

# ZEN-4/MKLP1 Is Required to Polarize the Foregut Epithelium

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## Summary

**Background:** Epithelial tubes are a key component of organs and are generated from cells with distinct apico-basolateral polarity. Here, we describe a novel function during tubulogenesis for ZEN-4, the *Caenorhabditis elegans* ortholog of mitotic kinesin-like protein 1 (MKLP1), and CYK-4, which contains a RhoGAP (GTPase-activating protein) domain. Previous studies revealed that these proteins comprise centralspindlin (a complex that functions during mitosis to bundle microtubules), construct the spindle midzone, and complete cytokinesis.

**Results:** Our analyses demonstrate that ZEN-4/MKLP1 functions postmitotically to establish the foregut epithelium. Mutants that lack ZEN-4/MKLP1 express polarity markers but fail to target these proteins appropriately to the cell cortex. Affected proteins include PAR-3/Bazooka and PKC-3/atypical protein kinase C at the apical membrane domain, and HMR-1/cadherin and AJM-1 within *C. elegans* apical junctions (CeAJ). Microtubules and actin are disorganized in *zen-4* mutants compared to the wild-type.

**Conclusion:** We suggest that ZEN-4/MKLP1 and CYK-4/RhoGAP regulate an early step in epithelial polarization that is required to establish the apical domain and CeAJ.

## Introduction

Epithelial tubes are a fundamental building block of organs and promote the efficient delivery of substances in and out of tissues. Epithelial tubes can arise by remodeling of preexisting epithelia, as occurs during branching morphogenesis of the lung [1, 2]. Epithelial tubes are also constructed by groups of cells induced to form epithelia de novo. For example, the nephrons of the kidney are generated by metanephric mesenchyme that undergo an epithelial transition in response to extracellular cues [3]. During epithelium formation, cells polarize and establish an apical domain, which faces the lumen of the tube, and a basolateral domain, which contacts neighboring cells and the extracellular matrix. In vertebrates, these membrane compartments are separated by two junctions, each composed of distinct protein components. For example, E-cadherin mediates homophilic adhesion and is localized within adherens junctions, while the PAR complex is a component of tight

junctions, which prevent mixing of apical and basolateral components. The apical cortex is associated with actin microfilaments as well as an antiparallel network of microtubules [4, 5]. Antiparallel microtubules flank the basal surface while a parallel array of microtubules exists along the longitudinal axis, with their minus ends oriented toward the apical surface [6, 7]. Our current challenge is to determine how these polarized domains are established and to understand the interplay between molecules located at the cell cortex and those within the cytoplasm. Here, we explore these issues for the *C. elegans* foregut, which is an example of a simple linear tube formed by de novo epithelialization [8–10].

The nematode digestive tract is an unbranched epithelial tube composed of a single layer of cells. Between the foregut (or pharynx) and the external epidermis lie nine cells, the arcade cells, organized into two epithelial rings [8, 11]. The arcade cell epithelium serves a critical purpose to link the digestive tract to the epidermis; without it, the anterior digestive tract develops into a sac confined to the interior of the animal [10].

The arcade cell epithelium is formed during midembryogenesis after the embryonic cell divisions are complete, and the epidermis and pharynx have become epithelialized. The cellular behaviors of the arcade cells raise the question of what mechanisms induce them to form an epithelial tube. In many other animals, epithelialization is initiated by cell-cell contact through cadherin activation. Cell contact seems to be an unlikely cue for the arcade cells since their position does not change before or during epithelialization. Consistent with this idea, proteins normally required for cell contact-induced epithelialization are not necessary to establish the arcade cell epithelium. These include E-cadherin/*hmr-1*,  $\alpha$ -catenin/*hmp-1*, the  $\beta$ -catenins *hmp-2* and *bar-1*, and the p120 homolog *jac-1* [12–22]. In this regard, the arcade cells resemble the kidney mesenchyme, for which single and double combinations of cadherin mutations fail to disrupt formation of epithelial nephrons [3, 23–25]. One explanation for the absence of dramatic kidney phenotypes associated with cadherin mutations is redundancy: multiple cadherins are expressed in the developing kidney, and these may substitute for each other. However, an intriguing alternative possibility raised by the *C. elegans* studies is that additional pathways may contribute toward tube formation during organ development.

*C. elegans* epithelia resemble those of other organisms with apical and basolateral domains [26]. LET-413/Scribble is localized to the basolateral domain, where it restricts the spread of components of adherens junction and the apical domain [27–29], while the PAR complex is confined to the apical domain [30]. A single junction, the CeAJ, separates the apical and basolateral domains and has features of both adherens and tight junctions. For example, the CeAJ contains proteins that mediate adhesion such as HMR-1/cadherin, HMP-1/ $\alpha$ -catenin, HMP-2/ $\beta$ -catenin, and VAB-9/claudin [21, 31]. Located

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slightly more basally but still within the CeAJ are DLG-1/ Discs large and the coiled-coil protein AJM-1 [28, 32].

