



PAR-6, but not E-cadherin and β -integrin, is necessary for epithelial polarization in *C. elegans*

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ABSTRACT

Cell polarity is a fundamental characteristic of epithelial cells. Classical cell biological studies have suggested that establishment and orientation of polarized epithelia depend on outside-in cues that derive from interactions with either neighboring cells or the substratum (Akhtar and Streuli, 2013; Chen and Zhang, 2013; Chung and Andrew, 2008; McNeill et al., 1990; Nejsum and Nelson, 2007; Nelson et al., 2013; Ojakian and Schwimmer, 1994; Wang et al., 1990; Yu et al., 2005). This paradigm has been challenged by examples of epithelia generated in the absence of molecules that mediate cell-cell or cell-matrix interactions, notably E-cadherin and integrins (Baas et al., 2004; Choi et al., 2013; Costa et al., 1998; Harris and Peifer, 2004; Raich et al., 1999; Roote and Zusman, 1995; Vestweber et al., 1985; Williams and Waterston, 1994; Wu et al., 2009). Here we explore an alternative hypothesis, that cadherins and integrins function redundantly to substitute for one another during epithelium formation (Martinez-Rico et al., 2010; Ojakian et al., 2001; Rudkouskaya et al., 2014; Weber et al., 2011). We use *C. elegans*, which possesses a single E-cadherin (Costa et al., 1998; Hardin et al., 2013; Tepass, 1999) and a single β -integrin (Gettner et al., 1995; Lee et al., 2001), and analyze the arcade cells, which generate an epithelium late in embryogenesis (Portereiko and Mango, 2001; Portereiko et al., 2004), after most maternal factors are depleted. Loss of E-cadherin^{HMR-1} in combination with β -integrin^{PAT-3} had no impact on the onset or formation of the arcade cell epithelium, nor the epidermis or digestive tract. Moreover, β -integrin^{PAT-3} was not enriched at the basal surface of the arcades, and the candidate PAT-3 binding partner β -laminin^{LAM-1} was not detected until after arcade cell polarity was established and exhibited no obvious polarity defect when mutated. Instead, the polarity protein par-6 (Chen and Zhang, 2013; Watts et al., 1996) was required to polarize the arcade cells, and par-6 mutants exhibited mislocalized or absent apical and junctional proteins. We conclude that the arcade cell epithelium polarizes by a PAR-6-mediated pathway that is independent of E-cadherin, β -integrin and β -laminin.

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Introduction

Work over the last three decades revealed the importance of E-cadherin and β -integrin to maintain the polarized organization of epithelial cells (Chen and Zhang, 2013; Nelson et al., 2013), but has not addressed whether these molecules are also critical to establish polarity, given that some epithelia appear to form in their absence (e.g. (Baas et al., 2004; Costa et al., 1998; Harris and Peifer, 2004; Raich et al., 1999; Roote and Zusman, 1995; Vestweber et al., 1985; Williams and Waterston, 1994; Wu et al., 2009)). Here we explore the roles of cadherin and integrin in the nematode *C. elegans*. Wild-type *C. elegans* epithelia resemble those from other organisms, with an apical membrane that faces a lumen, a

basolateral membrane that abuts a basement membrane, and an apical junctional domain that separates the two (Fig. 1A) (Nelson et al., 2013). The relative apical-basolateral distribution of ezrin/radixin/moesin^{ERM-1}, PAR-3, PAR-6, the cell adhesion receptor E-cadherin^{HMR-1}, Discs Large^{DLG-1}, the ECM receptor β -integrin^{PAT-3} and laminins is conserved between *C. elegans* and other animals (Fig. 1A) (Hohenester and Yurchenco, 2013; Kramer, 2005; Labouesse, 2006; Macara, 2004).

C. elegans embryos comprise two major epithelial organs – the epidermis, and the digestive tract (www.wormatlas.org) – neither of which requires either E-cadherin or β -integrin for their establishment (Costa et al., 1998; Raich et al., 1999; Williams and Waterston, 1994). Loss of E-cadherin^{HMR-1} leads to depletion of its binding partner catenins (α -catenin^{HMP-1}, β -catenin^{HMP-2}, and p120^{ICL-1}), as well as ZO1^{ZOO-1} and claudin^{VAB-9} from the apical junction (Costa et al., 1998; Lockwood et al., 2008; Simske et al., 2003), similar to vertebrates (Gumbiner et al., 1988; Herrenknecht et al., 1991). However, other apical and junctional proteins localize

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normally, including the apical marker ERM-1 (ezrin/radixin/moesin homolog) (Van Furden et al., 2004) and the junctional marker AJM-1 (Costa et al., 1998; Lockwood et al., 2008; Raich et al., 1999). β -integrin^{PAT-3} has mainly been studied in the context of focal adhesion formation and cell invasion, and a role in epithelial polarity has not been observed (Hagedorn et al., 2009; Moerman and Williams, 2006). Integrin's binding partner, β -laminin^{LAM-1}, is required to orient the pharynx properly, but all epithelia polarize (Rasmussen et al., 2012). Thus, factors that are often considered critical to generate polarity are dispensable in *C. elegans*. Despite generating epithelia, mutants in the sole E-cadherin gene *hmr-1*, the sole β -integrin gene *pat-3* or the sole β -laminin homolog *lam-1* each die during embryogenesis, revealing the importance of these genes for *C. elegans* development (Costa et al., 1998; Grana et al., 2010; Rasmussen et al., 2012; Williams and Waterston, 1994).

Studies with vertebrates have suggested that E-cadherin and integrins can function redundantly via adhesive crosstalk (reviewed in (Weber et al., 2011)). Outside-in signals from either the lamina or junctions could provide orienting cues. For example, several shared downstream effectors of cadherins and integrins, such as RhoGTPases (e.g. Rho, Rac), play a role in epithelial polarity (O'Brien et al., 2001; Playford et al., 2008; Shewan et al., 2005; Yeaman et al., 1999; Yu et al., 2005), raising the possibility that in the absence of one pathway the other could still regulate these proteins. We chose to examine the integrin and E-cadherin pathways in *C. elegans*, focusing on the nine arcade cells that link the foregut to the rostral epidermis (Portereiko and Mango, 2001; Portereiko et al., 2004) (Fig. 1B). E-cadherin^{HMR-1} and β -integrin^{PAT-3} were ideal candidates for double-mutant analysis because antibodies for each factor exist (Gettner et al., 1995; Zaidel-Bar et al., 2010), enabling us to monitor the degree of depletion for each protein.

