and HMP-1 and PAR-6 were apical in intestinal cells of embryos depleted of DLG-1 (n=118) (see Fig. S2C,E in the supplementary material). Depleting HMR-1 (stained with HMP-1, n=36) or DLG-1 (stained with HMP-1, n=40) in par-6(M/Z) embryos yielded phenotypes similar to those of par-6(M/Z) embryos: in each case junction proteins were apical but fragmented (see Fig. S2D,F in the supplementary material). In contrast to HMR-1 and DLG-1, the basolateral protein LET-413/Scribble is required for formation of continuous junctions and to maintain the apical localization of junction proteins and other apical proteins (Bossinger et al., 2001; Koppen et al., 2001; Legouis et al., 2000; McMahon et al., 2001). Similar to observations by others (Bossinger et al., 2001; Koppen et al., 2001; Legouis et al., 2000; McMahon et al., 2001), we noted that PAR-6 and DLG-1 spread laterally in intestinal cells of embryos depleted of LET-413 (87/89 embryos). let-413(RNAi) par-6(M/Z) embryos stained for LET-413 showed an indistinguishable phenotype (24/24 embryos). In contrast to HMR-1 and DLG-1, the basolateral protein LET-413/Scribble is required for formation of continuous junctions and to maintain the apical localization of junction proteins and other apical proteins (Bossinger et al., 2001; Koppen et al., 2001; Legouis et al., 2000; McMahon et al., 2001). Similar to observations by others (Bossinger et al., 2001; Koppen et al., 2001; Legouis et al., 2000; McMahon et al., 2001), we noted that PAR-6 and DLG-1 spread laterally in intestinal cells of embryos depleted of LET-413 (87/89 embryos). let-413(RNAi) par-6(M/Z) embryos stained for LET-413 showed an indistinguishable phenotype (24/24 embryos). 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**PAR-6 is required to condense apical junction proteins**

In wild-type embryos, puncta of junction proteins such as DLG-1 congregate at the apicolateral surface and condense into belt-like apical junctions (Bossinger et al., 2001; Koppen et al., 2001; McMahon et al., 2001). The abnormal organization of junction proteins in par-6(M/Z) embryos could result from a failure to initially condense junction proteins or a failure to prevent continuous junctions from fragmenting during the stresses of morphogenesis. To determine when PAR-6 function is first required during apical junction formation, we performed timelapse fluorescence microscopy experiments to follow the movements of GFP-tagged DLG-1 (DLG-1GFP) in par-6(M/Z) and control embryos (Fig. 7 and see Movies 1, 2 in the supplementary material). We imaged junctions...
formation in epidermal cells, which are superficial and have apical surfaces easily observed within the same focal plane. As others have described (Bossinger et al., 2001; Koppen et al., 2001; McMahon et al., 2001), we noted that puncta of DLG-1GFP in control embryos coalesced rapidly into continuous belt-like junctions (16/16 embryos). DLG-1GFP in par-6(M/Z) embryos was expressed at a similar stage and formed apical puncta, but these puncta failed to condense into continuous junctions (9/9 embryos). In addition, we never observed continuous DLG-1 in intestinal epithelial cells of par-6(M/Z) embryos at stages when DLG-1 becomes continuous in wild type (Fig. 7C,D). We conclude that PAR-6 is required for the initial formation of continuous apical junctions.

**DISCUSSION**

**PAR-6 functions reiteratively to control cell adhesion**

Our results show that PAR-6 is essential for epithelial cells to remain adherent during elongation. This is not the first stage of embryogenesis in which PAR-6 is needed for proper cell-cell adhesion – early embryonic cells lacking PAR-6 can separate from one another inappropriately. The different surfaces of early embryonic cells show contrasting patterns of adhesion with neighboring cells. For example, the lateral surfaces of cells (which contact adjacent cells) are very adherent, whereas the inner surfaces of cells (which face the interior of the embryo) separate from one another to form a small centrally positioned blastocoel cavity (Nance and Priess, 2002). PAR-6 localizes to the outer surfaces of early
embryonic cells (Hung and Kemphues, 1999; Nance and Priess, 2002) and is required to prevent lateral surfaces from forming blastocoeel-like separations (Nance et al., 2003).

Despite these analogies, the mechanism by which PAR-6 regulates adhesion in early embryonic cells and epithelial cells probably differs. Adhesion between epithelial cells is mediated in part by apical junctions. For example, removing both DLG-1 and HMP-1/α-catenin causes epithelial cells to separate from one another (Segbert et al., 2004). Because in par-6(M/Z) embryos apical DLG-1 and HMP-1 are fragmented rather than belt-like, it is likely that the adhesion defects we observed are caused by defective junctions. By contrast, early embryonic cells do not form apical junctions (Costa et al., 1998; Nance and Priess, 2002). Adherens junction proteins such as HMR-1/E-cadherin and HMP-1/α-catenin are instead found at all sites of cell-cell contact, are not needed for cell adhesion, and do not require PAR-6 to localize (Costa et al., 1998; Nance et al., 2003) (our unpublished observations). PAR-6 regulates early embryonic cell adhesion and epithelial cell adhesion independently, as embryos lacking PAR-6 in early embryonic cells but expressing PAR-6 in epithelial cells do not develop fragmented junctions or epithelial cell adhesion defects (Nance et al., 2003).