In this study we demonstrate that ZEN-4 and its partner CYK-4 are critical to form the arcade cell epithelium. Previous studies revealed that ZEN-4 and CYK-4 bundle antiparallel microtubules to establish the spindle midzone and that they are required to complete cytokinesis [33–39]. Our findings indicate that these proteins have an additional function during pharyngeal tubulogenesis that is independent of their role in cell division. We suggest that ZEN-4 and CYK-4 function upstream of the membrane-associated polarity proteins to build the apical domain and CeAJ.

## Results

### Mitotic Kinesin-like Protein *zen-4* and RhoGAP *cyk-4* Have a Role in Pharynx Attachment

To discover proteins required for gut tubulogenesis, we performed a genetic screen for arrested larvae with pharynges unattached to the epidermis (Pun phenotype; our unpublished data). Here, we focus on one gene identified by this screen and defined by the allele *px47*. In *px47* mutants, the arcade cell epithelium that normally bridges the pharynx and epidermis was absent, leading to arrested Pun larvae and late stage embryos (Figures 1A–1D).

Three lines of evidence demonstrate that *px47* is an allele of *zen-4*/MKLP1. First, *px47* mapped near the *zen-4* locus and failed to complement *zen-4(w35)* (Figures 1E and 1F). Second, the lethality associated with *px47* homozygotes could be rescued by a 7 kb DNA fragment sufficient for *zen-4* rescue in other studies (six of six transgenic lines; Experimental Procedures; [36, 40]). Third, we identified a G-to-A transition at the splice acceptor site of the third exon of *zen-4* in *px47* mutants. This mutation is predicted to inactivate the natural splice site (Figure S1). RT-PCR analysis has shown that there is only 30.7% of *zen-4* message in the *px47* mutants as compared to wild-type worms (Figure S2).

Previous studies focused on the mitotic roles of *zen-4* to establish midzone microtubules and complete cytokinesis [35, 36, 41]. Embryos lacking maternal *zen-4* activity arrest at the one-cell stage with multiple nuclei, while embryos lacking zygotic *zen-4* activity fail to complete embryonic enclosure, probably because the normal complement of epidermal cells is missing due to later defects in cytokinesis [36]. The *zen-4(px47)* mutation likely leads to reduced *zen-4* activity but is not null. While a subset of *zen-4(px47)* homozygotes arrested with enclosure defects like the homozygous null mutants, the remainder arrested as first-stage larvae, typically with an unattached pharynx (Figure 1B). We reasoned that the weaker phenotypes associated with *zen-4(px47)* reflected two effects. First, the maternal contribution of wild-type *zen-4* rescued the early embryonic defects, as seen for the null allele *zen-4(w35)* [36]. Second, *zen-4(px47)* was likely a weak loss-of-function mutation since *zen-4(px47)* larvae had reduced levels of *zen-4* RNA (Figure S2). Moreover, the temperature-sensitive, loss-of-function allele *zen-4(or153)* could produce a Pun phenotype virtually identical to *zen-4(px47)* (Table 1).