Results and discussion

Inactivation of *hmr-1/cadherin* and *pat-3/integrin*

Homozygous mutant embryos from heterozygous mothers were stained for HMR-1. This control indicated that residual E-cadherin^{HMR-1} protein remained but failed to accumulate at the plasma membrane of *hmr-1* mutant embryos prior to the onset of epithelial polarization or at cell-cell junctions of mature epithelia (Figs. 3,S1–S3). These data suggested that the maternal endowment of wild-type protein had been depleted and the remaining protein was zygotically-derived mutant protein that lacked HMR-1 activity. We performed two experiments to ensure that the residual HMR-1 was non-functional. First, we showed that α -catenin^{HMP-1}, which is an HMR-1 target, was not localized properly in *hmr-1* mutants (Fig. S1). Normally, HMP-1 requires HMR-1 to accumulate at the apical junction, but remains cytoplasmic when *hmr-1* activity is lost (Costa et al., 1998). Second, we compared the phenotype of *hmr-1* mutants with that of embryos lacking maternal and zygotic HMR-1 as a result of RNA interference (RNAi) against *hmr-1* in *hmr-1; ergo-1* mutants (Fig. S2). *ergo-1* mutations were used because they enhanced RNAi (Yigit et al., 2006) without altering the *hmr-1* phenotype (Fig. S1). Both *hmr-1* (*zu389*) and *hmr-1*(*zu389*); *ergo-1*; *hmr-1*(RNAi) exhibited the expected Hmr phenotype, with a failure of embryonic elongation and gaps visible between different organs such as a gap between the epidermis and the foregut/arcade cells (Figs. 1,S2,S3) (Costa et al., 1998; Rasmussen et al., 2013). These results confirmed previous studies that showed mutants lacking both maternal and zygotic HMR-1 had no polarity defects (Raich et al., 1999), and that all epithelia were generated (Bossinger et al., 2001; Costa et al., 1998; Firestein and Rongo, 2001; Raich et al., 1999; Segbert et al., 2004;

Simske et al., 2003; Totong et al., 2007; Van Furden et al., 2004). We note that like *hmr-1*(*zu389*), embryos homozygous for the deletion allele *hmr-1*(*tm5601*) also localize DLG-1 appropriately to junctions (Fig. S1). We conclude that cortical polarity in epithelia is normal in *hmr-1*/cadherin mutants.

The strongest epithelial defects associated with HMR-1 that we observed were within the intestinal cytoplasm. As the intestinal epithelium is formed, the cells undergo cytoplasmic polarization (Leung et al., 1999) in which intestinal nuclei migrate to the nascent apical surface. This process occurred normally in *hmr-1* (*zu389*) mutants; however, in some late stage Hmr embryos we noted that the nuclei failed to remain tightly associated with the apical surface (Fig. 1). These data suggest that E-cadherin^{HMR-1} is dispensable for the initiation of cytoplasmic polarity but necessary, in part, for maintenance. Taken together, these experiments suggest that *hmr-1*(*zu389*) is a very strong loss of function allele. Nevertheless, we recognize that neither *zu389* nor *tm5601* may be null.

Next we examined PAT-3. In *pat-3* single mutants, residual β -integrin^{PAT-3} signal was detectable at the time of epithelial polarization due to a stable maternal contribution (Rasmussen et al., 2012) (Fig. S1). Therefore, we used RNAi to deplete *pat-3* and ensure PAT-3 protein was absent before the onset of epithelial polarization. This approach generated the expected Paralyzed at Twofold (Pat) phenotype (Fig. S1) (Williams and Waterston, 1994). RNAi against *pat-3* and *hmr-1* together failed to deplete either PAT-3 or HMR-1 completely (data not shown). Consequently, we relied on RNAi against *pat-3* combined with *hmr-1; ergo-1* to inactivate both cadherin and integrin.

Cadherin-Integrin double mutant analysis reveals no redundant roles in polarity

We analyzed the arcade cells of *hmr-1; ergo-1; pat-3*(RNAi) mutant embryos (hereafter referred to as *hmr-1; pat-3* mutants) for defects in the localization of apical and junctional markers. The PDZ-containing PAR-6 polarity protein ($n > 20$), ERM-1 ($n > 20$) and F-actin ($n = 20$, visualized by phalloidin) are enriched at the apical membranes of worm epithelia (Gobel et al., 2004; Leung et al., 1999; McMahon et al., 2001; Nance et al., 2003; Priess and Hirsh, 1986; Van Furden et al., 2004), and these factors localized normally in single and multi-mutant arcade cells (Figs. 1,S1,S3). Junctional proteins Discs Large^{DLG-1} ($n > 50$) and AJM-1/coiled-coil protein ($n > 30$) (Bossinger et al., 2001; Firestein and Rongo, 2001; Francis and Waterston, 1991; Koppen et al., 2001; McMahon et al., 2001; Podbilewicz and White, 1994) were restricted to the apical-basolateral boundary in mutant embryos similar to the wild type (Fig. 1, Fig. S3). Interestingly, we did not observe a break in the connection between the epidermis and the arcade-cell epithelia of many (Fig. 1F,J; Fig. S3K,M) *hmr-1; pat-3* embryos, unlike *hmr-1* single mutants (Fig. 1H,L,P; Fig. S3H). One possible explanation is that *pat-3* mutations, which lead to paralysis (Williams and Waterston, 1994), reduce the pulling forces within the embryo and thereby leave the epidermis-arcade cell attachment intact when both *hmr-1* and *pat-3* are inactivated.

To confirm that junctional staining at the anterior of the embryo represented arcade cells, we stained *hmr-1* and *hmr-1; pat-3*(RNAi) embryos with antibodies against PHA-4/FoxA (the forkhead transcription factor that defines the foregut fate (Horner et al., 1998; Kaltenbach et al., 2005; Mango et al., 1994)), to position the arcade cells, and with DLG-1 antibodies to label junctions. This staining indicated that the location of the arcade cells and accumulation of DLG-1 were normal in *hmr-1* (Fig. 1S) and *hmr-1; pat-3*(RNAi) mutant embryos (Fig. 1T). Similarly, the distribution of ERM-1, DLG-1 and basement membrane marker α -laminin^{LAM-3} were appropriately localized in mature epithelia of

the digestive tract (pharynx and intestine) and the epidermis (Fig. S3). We conclude that E-cadherin^{HMR-1} and β -integrin^{PAT-3} are dispensable to generate the polarized cortex of epithelia in *C. elegans*. Instead, we observed mis-localized nuclei, particularly in the mature intestine of some *hmr-1* mutants, which was not suppressed by *pat-3*. In addition, we detected weak linkages between organs in *hmr-1* mutants (e.g. epidermis to foregut and

foregut to intestine), and this phenotype was partially suppressed by loss of *pat-3*.

The arcade cells polarize before the laminin-enriched basement membrane is visible

As a complementary experiment, we examined whether a presumed ligand for β -integrin^{PAT-3}, namely β -laminin^{LAM-1}, was required for arcade cell polarity. It was recently reported that β -laminin^{LAM-1} is necessary to orient the foregut epithelium and coordinate organ architecture (Rasmussen et al., 2012). To examine the role of laminin^{LAM-1}, we first examined the timing and localization of α -laminin^{LAM-3} and β -laminin^{LAM-1} during arcade cell polarization. Normally, the arcade cells polarize at mid-embryogenesis (1.25fold stage), when they form a linear array between the outer epidermis and the foregut (Fig. 1B). As development proceeds, the arcade cells move posteriorly and shrink their apical surfaces dramatically (Fig. 1B).

Just prior to the time of arcade cell epithelialization (comma stage), no α -laminin^{LAM-3} or GFP-tagged β -laminin^{LAM-1} was detected at the presumptive arcade cell basement membrane, although the polarized foregut primordium was already surrounded by basement membrane (our positive control for staining) (Fig. 2A, Fig. S4A). LAM-1 and LAM-3 remained undetectable after the arcade cells first displayed polarized localization of the junctional marker DLG-1 (1.25fold stage) (Fig. 2B, Fig. S4B). In addition, the integrin PAT-3 was not enriched in the basal surface of the arcade cells after polarization (Fig. S4D). By the time the basement membrane had extended into the anterior of the embryo (2-fold stage), the arcade cell bodies had relocated away from their initial position and were positioned outside the enclosure, while their apical surfaces had contracted to form two small rings between the epidermis and the pharynx (Figs. 1B, 2C, S4C). These data indicate that the arcade cells become polarized prior to laminin accumulation in nascent basement membranes.