Continuous apical junctions never form in par-6(M/Z) embryos, so why do epithelial cells only show adhesion defects at later stages after elongation has begun? One possibility is that apical junctions become more defective over time. Although this is difficult to exclude, we observed that the fragmented organization of DLG-1GFP in par-6(M/Z) epithelial cells does not worsen in timelapse movies as long as 2 hours (our unpublished observations). Instead, we propose that junctions are not needed to hold cells together during the sheet-like epithelial cell movements of epidermal enclosure, but are required to resist tension known to develop during elongation (Priess and Hirsh, 1986). Studies of morphogenetic movements in other organisms indicate that epithelial cells do not always require intact junctions to adhere or migrate. For example, sheet-like movements of zebrafish myocardial precursor cells can still occur when junctions are disrupted by mutation of the pck-3 homolog heart and soul (prkci) (Rohr et al., 2005).

PAR-6, PKC-3 and epithelial polarity

Our results show that full-length PAR-6 and asymmetrically localized PKC-3 are not needed for embryonic epithelial cells to polarize. For example, we observed that apical cytoskeletal markers (microtubules, ACT-5, IFB-2), apical junction proteins (PAR-3, HMR-1, HMP-1, DLG-1 and AJM-1), and the basolateral protein LET-413 all localize asymmetrically in par-6(M/Z) embryos. In principle, the ability of par-6(M/Z) epithelial cells to polarize could be explained by incomplete removal of PAR-6 or PAR-6GFP. Several observations suggest that this is very unlikely. First, we were unable to detect PAR-6 or PAR-6GFP in epithelial cells of par-6(M/Z) embryos. Second, phenotypes of par-6(M/Z) embryos did not become stronger when we used RNAi to knockdown any hypothetical remaining PAR-6. Third and most convincing, PKC-3 never localized apically in par-6(M/Z) epithelial cells; in Drosophila epithelial cells and C. elegans early embryonic cells, aPKC/PKC-3 apical positioning requires PAR-6 (Hutterer et al., 2004; Nance et al., 2003). As par-6(tm1425) does not remove the complete par-6 coding sequence, we cannot exclude the possibility that par-6(M/Z) embryos express alternative isoforms of PAR-6 not detected by PAR-6 antibodies. However, as tm1425 deletes much of the PKC-3-binding (PB1) domain, any alternative isoforms would not likely bind PKC-3.

The epithelial phenotype of C. elegans par-6(M/Z) embryos contrasts with that of Drosophila blastoderm epithelial cells lacking Par-6. Blastoderm epithelial cells begin to polarize as they form by cellularization, when membranes invaginate to separate nuclei in the syncytial embryo (Lecuit, 2004). In embryos lacking both maternal and zygotic Par-6, the apical protein Patj, the apical junction protein Arm/β-catenin, and Baz/PAR-3 are all mislocalized to lateral surfaces (Hutterer et al., 2004; Petronczki and Knoblich, 2001). The different phenotypes of fly and worm epithelial cells lacking PAR-6 might reflect an evolving role for PAR-6 in epithelial polarity. Alternatively or in addition, PAR-6 might be utilized differently in epithelial cells that form by cellularization (Drosophila blastoderm cells) versus epithelial cells that differentiate from groups of nonpolarized precursors (C. elegans embryonic cells). Cultured mammalian epithelial cells such as MDCK cells also form from nonpolarized precursor cells, and Par6 regulates tight junction formation in these cells (see Introduction). However, because cell lines lacking all Par6 activity have not been described, it is not yet known if mammalian Par6 also controls other epithelial asymmetries.