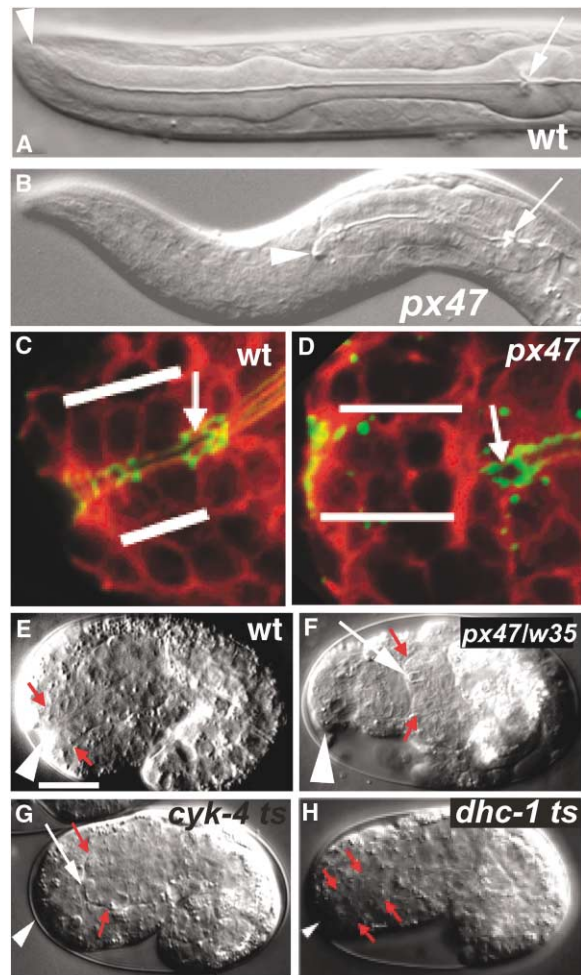


Figure 1. Centralspindlin Components *zen-4*/MKLP1 and *cyk-4*/RhoGAP Are Required for Foregut Tubulogenesis

(A) Differential interference contrast (DIC) image of a wild-type larva with foregut (pharynx) attached to the buccal cavity (arrowhead). (B) DIC image of a pharynx unattached (Pun) mutant where the tip of the pharynx (arrowhead) has failed to reach the buccal cavity. Both wild-type and mutant larvae have hallmarks of well-differentiated, organized pharynges (arrows).

(C and D) Embryos stained with anti-UNC-70/β-spectrin to visualize cell boundaries (red) and anti-GFP to detect AJM-1::GFP in adherens junctions (green). (C) Wild-type arcade cells (between white lines) express AJM-1::GFP, illustrating that they are epithelialized. (D) Mutant *px47* arcade cells (bracket) do not form an epithelium. Arrow denotes pharynx.

(E–G) Embryos at the 1.5-fold stage of development. White arrowheads indicate the buccal cavity. White arrows show the anterior end of Pun pharynges and red arrows highlight the pharynx tissue. (E and F) *px47* is an allele of *zen-4*. (E) Wild-type embryo in which the pharynx and arcade cells (red arrows) are connected to the buccal cavity (arrowhead). (F) *px47/zen-4(w35)* trans-heterozygotes fail to complement and have ventral enclosure and pharynx attachment defects identical to *zen-4(w35)* homozygotes. The anterior edge of pharynx (white arrow) fails to connect to the buccal cavity (arrowhead). (G) Pun phenotype of *cyk-4(t1689ts)* mutant shifted to restrictive temperature at ~225 min of development. The pharynx is outlined in red arrows.

(H) The pharynx is attached in *dhc-1(ct42)* mutants as monitored under the light microscope or by MH27 antibody staining for CeAJs (data not show). Bar = 5 μm. Anterior is left, dorsal is up in all figures.

Table 1. TS Mutations of *zen-4* and *cyk-4* Show Pun Phenotypes

TS mutant		Embryonic		
		Enclosure Defects (n)	Pharynx Unattached (n)	Pharynx Attached (n)
<i>zen-4(or153)</i> 180 min	14	20	0	
<i>zen-4(or153)</i> 240 min	0	14	6	
<i>cyk-4(t1689)</i> 225 min	7	20	0	
<i>cyk-4(t1689)</i> 240 min	0	6	16	

Temperature-sensitive alleles of *zen-4(or153)* or *cyk-4(t1689)* were kept at permissive temperature (15°C and 18°C) for 180, 225, or 240 min, and then shifted to restricted temperature (26°C) for 195 min. Embryo phenotypes were assessed under the microscope.

Wild-type ZEN-4 regulates microtubule dynamics during mitosis in a complex with the RhoGAP protein CYK-4 [38, 39]. CYK-4 promotes the microtubule bundling activity of ZEN-4 in vitro [39], and loss of *cyk-4* leads to incomplete cytokinesis in vivo [38]. We used a temperature-sensitive allele of *cyk-4* to determine whether *cyk-4* was also required to form the arcade cell epithelium (Table 1). 20/27 embryos were Pun, and 7/27 had embryonic enclosure defects when *cyk-4(t1689ts)* embryos were shifted to nonpermissive temperature 225 min after the first embryonic cell division (~8E stage). When *cyk-4(t1689ts)* mutants were shifted to restrictive temperature 15 min later (240 min), we observed no enclosure defects. A subset was still Pun, but most worms were wild-type. We conclude that CYK-4 is required for pharyngeal morphogenesis and likely functions in conjunction with ZEN-4.

### Normal Cytokinesis in *zen-4(px47)* Mutant Embryos

Given the role of *zen-4* during cytokinesis, we examined whether *zen-4(px47)* homozygotes were Pun because arcade cells were absent or because the arcade cells could not form an epithelium. Four observations demonstrate that *zen-4(px47)* mutants produce arcade cells normally and that therefore *zen-4* plays a more direct role during epithelialization.