To test whether laminins are required for arcade cell polarity, we analyzed *lam-1(ok312)* mutants, which were previously reported to have pharynges with a bifurcated lumen (Rasmussen

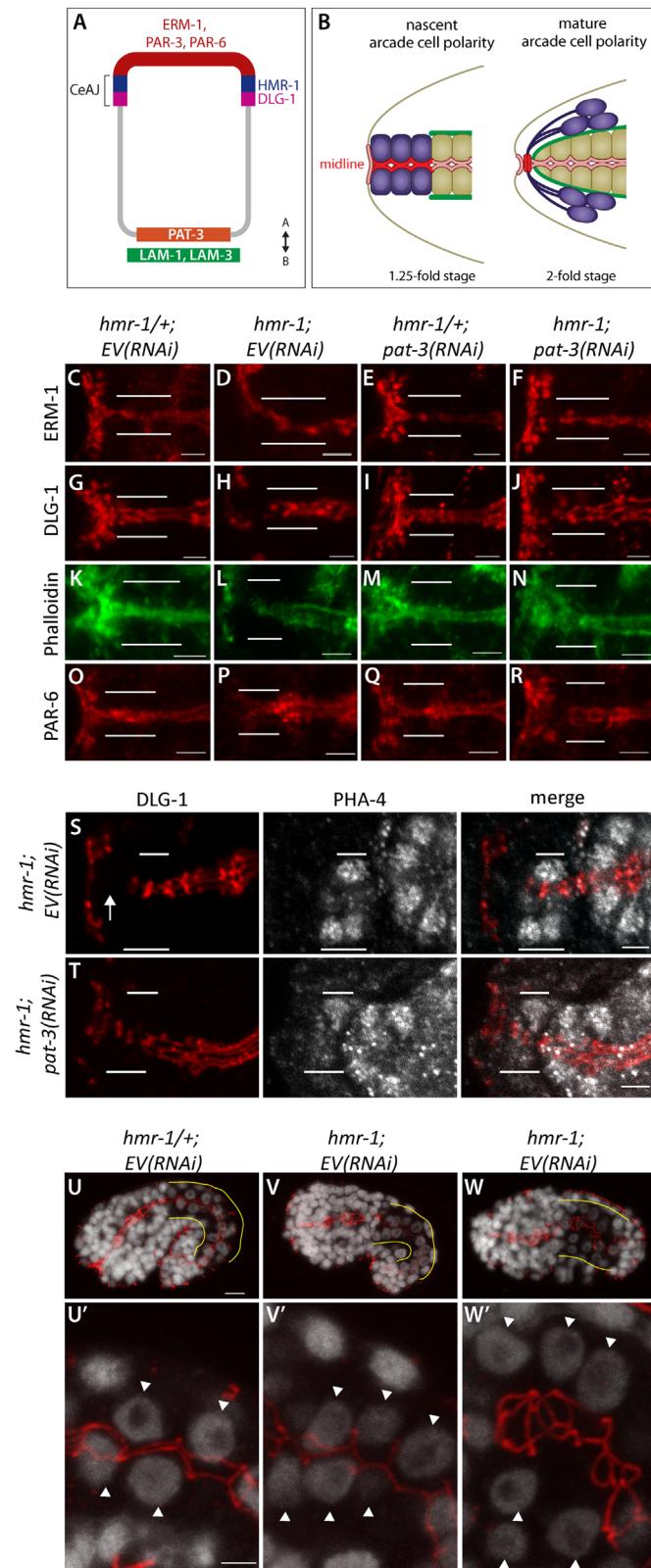


Fig. 1. E-cadherin and β integrin are dispensable for epithelial polarity of arcade cells. A. A generic *C. elegans* epithelial cell showing the apical (A, top) to basal (B, bottom) localization of polarized proteins used in this study. ERM-1, PAR-3 and PAR-6 localize to the apical domain. The *C. elegans* apical junction (CeAJ) is composed of two domains, the more apical with HMR-1/E-cadherin and basal with DLG-1/Discs large. PAT-3/ β -integrin localizes basally to contact the basement membrane, which contains LAM-1/ β -laminin and LAM-3/ α -laminin. B. When arcade cells first polarize, they form three rows of three, with apical surfaces (red) at the midline (two rows are drawn). As the epithelium matures, the arcade cell bodies migrate posteriorly, and their apical surfaces constrict. Pink lines denote epidermal and pharyngeal junctions, green line is the pharyngeal basement membrane. C-R. Maximum intensity projections through the arcade cell epithelium. The arcade cells are denoted by white lines. C-F. ERM-1 is enriched at the apical surface of the arcade cells. G-J. DLG-1 is localized to the junctional domain in arcade cells. K-N. F-actin, as visualized by Phalloidin-Alexa488, is apically enriched in arcade cells. O-R. PAR-6 is properly localized at the apical-junctional interface of the arcade cells. S-T. PHA-4 (white) marks the arcade cells (white lines) and other foregut cells. DLG-1 (red) is localized to the junctional domain of the arcades in both *hmr-1* (S) and *hmr-1; pat-3(RNAi)* (T) mutants. Note that in the *hmr-1* mutant embryo in K the arcades have detached from the epidermis (arrow). U-W. Nuclei are labeled with DAPI (white) and junctions are marked by DLG-1 (red). Yellow lines outline the intestinal epithelium. U'-W'. Close-up of the anterior intestine from U-W. Arrowheads point to intestinal nuclei. U, U'. Intestinal nuclei are tightly associated with the apical surface in *hmr-1*/+ embryos, as shown by the proximity of the nuclei to DLG-1 stained apical junctions. V, V'. Early *hmr-1* mutant embryo, where the intestinal nuclei have migrated as normal. W, W'. Late *hmr-1* mutant embryo, where the intestinal nuclei have drifted away from the apical surface. Note the large gaps between the nuclei and DLG-1-marked junctions. Scale bar is 2 μ m (except in U-W, where it is 5 μ m). Anterior is to the left. N > 20 embryos each stain. See also Figs. S2, S3.