If PAR-6 is not required to polarize C. elegans epithelial cells, which pathways are utilized? One possibility is that C. elegans epithelial cells rely on a combination of polarization pathways, and inactivation of any single pathway might not fully block polarization. In Drosophila, PAR proteins function coordinately with other pathways to regulate epithelial polarity (Humbert et al., 2003; Macara, 2004). For example, adherens junction proteins such as E-cadherin, basolateral proteins such as Discs large and Scribble, and apical proteins such as Crumbs function in different pathways that each contribute to epithelial polarization. Homologues of these proteins are not essential for the initial polarization of epithelial cells in C. elegans (Bossinger et al., 2001; Costa et al., 1998; Firestein and Rongo, 2001; Koppen et al., 2001; McMahon et al., 2001), although LET-413/Scribble is required for proper positioning and compaction of junction proteins and for preventing the progressive lateral spread of apical proteins such as PAR-6 (Bossinger et al., 2001; Koppen et al., 2001; Legouis et al., 2000; McMahon et al., 2001). Our double-mutant experiments suggest that simple redundant interactions between PAR-6 and HMR-1, DLG-1, or LET-413 are not required for epithelial cells to polarize. Additionally, in RNAi experiments we failed to detect redundant interactions between PAR-6 and CRB-1/Crumps (our unpublished observations), although we could not verify depletion of CRB-1 because CRB-1 antisera is no longer available (O. Bossinger, personal communication). Despite these observations, it is possible that complex redundant interactions between combinations of these pathways mediate epithelial polarization.

Alternatively, epithelial polarity could be mediated by PAR-3, independently of PAR-6 and PKC-3. Indeed, Baz/PAR-3 acts upstream of aPKC to polarize Drosophila blastoderm epithelial cells and is also required in MDCK cells for tight junction formation (Chen and Macara, 2005; Harris and Peifer, 2005). C. elegans PAR-3 is required for the apical localization of AJM-1 and microfilaments in epithelial cells of the distal spermatheca (Aono et al., 2004), suggesting that it might be essential for polarity in all epithelial cells. Interestingly, Drosophila Baz/PAR-3 localizes to a different apical domain than Par-6 or aPKC/PKC-3 (Harris and Peifer, 2005), and we noted a similar spatial organization of PAR-3, PAR-6, and PKC-3 in C. elegans embryonic epithelial cells.

PAR-6 and apical junctions

PAR-6 appears to have at least partly different functions than LET-413 or DLG-1 in regulating junction biogenesis. Whereas LET-413 is required to properly compact junction proteins such as HMP-1
and to prevent their lateral spread (Koppen et al., 2001; Legouis et al., 2000; McMahon et al., 2001), DLG-1 plays only a minor role in compacting HMP-1 and is not needed for HMP-1 apical localization (Bosinger et al., 2001; Firestein and Rongo, 2001; Koppen et al., 2001; McMahon et al., 2001). par-6(m/z) embryos have a phenotype distinct from that of let-413 mutant embryos and dlg-1(RNAi) embryos. For example, both PAR-6 and LET-413 are required for the integrity of apical junctions, but apical proteins do not spread laterally in par-6(m/z) mutant embryos as they do in let-413 mutant embryos. Additionally, PAR-6 and DLG-1 are each required for proper compaction of HMP-1 in apical junctions, but HMP-1 fragmentation is more severe in par-6(m/z) mutant embryos than that reported for dlg-1(RNAi) embryos (Bosinger et al., 2001; Firestein and Rongo, 2001; Koppen et al., 2001; McMahon et al., 2001). These observations suggest that PAR-6, LET-413 and DLG-1 regulate, at least partially, different aspects of junction biogenesis and maintenance. Because we found that junction proteins show an equivalent lateral spread in let-413(RNAi) embryos and let-413(RNAi) par-6(m/z) double-mutant embryos, the ectopic junctions observed in embryos lacking LET-413 are not caused by ectopic PAR-6 activity at the lateral cortex.

Our imaging experiments on DLG-1\(^{3\text{PP}}\) in par-6(m/z) embryos indicate that PAR-6 is required for the initial condensation of apical junction proteins into belt-like structures. The localization and timing of expression of PAR-6 is consistent with such a role. For example, we observed that levels of PAR-6\(^{3\text{PP}}\) increase at apical surfaces earlier than DLG-1 and AJM-1 and at the same time as HMP-1 and HMR-1. During the early stages of polarization, lateral and apical spots of PAR-6\(^{3\text{PP}}\) showed extensive colocalization with spots of HMR-1 and HMP-1, suggesting that they may be targeted to the apical cortex together via a common mechanism.

**Selective inhibition of maternal expression by par-6 maternal-effect-lethal mutations**

Original alleles of par-6 were identified in maternal-effect lethal screens requiring that homozygous mutant animals survive to adulthood (Watts et al., 1996). Our experiments demonstrate that these par-6 alleles prevent maternal but not zygotic expression of PAR-6. Both zu170 and zu222 are insertions of the Tc1 transposable element into the par-6 3′ UTR and do not alter par-6 coding sequences (Hung and Kemphues, 1999). A Tc1 insertion within sequences 3′ of the pop-1 stop codon also prevents maternal but not zygotic expression of POP-1 (Lin et al., 1995). Tc1 elements are targets of RNAi within the germline (Sijen and Plasterk, 2003), suggesting that maternal mRNAs containing Tc1 elements may be inactivated by RNAi. Thus introducing Tc1 sequences into the UTR of a gene might be a general strategy to selectively eliminate its maternal expression.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/7/1259/DC1