First, we counted the number of pharyngeal and arcade cells in *zen-4(px47)* mutants by staining Pun embryos with an antibody that recognizes PHA-4, a FoxA transcription factor expressed in the nuclei of pharyngeal and arcade cells [42, 43]. The same number of PHA-4-expressing nuclei were observed in wild-type and mutant embryos (both had  $93 \pm 2$ ,  $n = 4$  embryos each). In *zen-4(px47)* mutants, all nine arcade nuclei could be identified and were located appropriately between pharyngeal and epidermal cells (Figures 2A and 2B, white brackets).

Second, we examined each arcade cell to determine whether it was mononucleate. To test the possibility that nuclear division occurred without cellular division, we stained *zen-4(px47)* mutants with antibodies that recognize UNC-70/ $\beta$ -G spectrin, which is associated with the plasma membrane [44, 45] and PHA-4/FoxA, which is nuclear [43]. Each arcade cell nucleus was confined within a single cell body, demonstrating that the arcade cells were mononucleate in *zen-4(px47)* mutants (Figures 2C and 2D).

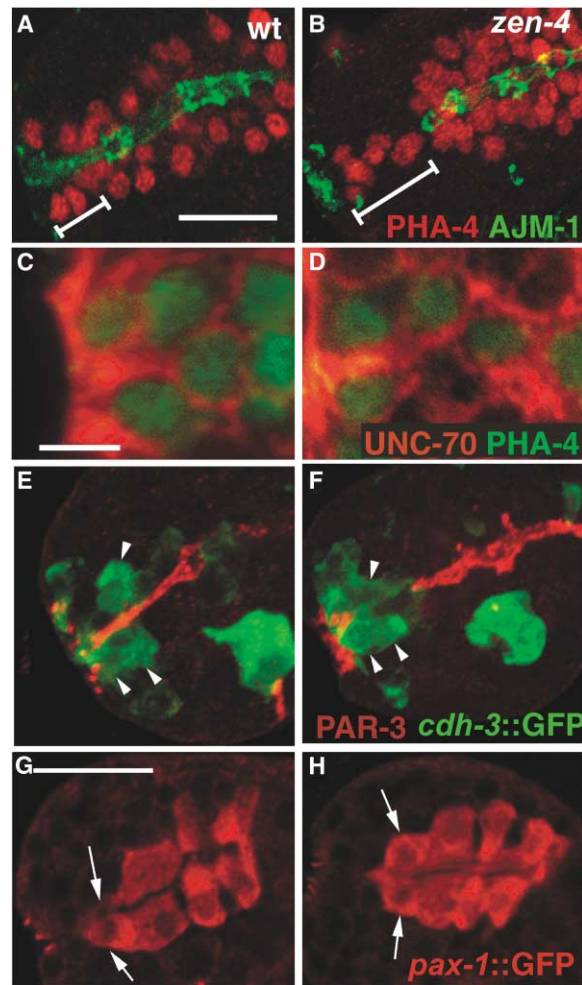
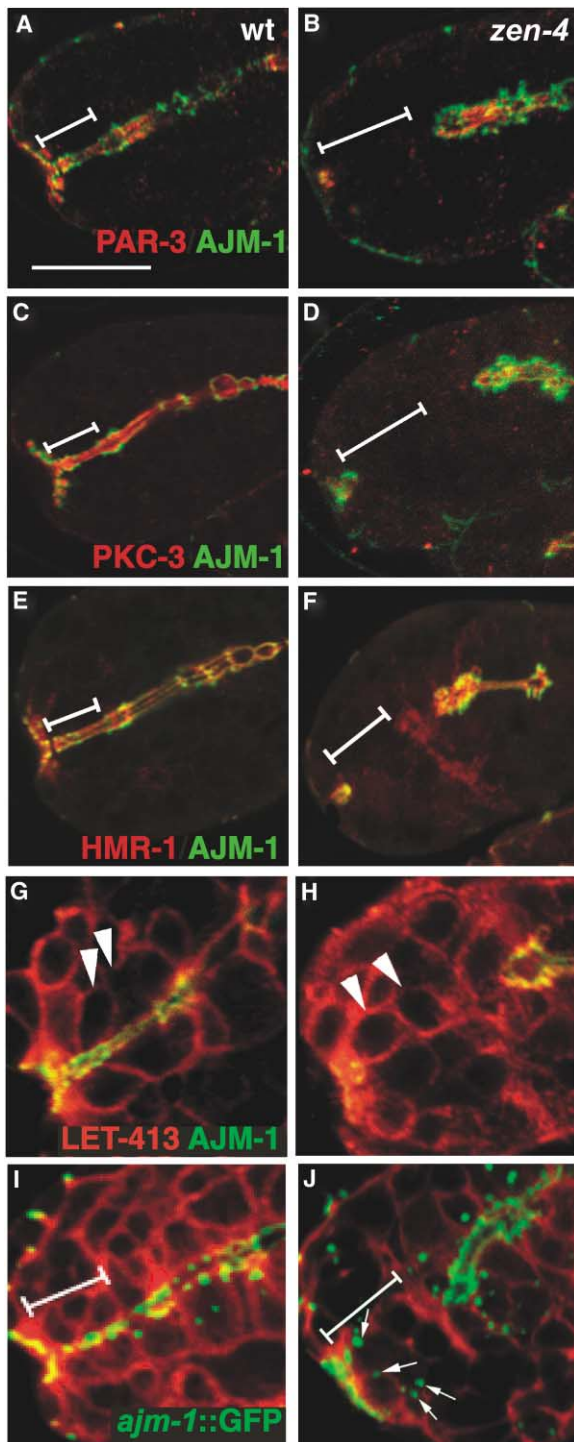