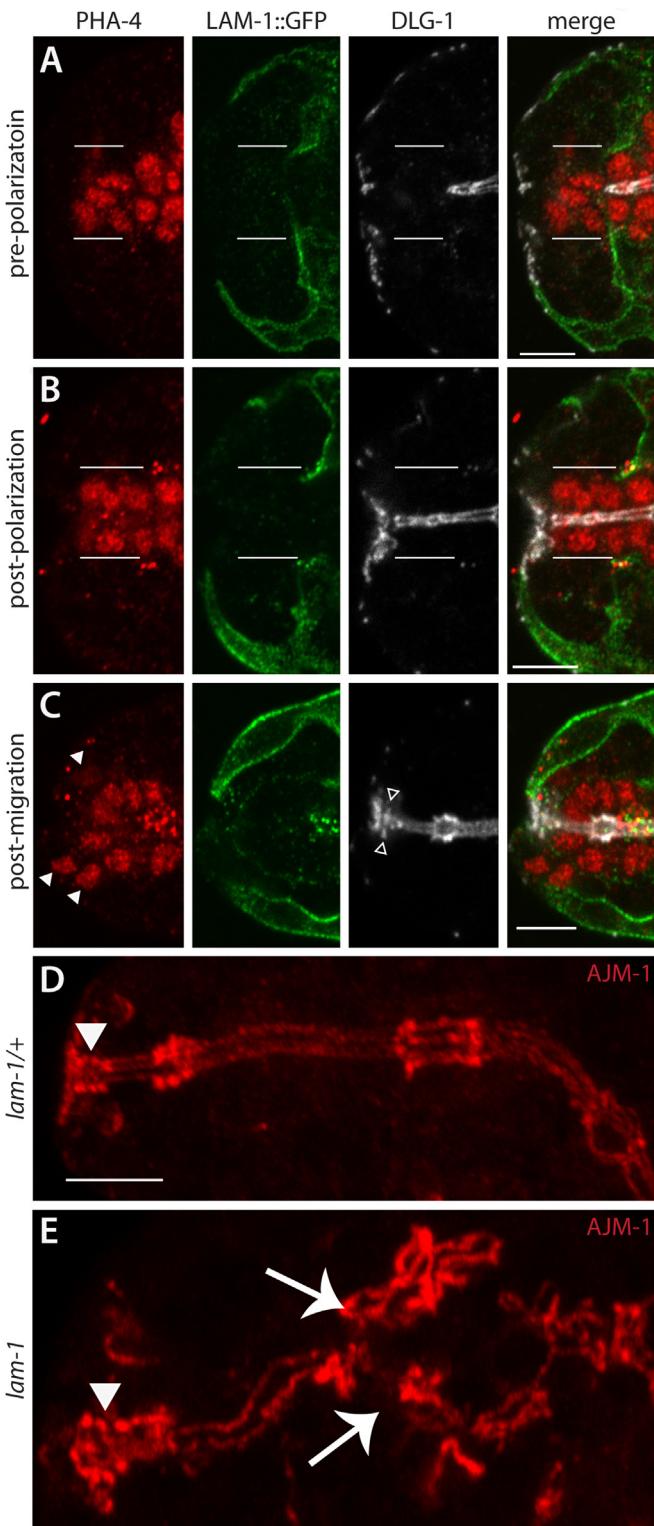


Fig. 2. Arcade cells do not contact laminin in the wild type and polarize normally in laminin mutants. A-C. PHA-4/FoxA (red) is expressed in arcade cells (white brackets) and other foregut cells. LAM-1: GFP (green) is expressed in the developing basement membrane. DLG-1 (white) localizes junctionally in polarized arcade cells. A, B. The arcade cells do not contact LAM-1: GFP prior to polarization (A) or after (B). Compare B to Fig. 1B, left. C. The arcade cells (closed arrowheads) migrate posteriorly and shrink their apical surfaces (open arrowheads). Compare to Fig. 1B, right. D-E. Junctional AJM-1 (red, arrowhead) in arcade cells of *lam-1*/+ control embryos (D) and *lam-1* mutants (E). Note the bifurcated pharynx (arrows) in *lam-1*. Anterior to the left, scale bar is 2 μ m (A-C) or 5 μ m (D-E). $N \geq 15$ embryos each stain. See also Fig. S4.

We detected the arcade cells and foregut cells using antibodies against PHA-4/FoxA (Horner et al., 1998; Kaltenbach et al., 2005; Mango et al., 1994). Antibodies against the junctional proteins AJM-1, DLG-1, and HMR-1 were used to determine if the arcade cells were polarized in *lam-1* mutants and to monitor the dual lumen phenotype (our positive control for *lam-1* mutations). Our results indicated that > 90% embryos with dual-lumen foreguts exhibited unambiguous enrichment of AJM-1 ($n > 30$), DLG-1 ($n > 90$) and HMR-1 ($n = 90$) at the apical junction of the arcade cells (Fig. 2E). We also confirmed previously published results that these markers localized normally to the single lumen of the intestine and to the cell junctions of the epidermis (Rasmussen et al., 2012) (Fig. S4). Taken together, these data reveal that the nascent arcade cells do not contact the predicted β -integrin^{PAT-3} ligands (α - and β -laminin), do not have detectable levels of β -integrin^{PAT-3} at the basal surface, and do not require *lam-1* activity to generate a polarized epithelium with the proper apicobasal orientation.

The initiation of epithelial polarity is intact in cadherin-integrin double mutants

We next considered whether the timing of epithelial polarization was abnormal or delayed in *hmr-1*; *pat-3*(RNAi) mutants. The arcade cell epithelium forms extremely quickly, in less than ten minutes (Portereiko and Mango, 2001). Therefore, we chose to focus on the well-described initiation of polarity of the intestine and epidermis, which occurs more slowly and allows intermediate stages to be visualized (Achilleos et al., 2010; Leung et al., 1999; Podbilewicz and White, 1994; Totong et al., 2007).

Normally, the epidermis forms 'spot' junctions of DLG-1 and AJM-1 at the late 8E stage (when there are eight Endodermal intestinal precursor cells) (Chisholm and Hardin, 2005; Podbilewicz and White, 1994). These junctions coalesce to form circumferential belts at the beginning of the 16E stage. At these stages, *hmr-1* mutants do not exhibit any morphological defect. Therefore, we used antibody staining against HMR-1 to identify mutant embryos. Mutants have reduced HMR-1 expression and the remaining signal is not localized to the cell membrane but appears punctate in the cytoplasm. We examined *hmr-1*; *pat-3*(RNAi) embryos ($n > 20$) at the 8E stage and saw spot junctions form at the appropriate time (Fig. 3). In the wild type, the intestinal precursor cells initiate polarization at the 16E stage, with clusters of polarity proteins at the nascent apical surface that mature into continuous bands (Leung et al., 1999; Totong et al., 2007). PAR-3 is one of the earliest polarity markers of the *C. elegans* intestinal precursors and is important for other polarity markers to coalesce at the apical junctional compartment (Achilleos et al., 2010). We assayed PAR-3 in *hmr-1*; *pat-3*(RNAi) mutant embryos at the 16E stage ($n > 20$) and observed foci followed by unbroken bands of apical domains, as in wild-type embryos (Fig. 3). We observed similar results for the apical markers ERM-1 ($n > 20$) and actin/phalloidin ($n = 15$) (Fig. S1). Taken together, these data suggest that the canonical outside-in signaling molecules E-cadherin and β -integrin are not redundantly required to initiate polarity for *C. elegans* epithelial cells.

Arcade cell polarization depends on PAR-6

Given that the arcade cells polarize without β -integrin or E-cadherin, we wondered what molecules are important for arcade cell epithelialization. The PAR polarity proteins were appealing candidates, given their importance for polarizing many different cell types in metazoans (Nance and Zallen, 2011). For example, in MDCK cells, the PDZ protein *par-6* is necessary for proper tight junction assembly and functions with aPKC to

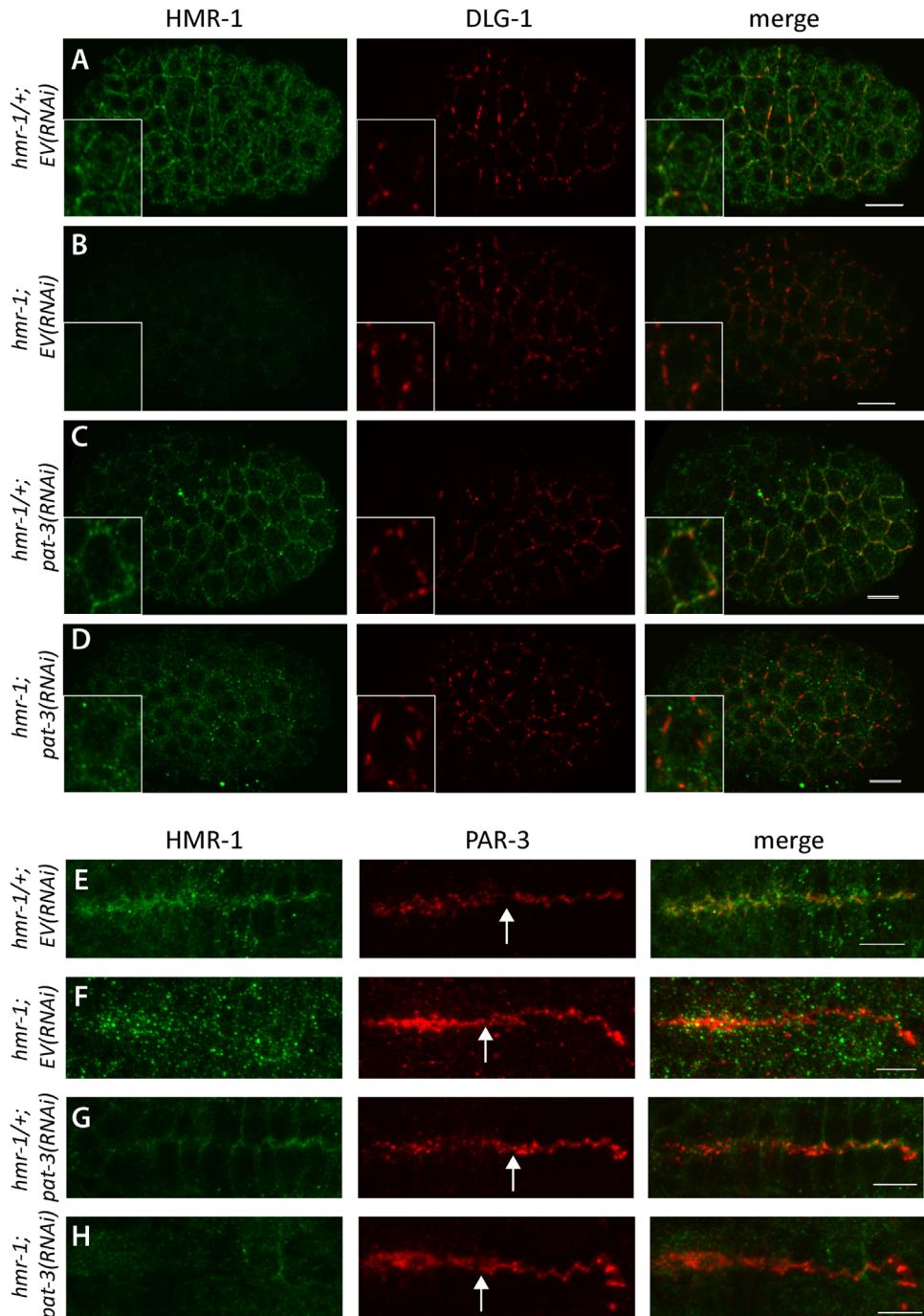


Fig. 3. Initiation of epithelial polarity is normal in E-cadherin/ β -integron single and double mutants. A-K. HMR-1 (green) distinguishes *hmr-1*⁺ from *hmr-1* mutants. A-D. Maximum intensity projections of dorsal focal planes showing polarizing epidermal cells. DLG-1 (red) is localized in discrete foci along the polarizing epidermal membrane. Insets show a single epidermal cell. E-K. Maximum intensity projections of central focal planes with polarizing foregut and intestine, which are to the left and right, respectively, of the arrow that denotes their junction. PAR-3 (red) is enriched at the midline, which is the nascent apical domain. Anterior to the left, scale bar is 5 μ m. N > 50 embryos each stain. See also Fig. S1.

maintain the apical compartment (Chen and Zhang, 2013). In *C. elegans*, maternal PAR-6 is required to polarize the one-cell embryo, but loss of maternal *par-6* produces disorganized embryos that have not been analyzed for arcade cell polarity. Totong and colleagues (2007) developed an elegant strategy to bypass the early role and focus on later phenotypes. Using a modified PAR-6 protein, maternally-deposited PAR-6 is available to fulfill the early functions of *par-6*, but is degraded shortly afterwards, thereby allowing discovery of later activities (called *par-6(MZ)*) (Totong et al., 2007). We elected to examine *par-6(MZ)* mutant embryos for

arcade cell polarity defects, looking both at the time the arcade epithelium is established and at slightly later stages.

The apical marker ERM-1 was strongly affected, as ERM-1 was targeted to the cortex of mutant arcade cells but was not apically enriched in 19/23 embryos (Fig. 4B). In the remainder (4/23), we observed apically-enriched ERM-1 with gaps between neighboring epithelial cells (Fig. 4C). HMR-1 and AJM-1 failed to localize to the junctional domain in ~50% of *par-6(MZ)* mutant arcade cells. AJM-1 was strongly affected, as 16/30 embryos had no detectable AJM-1 in mutant arcade cells (Fig. 4H). In the remainder (14/30), AJM-1