Figure 2. Normal Cytokinesis in *zen-4(px47)* Embryos

(A) wild-type (single focal plane) and (B) *zen-4(px47)* embryos (Z series build) stained for PHA-4 (red) to visualize arcade cells and AJM-1 (green) to distinguish wild-type from mutant embryos. Both had  $93 \pm 2$  PHA-4+ cells, which includes nine arcade cells, identified by position (bracket). (C) Wild-type and (D) *zen-4(px47)*; *pxls*[PHA-4::GFP] embryos stained for UNC-70 (red) and PHA-4::GFP (green) to examine arcade cell boundaries. Each arcade nucleus is confined within a single plasma membrane. Arcade cells (arrowheads) from (E) wild-type and (F) *zen-4(px47)* embryos express *CDH-3::GFP* (green) in arcade cells. Mutants were identified with  $\alpha$ -PAR-3 antibodies (red). (G) wild-type and (H) *zen-4(px47)* embryos express PAX-1::GFP in pharyngeal e2 epithelial cells (red; arrows) and marginal cells. Only two of three e2 cells can be seen in the focal plane for panel H. Bar = 5  $\mu$ m.

Third, we determined whether the cell division that produced the arcade cells occurred normally in mutant embryos by examining cell fate markers for the arcade cells and their sisters. Two arcade cells, posterior arcade DR and DL, have pharyngeal epithelial cells e2DR and e2DL as siblings [9]. In both wild-type and mutant *zen-4(px47)* embryos, a *CDH-3::GFP* reporter was expressed in the arcade cells (Figures 2E and 2F) and a PAX-1::GFP reporter was activated in the e2 pharyngeal epithelial cells (Figures 2G and 2H; [15]). These data indicate that the final cell division that generated the posterior arcade cells and their pharyngeal e2 sisters



**Figure 3. Polarity Defects in *zen-4(px47)* Mutants**  
Mutant embryos identified with AJM-1 (green) staining. (A, C, E, G, and I) Wild-type and (B, D, F, H, and J) *zen-4(px47)* embryos stained with antibodies that recognize polarity markers. The positions of the arcade cells are marked with white brackets. (A and B) apical marker PAR-3 (red) is present in wild-type, but not mutant, arcade cells as is (C and D) apical marker PKC-3/atypical protein kinase C. (E and F) Adherens junction markers AJM-1 (green) and HMR-1/cadherin are also disrupted in *zen-4(px47)* mutants. (G and H) The basolateral marker LET-413 (red) appears uniformly distributed in mutant arcade cells. Arcade cells (arrowheads) are cuboidal in (G) wild-type but round in (H) *zen-4(px47)* mutant embryos. Arcade cells from (I) wild-type and (J) *zen-4(px47)* embryos express AJM-1::GFP (arrows; green) even though the junctional protein is not localized to adherens junctions. UNC-70/ $\beta$ -spectrin (red) demarks cell boundaries. Bar = 5  $\mu$ m.

occurred in *zen-4(px47)* embryos and that these cells differentiated appropriately.

Fourth, we used the conditional allele *zen-4(or153ts)* [41] to demonstrate that the temperature-sensitive periods for cytokinesis and pharyngeal tubulogenesis were distinct (Table 1). *zen-4(or153ts)* embryos were grown at permissive temperature and shifted to restrictive temperature at different stages of embryogenesis. Embryos shifted at 180 min after the first cell division ( $\sim$ 200 cell stage; see [9] for developmental stages) arrested with large, undivided cells and embryonic enclosure defects (14/34 embryos). The remaining embryos (20/34) were Pun, with no obvious cell division defects. Embryos shifted at 240 min after the first cell division ( $\sim$ 8E stage) developed into larvae with a Pun phenotype and without any apparent undivided cells, as monitored under the light microscope (14/20). The remaining embryos (6/20) were phenotypically wild-type. These findings indicate that the cytokinesis and pharyngeal phenotypes were temporally separable.