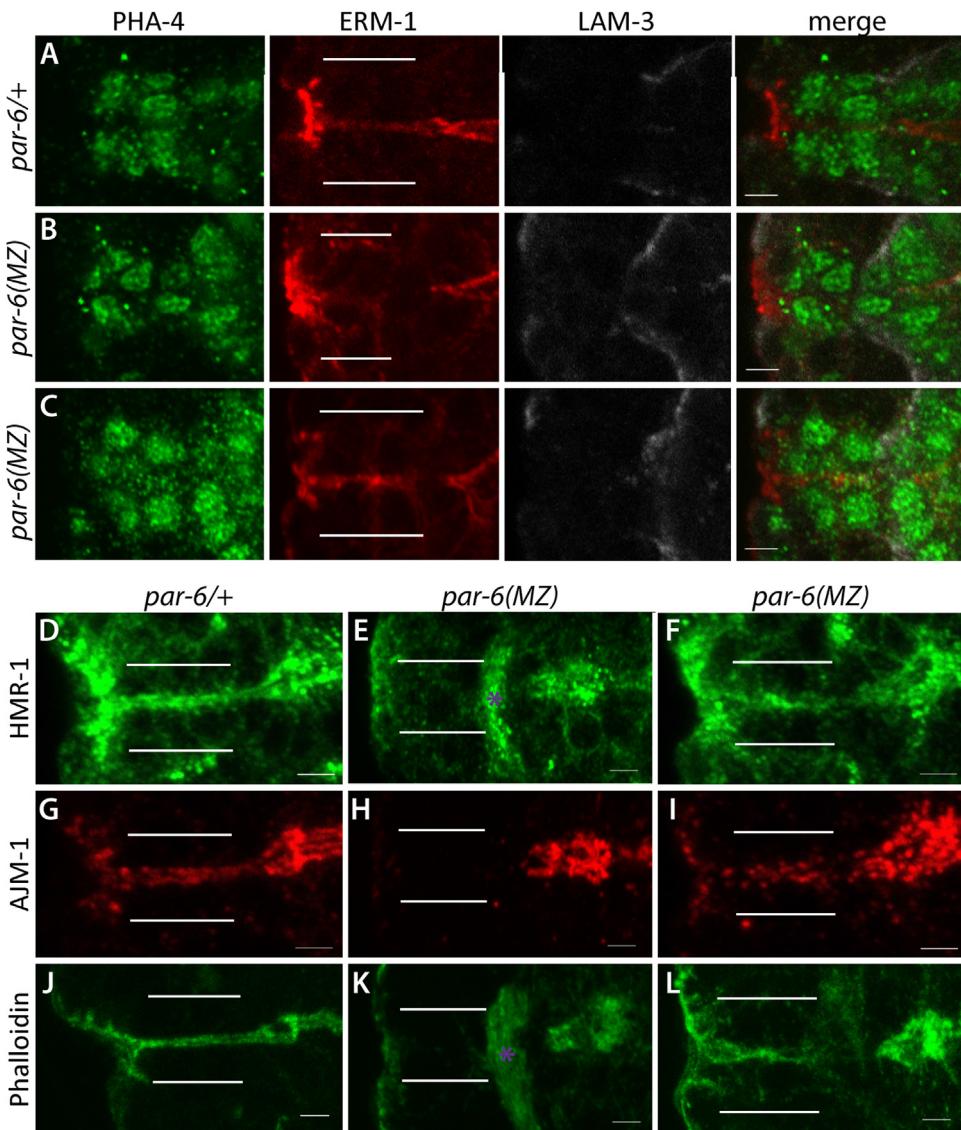


Fig. 4. PAR-6 promotes arcade cell polarization. A-C. PHA-4/FoxA (green) denotes the position of the arcade cells and adjacent foregut cells, which are enclosed by LAM-3⁺ basement membrane (white). A. In control sibling embryos, ERM-1 (red) is enriched at the apical membrane in arcade cells. B. In the majority of *par-6*(*MZ*) mutants, ERM-1 is not apically enriched in arcade cells. C. A minority of *par-6*(*MZ*) mutant embryos display ERM-1 at arcade cell apical membranes. D-I. Junctional markers HMR-1 (D-F) and AJM-1 (G-I) are enriched at arcade cell junctions in control sibling embryos (D, G). In ~50% of *par-6*(*MZ*) mutants, both markers are absent from arcade cell junctions (E, H). In the other half, both markers are enriched in the arcade cell junctions (F, I), but AJM-1 is fragmented (I). J-L. Actin, as visualized by phalloidin, localizes to the apical surface of arcade cells in control embryos (J). In half of the analyzed *par-6*(*MZ*) mutant embryos actin failed to enrich in the arcades (K) while in the other half apical actin was observed (L) but was discontinuous with neighboring epithelia. White brackets denote arcade cell positions. Anterior to the left, scale bars 2 μm. Purple asterisks in E and K denote HMR-1 and actin enrichment in the nerve ring (main neuropil in *C. elegans*). See also Fig. S5.

was apically enriched in the arcades but failed to coalesce into mature junctions (Fig. 4I, Fig. S5). HMR-1 failed to enrich at the junctional domain in 4/9 mutant embryos (Fig. 4E). In the remainder (5/9), HMR-1 was localized to junctions, and the junctional staining appeared more normal than that of AJM-1 (Fig. 4F). For example, we observed a more continuous band for HMR-1 in one embryo that also exhibited fragmented AJM-1 (Fig. 4F vs I). These data resemble what was observed previously in *par-6*(*MZ*) mutant intestines, where HMR-1, unlike AJM-1, lacked breaks between cells (Totong et al., 2007). Finally, we analyzed the polarized enrichment of actin using Phalloidin. In 5/9 embryos, phalloidin was not prominent at the apical surface of arcade cells (Fig. 4K), while in the rest (4/9) we detected apical enrichment (Fig. 4L). We note that the *par-6*(*MZ*) phenotype was stronger for the arcade cells than that of the epidermis, intestine or foregut where AJM-1 and ERM-1 are apically enriched (Totong et al., 2007) (Fig. S5). The weaker phenotype for these organs may

indicate a reduced need for PAR-6 compared to the arcade cells, or higher levels of residual PAR-6 in *par-6*(*MZ*) mutants.

The incomplete expressivity of *par-6*(*MZ*) mutants for the arcade cells may reflect a partial requirement for PAR-6 during wild-type epithelialization, or lingering PAR-6 protein in *par-6*(*MZ*) embryos. Supporting the latter hypothesis, mutant embryos with no arcade cell junctions lacked PAR-6 protein in these cells, whereas *par-6*(*MZ*) embryos with puncta of junctional proteins exhibited low levels of PAR-6 (Fig. S5). We note that the *par-6*(*MZ*) configuration is predicted to affect the full-length PAR-6 protein (PAR-6L), but not a shorter isoform generated by alternative splicing (PAR-6S) (Fig. S5). PAR-6S is expected to have reduced or altered function compared to PAR-6L, as it lacks the PB1 domain, which binds PKC-3/aPKC (Lin et al., 2000; Suzuki et al., 2001) and is deleted by the *tm1425* loss-of-function mutation (Totong et al., 2007). In sum, although some *par-6*(*MZ*) embryos contain residual or truncated PAR-6 protein, the complete absence of apical or

junctional polarity proteins in the majority of *par-6(MZ)* mutant arcade cells indicates that PAR-6 is a key player for arcade cell polarization.

Concluding remarks

Our findings demonstrate that the *C. elegans* arcade cells generate an epithelium independent of E-cadherin and β -integrin, and instead require *par-6*. In *hmr-1(zu389); pat-3(RNAi)* mutants, the arcade cells display polarized localization of all tested apical and junctional markers. While there is residual mutant HMR-1 protein in the double mutant, our data indicate this protein is non-functional because neither mutant HMR-1 protein (Figs. 3, S1–S3) nor its binding partner α -catenin (Fig. S1) are enriched at junctions. In addition, we showed that further depletion of maternal and mutant, zygotic HMR-1 via RNAi in *hmr-1* mutants does not produce a polarity defect (Fig. S2).

Loss of *par-6* activity completely abolished polarization of the arcade cells in a majority of mutant embryos, suggesting that *par-6* plays an important role in this process (Figs. 4, S5). As noted above, this is a stronger phenotype than what is seen in other worm epithelia. Why is PAR-6 so important for the arcades cells? We speculate that perhaps because the arcades polarize more rapidly than other epithelia additional proteins may be needed to ensure timely delivery/enrichment of polarity proteins at the apical surface, rather than assign this importance to a single factor (e.g. PAR-3 in the intestine (Achilleos et al., 2010)). In agreement with this idea, we note that the *par-6(MZ)* phenotype is incompletely penetrant, suggesting other factors function in parallel to PAR-6.

In other species, PAR-6 has important roles in epithelial polarity. In *Drosophila*, PAR-6 is necessary to maintain the first epithelium of the embryo (Harris and Peifer, 2005; Hutterer et al., 2004). In mutants lacking both maternal and zygotic *par-6*, polarity markers fail to accumulate appropriately along the apical and lateral surfaces (Hutterer et al., 2004). In older *Drosophila* embryos, epithelia lose their normal columnar appearance, and a partially penetrant polarity defect is observed in neuroepithelial-derived dividing neuroblasts (Petronczki and Knoblich, 2001). In vertebrates, Par6 is a key regulator of tight junction formation (Alarcon, 2010; Gao et al., 2002; Joberty et al., 2000; Yamanaka et al., 2001) and apical identity (Hayase et al., 2013). Our work demonstrates that PAR-6 also regulates epithelial polarity in *C. elegans* and lies upstream of HMR-1/E-cadherin in the arcade cells. In the future, we would like to examine the role of PAR-3 in arcade cell epithelium formation, as it is a key regulator of polarity in many contexts (Nance and Zallen, 2011).