As a complementary approach, we examined whether we could generate a Pun phenotype by disrupting cytokinesis with a mutation in a different gene. *dhc-1(ct42)* carries a temperature-sensitive mutation in the dynein heavy-chain locus [46]. By shifting to nonpermissive temperature, we could generate large cells with cytokinesis defects and aberrant embryonic enclosure, but were unable to produce a Pun phenotype (Figure 1H). We conclude that the pharyngeal tubulogenesis defects observed in *zen-4(px47)* mutants do not reflect impaired cytokinesis. Rather, our data suggest a new role for *zen-4*, and by implication *cyk-4*, during morphogenesis and distinct from their known activities in cell division.

#### ZEN-4 Is Required to Polarize Arcade Cells

A survey of polarity markers indicates that *zen-4* is essential for arcade cells to form a polarized epithelium. Homozygous *zen-4(px47)* embryos failed to accumulate components of the apical membrane domain including PAR-3/*Bazooka* (Figures 3A and 3B) [32, 47] and PKC-3, an atypical protein kinase C (Figures 3C and 3D) [32, 47]. Adherens junctions were not generated in mutant embryos, judging by the absence of the coiled-coil protein AJM-1 [48, 49] and HMR-1/cadherin [21] at the plasma membrane (Figures 3E and 3F). Whereas arcade cells became columnar and elongated during wild-type epithelium formation [10], these cells remained rounded in *zen-4(px47)* mutants (Figures 1C and 1D and Figures 3G and 3H, arrowheads). We conclude that the arcade cells do not generate an apical membrane domain or adherens junctions in *zen-4(px47)* mutant embryos. Similarly, AJM-1, HMR-1, and PAR-3 were also disrupted in *cyk-4* temperature-sensitive mutants shifted to the restrictive temperature (Figure S3). The absence of polarity markers represents the most severe epithelial phe-

wild-type but round in (H) *zen-4(px47)* mutant embryos. Arcade cells from (I) wild-type and (J) *zen-4(px47)* embryos express AJM-1::GFP (arrows; green) even though the junctional protein is not localized to adherens junctions. UNC-70/ $\beta$ -spectrin (red) demarks cell boundaries. Bar = 5  $\mu$ m.

Table 2. Rescue of *zen-4* Mutants

Pharynx Phenotype	<i>zen-4(px47)</i> (n = 120)	Txg Line 1 (n = 118)	Txg Line 2 (n = 154)
Embryonic enclosure defects	5.8%	6.2%	5.7%
Pharynx unattached	11.8%	2.8%	3.2%
Pharynx attached	6.7%	15%	14.9%
Wild-type	75.7%	76%	76.2%

Hermaphrodites from SM1077 *zen-4(px47) dpy-20(e1282)/bli-6(sc16) unc-24(e138)* with and without the *cdh-3P::zen-4* transgene were permitted to lay embryos on plates for 24 hr. The hermaphrodites were removed and the progeny were assessed for phenotypes 24 hr later. Two independent transgenic lines (lines 1 and 2) were examined.

notype observed in *C. elegans*, demonstrating a critical role for *zen-4* and *cyk-4* during epithelialization.

We examined LET-413/Scribble and UNC-70/ $\beta$ -G spectrin to determine if markers of basolateral polarity were affected by *zen-4(px47)*. Normally, LET-413/Scribble and UNC-70/ $\beta$ -G spectrin are restricted to the basolateral (LET-413) or lateral (UNC-70) surfaces in epithelial cells [27, 45]. These proteins are also localized to the cell cortex of all embryonic blastomeres during early development. In *zen-4(px47)* mutants, both LET-413::GFP and UNC-70 were associated with the plasma membrane and appeared uniformly distributed (Figures 3G and 3H). The expression we observed was consistent with either a distended basolateral domain or a complete absence of polarity in mutant arcade cells. Importantly, these data demonstrated that not all targeting to the cell cortex was disrupted by *zen-4* mutations but that *zen-4* was selective for the apical domain and CeAJ.

Given the absence of a CeAJ or apical domain, we wanted to determine whether junctional proteins were expressed in *zen-4(px47)* mutants. We assessed expression with GFP reporter constructs in live embryos and by immunohistochemistry. These GFP strains allowed us to circumvent possible problems with dilution or degradation of endogenous proteins mislocalized in the cytoplasm, which could preclude their visualization. This analysis revealed that junctional proteins were expressed in mutant embryos as in the wild-type. AJM-1::GFP accumulated in the cytoplasm of mutant arcade cells in small puncta (10/10 embryos; Figures 3I and 3J). Examination of AJM-1::GFP in living embryos indicated that this protein never associated with the plasma membrane, even at the earliest stages of epithelialization. Similarly, we observed expression of a transcriptional reporter for the cadherin *cdh-3* in the arcade cells of ten out of ten *zen-4(px47)* mutant embryos (Figure 2F). Neither AJM-1::GFP nor CDH-3::GFP was observed in nonepithelial cell types such as body wall muscles (zero of eight embryos each), revealing that the presence of GFP in arcade cells reflected their normal epithelial identity. We conclude that arcade cells in *zen-4(px47)* mutants express at least some proteins associated with polarity but that they cannot assemble these proteins into polarized domains at the cell surface.

#### ZEN-4 Functions Postmitotically

We envisioned two models for ZEN-4 and CYK-4 function during cellular polarization. One possibility was that the association of these proteins with midzone microtubules and the cell cortex during mitosis established an

asymmetric mark subsequently used by daughter cells to define the apicobasal axis. For example, after the completion of cytokinesis, ZEN-4 and CYK-4 remain within the division remnant, the region of the cell membrane where the cytokinetic furrow pinches during abscission [36, 38–41]. However, when we examined AIR-2, an aurora kinase localized within division remnants, we found no difference in the appearance or duration of division remnants between wild-type and *zen-4(px47)* homozygous embryos, suggesting this model less likely (data not shown). An alternative model was that ZEN-4 and CYK-4 functioned later, within the arcade cells after their birth. For example, these proteins have known microtubule-organizing activities, suggesting that the configuration of microtubules within arcade cells could be important to localize additional components such as exocytic vesicles or actin.

To distinguish between these models we tested whether we could rescue arcade cell epithelium formation by expressing *zen-4* postmitotically in mutant arcade cells. We placed the *zen-4* gene under control of the *cdh-3* promoter (*cdh-3P::zen-4*), which is active in the arcade cells and some epidermal cells, but not pharyngeal cells [15] (Figure S4). Examination of living embryos expressing CDH-3::GFP [15] revealed that GFP was first visible weakly, in the arcade cells 30 min after these cells were born (Figure S4).

Postmitotic expression of *zen-4* was sufficient for epithelium formation in mutant arcade cells (Table 2). In the parental strain *zen-4(px47)/+*, we observed the expected 25% *zen-4* homozygous lethality and 11.8% Pun. In animals carrying an extragenic array of *cdh-3P::zen-4*, we observed the same degree of lethality but only 2.8% (line 1) or 3.2% (line 2) Pun animals. This difference was statistically significant: line 1,  $p = 0.0057$ , and line 2,  $p = 0.014$ , by Fisher's Exact Test. The *cdh-3P::zen-4* construct was unable to rescue *zen-4(px47)* embryos to adulthood, presumably because *zen-4* has essential roles in cells that do not activate the *cdh-3* promoter. These data support the second model, in which ZEN-4 functions postmitotically to organize the microtubule cytoskeleton during polarization.

#### ZEN-4 Is Required to Organize Cytoskeleton

How might ZEN-4 function within arcade cells? ZEN-4 and its orthologs are required for the normal arrangement of microtubules during mitosis and can form bundles of microtubules in vitro [34, 39, 50, 51]. We examined wild-type and *zen-4(px47)* embryos to determine whether ZEN-4 also influenced microtubule organization