Our study also revealed that E-cadherin^{HMR-1} and β -integrin^{PAT-3} are not required individually or redundantly to polarize *C. elegans* arcade cells. In the *hmr-1/cadherin-pat-3/integrin* double mutant, we observed normal localization of all tested apical and junctional markers, such as ERM-1 and DLG-1 (Fig. 1), in the arcade cell epithelium. Proper localization of polarity markers was also observed for the digestive tract and epidermis, both at the early stages of polarity and in fully mature epithelia (Figs. 2, S3).

The independence from cadherins and integrins is not limited to *C. elegans* epithelia, and there is growing evidence that these factors are not essential to polarize at least some epithelia in other organisms. During mammalian kidney organogenesis, for example, the metanephric mesenchyme (MM) undergoes a mesenchymal-to-epithelial transition (MET) in the presence of E-cadherin blocking antibodies (Vestweber et al., 1985) and in cells without the downstream cadherin target p120-catenin (Marciano et al., 2011). Other studies have shown that conditional inactivation of E-cadherin does not affect polarization of the liver epithelium

(Battle et al., 2006) or thyroid follicular epithelium (Cali et al., 2007), as evidenced by junction formation. In *Drosophila*, epithelial cells polarize in the absence of a functional cadherin pathway. In mutants that affect the cadherin pathway (e.g. *armadillo*/ β -catenin mutant), for example, polarized epithelial cells are formed, although they are not entirely normal due to an expanded apical domain with Bazooka/PAR-3 targeted to the base of the apical surface (Harris and Peifer, 2004). In another *Drosophila* tissue, the follicular epithelium, the cadherin-catenin system was not required for establishing polarity but was important for its maintenance (Tanentzapf et al., 2000). Instead of polarization, E-cadherin is necessary for proper tissue integrity (similar to *C. elegans* (Costa et al., 1998)) and architecture, to orient the mitotic spindle, to function as a mechanotransducer and to activate downstream signaling events (den Elzen et al., 2009; Heisenberg and Bellaiche, 2013; Letizia and Llimargas, 2012; Morais-de-Sa and Sunkel, 2013; Priya et al., 2013; Shamir et al., 2014; Stephenson et al., 2010; Twiss and de Rooij, 2013; Weber et al., 2011).

Integrins can also be dispensable for polarization and apical membrane specification. For example, $\beta 1$ -integrin mutant kidneys contain tubules with normal polarity (Wu et al., 2009). *In vitro*, loss of integrin signaling during cyst formation results in cysts that lack a lumen, but with apical markers that are still discreetly localized suggesting these cells are polarized (Akhtar and Streuli, 2013; Yu et al., 2005; Bryant et al., 2014). The *Drosophila* midgut has a normal columnar appearance in integrin mutants (Tepass, 1997), similar to E-cadherin mutants (Tepass and Hartenstein, 1994). Finally, single intestinal cells cultured in suspension (i.e. without cell-cell or cell-matrix contacts) can polarize if the LKB1/Par4 polarity kinase is activated (Baas et al., 2004). Instead, a critical role of the $\beta 1$ -integrin/laminin pathway is to orient the apicobasal axis properly, similar to the role for *C. elegans lam-1* (Rasmussen et al., 2012), and to promote signaling pathways (Bryant et al., 2014; Akhtar and Streuli, 2013; Martinez-Rico et al., 2010; Ojakian et al., 2001; Ojakian and Schwimmer, 1994; Roote and Zusman, 1995; Weber et al., 2011; Wu et al., 2009; Yu et al., 2005). Together the data point to an E-cadherin- or integrin-independent polarization pathway for epithelia in multiple animals.

Prior studies did not address whether E-cadherin and integrins could function redundantly to establish epithelia. Moreover, interpretation of the phenotypes in other species was often complicated either by the existence of multiple cadherin and integrin genes, which could function redundantly. Our analysis took advantage of the sole E-cadherin and β -integrin genes in *C. elegans*, both of which were strongly inactivated. Our results show that epithelia can polarize independent of these canonical signals. It is likely that other “outside-in” signaling factors will be important for initiating polarity in contexts where E-cadherin and β -integrin are dispensable. We suggest that the arcade cell epithelium is an ideal system in which to uncover these additional factors. In fact, the arcade system has found a novel regulator of polarity, the mitotic kinesin ZEN-4. In *zen-4* mutants, the arcade epithelium fails to form (Portereiko et al., 2004). Future studies with the arcade system should continue to reveal players in cadherin-integrin independent epithelium formation.

Experimental procedures

Strains and maintenance

Nematodes were grown at 20 °C using standard conditions (Brenner, 1974). Some strains were provided by the CGC. Strains used: JJ1079 [*hmr-1(zu389)/lin-11(n566)* *unc-75(e950)* I] (Costa et al., 1998) (*hmr-1(zu389)* contains a premature stop codon

located prior to the transmembrane domain, generating a truncated protein that fails to localize to cell membranes (Broadbent and Pettitt, 2002), (Figs. 3, S1–S3)); JJ1743 [*par-6(tm1425)/hln1 [unc-54(h1040)] I; him-8(e1489) IV*]; FT36 [*unc-101(m1) par-6 (zu170)I; zuls43 [pie-1: GFP; PAR-6: ZF1+unc-119(+)]*] (Totong et al., 2007); RW3600 [*pat-3(st564)/qcC III*] (Williams and Waterston, 1994); SM1052 [*zen-4 (px47) dpy-20 (e1282)/bli-6(sc16) unc-24 (e138) IV*] (Portereiko et al., 2004); WM158 [*ergo-1(tm1860) V*] (Yigit et al., 2006); NK248 [*qyls10 [lam-1p: lam-1: GFP+unc-119 (+)]*] (Ziel et al., 2009).

Strains generated in this study: SM2238 [*pat-3(st564)/qcC dpy-19 (e1259) glp-1 (q339) qls26 (lag-2: GFP) III; ergo-1(tm1860) V*]; SM2269 [*hmr-1(zu389)/hln1 [unc-54(h1040)] I; ergo-1(tm1860) V*]; SM2342 [*pat-3(st564)/qcC dpy-19 (e1259) glp-1 (q339) qls26 (lag-2: GFP) III*]; SM2377 [*lam-1(ok3221)IV/nt1[qls51] IV/V*]; SM2472 [*hmr-1(zu389)/lin-11(n566) unc-75(e950) I; pxEx618 (HMP-1: GFP; rol-6 (d); salmon testes DNA)*]; SM2475 [*hmr-1(tm5601)/hln1 [unc-54 (h1040)] I; ergo-1 (tm1860) V*].

par-6(MZ) genetics

To generate *par-6(MZ)* mutants the following mating was performed, modified from (Totong et al., 2007): *par-6(tm1425)/hln1[unc-54(h1040)]; him-8(e1489)* males were mated with *unc-101(m1) par-6(zu170); zuls43* hermaphrodites. Non-Unc F1 L4 progeny were picked and incubated overnight. The following day the non-Unc F1 worms were transferred to a new plate and allowed to lay eggs for 6–8 h. Embryos were harvested and processed for immunostaining, as outlined below. *par-6(MZ)* mutant embryos were identified by having gaps in the pharyngeal/intestinal apical junctional domain and by immature junctions in the epidermis, as reported (Totong et al., 2007) (Fig. S5).

Molecular biology

pat-3 RNAi: A 1067 bp fragment of *pat-3* coding sequence (exons 3 and 4 with intervening intron) was amplified from genomic DNA using the sjj_ZK1058.2 primer pair (www.wormbase.org), modified with attB1 and attB2 Gateway sites. The resulting PCR product was used in a Gateway Recombination reaction using pPR244 ((Reddien et al., 2005), kind gift of Alejandro Sanchez Alvarado) as the template and BP Clonase II (Invitrogen) as the enzyme. pPR244 (also known as pDONRdT7) has two T7 polymerase promoters flanked by T7 terminators, to make RNAi more effective (Reddien et al., 2005). The recombinant plasmid was transformed into HT115 cells (Timmons et al., 2001) and used for RNAi by feeding.

Construction of HMP-1: GFP transgenic line

pJS435 (HMP-1: GFP, kind gift of Jeff Simske) was linearized with SpeI (New England Biolabs) and purified using the Qiagen PCR purification kit. 2 ng/ul of linearized pJS435, 55 ng of linearized pRF4 (*rol-6(d)* co-selection marker), and 43 ng/ul of Salmon Testes DNA (Sigma D1626) were mixed together and injected into JJ1079 [*hmr-1(zu389); lin-11(n566) unc-75(e950)*] animals. A single transgenic line (SM2472, see above) was generated and used to analyze HMP-1: GFP localization in *hmr-1(zu389)* mutant embryos.

RNA interference

RNAi was performed as described (Timmons et al., 2001), with minor modifications. A single bacterial colony was picked to grow in 6 ml of Luria broth+antibiotic (50ug/ml carbenicillin for pPD129.36/empty vector or 25ug/ml kanamycin for *pat-3*) for 8 h at 37 °C. Bacteria were pelleted and resuspended in an IPTG/antibiotic mixture (8 mM IPTG, 50ug/ml carb or 25ug/ml kan),

and 40ul was spread onto a 35 mm NGM (Nematode Growth Media (Stiernagle, 2006)) plate. The plates were covered with foil and incubated at room temperature for 48 h before use. 30–40 L4 animals (either WM158, SM2238, or SM2269) were transferred briefly (10 minutes) to a plate without food to remove residual OP50 bacteria, and then moved to the RNAi plate. Animals were incubated at 25 °C for 24 h, transferred to a new RNAi plate and incubated for an additional 6 h at 25 °C. Laid embryos were collected and either incubated overnight at 25 °C on NGM plates to score terminal phenotypes or used immediately for immunostaining. To ensure that we were observing nascent arcade cell polarity in *hmr-1* or *hmr-1;pat-3(RNAi)* mutants, mothers were dissected after 24 h of feeding RNAi, and 1–4cell embryos were collected. These embryos were aged for 5.5 h at 25 °C, giving a range of 1.25–1.75-fold embryos (for wild-type), suggesting that any *hmr-1* containing embryos that failed to elongate would be similarly aged.

Immunohistochemistry/microscopy/embryo staging

Embryos were placed on poly-L-lysine (Sigma P-8920) coated slides in 50 µl of water, and either fixed in 2% paraformaldehyde in 1X PBS if PHA-4 was included as one of the antibodies (those listed in Figs. 1,3,4, S4) (Kiefer et al., 2007) followed by freeze-crack or immediately processed using the freeze-crack method (Duerr, 2006). Following cracking, slides were incubated in ice-cold MeOH for either 3 min (PFA-fixed) or 8 min, or fixed in buffered paraformaldehyde [4% paraformaldehyde, 0.2% Triton X-100 (Sigma), 50 mM PIPES pH 6.8, 25 mM HEPES pH 6.8, 2 mM MgCl₂] (for phalloidin staining, modified from (Costa et al., 1997)), then rehydrated/permeabilized by three 5 m washes in TBS or two 5 m washes in TBS (for phalloidin staining). Slides were blocked in TNB Buffer (100 mM Tris-HCl pH 7.5, 200 mM NaCl, 1% BSA) with 10% normal goat serum (NGS; VWR #102643–580). All antibodies were also diluted using TNB+NGS. The following antibodies were used: rabbit anti-HMR-1 (1:1000, gift of Jeff Hardin, University of Wisconsin – Madison) (Zaidel-Bar et al., 2010); rabbit anti-DLG-1 (1:400, gift of Olaf Bossinger, RWTH Aachen University) (Segbert et al., 2004); rabbit anti-PHA-4 (1:400) (Kaltenbach et al., 2005); rabbit anti-PAR-6 (1:1000, gift of Tony Hyman, Max Planck Institute) (Hoeg et al., 2010); MH25 (detects PAT-3, DSHB) (1:10) (Francis and Waterston, 1985; Gettner et al., 1995); MH27 (detects AJM-1, 1:100, gift of Bob Waterston, University of Washington) (Francis and Waterston, 1991); mouse anti-ERM-1 (1:10, DSHB) (Hadwiger et al., 2010); mouse anti-PSD95 (detect DLG-1; Pierce MA1-045; 1:250) (Firestein and Rongo, 2001); P4A1 (detects PAR-3, 1:10, DSHB) (Nance et al., 2003); chicken anti-LAM-3 (1:100, gift of Bill Wadsworth, Rutgers University) (Huang et al., 2003), and chicken anti-GFP (1:200, Millipore # AB16901). As noted, some antibodies were obtained from the Developmental Studies Hybridoma Bank (DSHB) developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. Secondary antibodies were obtained from Invitrogen and conjugated to Alexa488, Alexa568 or Alexa647. Phalloidin conjugated to Alexa488 (Invitrogen A12379) was diluted 20-fold and incubated on slides at RT for 1–2 h. Embryos were scored for polarity phenotypes on either an AxioImagerM2 compound microscope with an HXP lightsource (Zeiss) or LSM780 confocal microscope (Zeiss). Images were obtained on a Zeiss LSM700 or LSM780 confocal microscope, images were exported from ZEN Blue (Zeiss), and maximum projections generated in Image J (NIH).

Embryos were staged based on the number of endodermal (e.g. intestinal) precursors (Leung et al., 1999) or by the shape of the embryo (Chisholm and Hardin, 2005). The epidermis begins to polarize during the 8E stage, around 230–250 min after first

cleavage (Chisholm and Hardin, 2005; Podbilewicz and White, 1994). The intestine begins to polarize during the beginning of the 16E stage, around 270 min after first cleavage (Achilleos et al., 2010; Chisholm and Hardin, 2005; Leung et al., 1999). The arcade cells initiate polarization at the late comma stage (when the epidermis has begun to constrict to shape the embryo into a worm, and thus the embryo resembles a comma), around 395 min after first cleavage (Chisholm and Hardin, 2005; Portereiko and Mango, 2001). The 1.25fold stage, at which time the arcades are fully polarized, occurs ~420 min after first cleavage (Chisholm and Hardin, 2005). The 2-fold stage, which is the terminal stage for *pat-3* arrest, is reached ~450 min after first cleavage (Chisholm and Hardin, 2005). Timing at 20 °C, based on (Sulston et al., 1983).

Terminal polarity was assessed by enrichment of markers at apical/junctional domains. The arcade cells were scored as positive if any marker was seen anterior to the basement membrane-contained pharynx, even if the pharynx appeared detached from the epidermis (e.g. Fig. 1H,L,P,S).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ydbio.2015.03.002>.

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