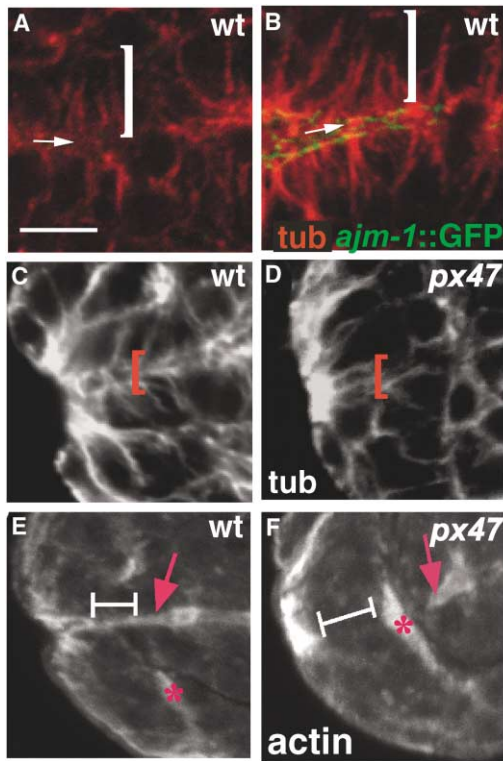


Figure 4. Cytoskeletal Defects in *zen-4(px47)* Mutants

(A) Microtubules (red) are uniformly distributed in pharyngeal cells before epithelialization (same level of staining along the length of the bracket) and (B) become enriched at the apical cortex (arrow) coincident with epithelialization as it can be seen that they are more microtubule staining at the bottom of the bracket (the apical side of the arcade cell) than at the top of the bracket (the basal side of the cell). Epithelialization was monitored by the appearance of AJM-1 (green) in adherens junctions (arrow). (C and D) Apical enrichment of microtubules (bracket) during epithelialization of the arcade cells in (C) wild-type, but not (D) *zen-4(px47)*, embryos. (E and F) Actin concentrated apically (arrow) in (E) wild-type arcade cells but dispersed in (F) *zen-4(px47)* embryos. Actin-rich neuronal processes (asterisks) are not part of the foregut. Actin is present in anterior pharynx of both wild-type and *zen-4(px47)* embryos (arrow). Bar = 2  $\mu$ m.

in epithelia. Prior to epithelialization, as monitored by AJM-1 localization, microtubules were evenly distributed in wild-type cells (30/30; Figure 4A, bracket). During epithelialization, we observed a dramatic increase of microtubules near the nascent apical surface of pharyngeal and midgut cells, coincident with the initial targeting of AJM-1 to adherens junctions (30/30; Figure 4B). In *zen-4(px47)* mutants, the overall level of microtubules remained similar to the wild-type, but they were not concentrated at the erstwhile apical region of the cell (red brackets in Figures 4C and 4D). These data suggest that ZEN-4 is required to establish the proper configuration of microtubules at the nascent apical surface of arcade cells.

*cyk-4* contains a RhoGAP domain and exhibits GAP activity in vitro [38], suggesting that ZEN-4 and CYK-4 regulate RhoGTPase activity and downstream effectors such as actin [52]. We used phalloidin staining to examine the actin filament network in wild-type and *zen-*

*4(px47)* embryos (Figures 4E and 4F, brackets). Whereas wild-type embryos accumulated actin at the nascent apical surface at the onset of epithelialization, actin remained dispersed in the arcade cells of *zen-4(px47)* mutants (Figures 4E and 4F). In addition, the level of stable microfilaments appeared lower in *zen-4(px47)* mutants compared to wild-type. We conclude that *zen-4* is essential to organize the apical microfilament lattice during arcade cell epithelium formation.

## Discussion

Identification of the factors required for cellular polarization is critical to understand how epithelia are formed and molded into tubes. Our genetic analysis has uncovered a new function for the kinesin-like protein ZEN-4/MKLP and its partner CYK-4/RhoGAP to polarize the arcade cells in the nematode foregut. The phenotypes we observe are the strongest known for any *C. elegans* epithelium, indicating this function is essential. We suggest ZEN-4/MKLP1 and CYK-4/RhoGAP regulate an early step in epithelial polarization that is selectively required to establish the apical surface and CeAJ.

## Two Models for *zen-4* and *cyk-4* Function

A surprising discovery is that *zen-4*, and by extension *cyk-4*, are critical for the earliest stages of epithelium formation. Polarity proteins never accumulated at the cell surface of *zen-4* mutant arcade cells, which maintained a round, nonadherent morphology. This phenotype contrasted with those of proteins involved in epithelial maintenance, such as *let-413/scribble*. In epidermal cells lacking *let-413/scribble*, apical and CeAJ-associated proteins are positioned normally at the early stages of epithelium formation but remain along the lateral surface rather than becoming condensed into the junctional region as occurs in wild-type embryos [29]. Visualization of AJM-1::GFP in living *zen-4(px47)* embryos, on the other hand, revealed that AJM-1::GFP was expressed in arcade cells but failed to associate with cell cortex even at the earliest stages examined. Temperature-shift experiments with conditional mutants support a role in initiation rather than maintenance of epithelia. Incubation of *zen-4* or *cyk-4* temperature-sensitive mutants at restrictive temperature for 12 hr or more after arcade cell epithelium formation did not disrupt epithelial integrity. A simple explanation for these results is that these proteins are required at a distinct stage during polarization and once that stage has passed, inactivation has no phenotypic consequences for the arcade cell epithelium. The alternative explanation that ZEN-4 or CYK-4 protein perdures for extended periods of time seems unlikely given the long incubation times at nonpermissive temperature (12–18 hr) and the rapid inactivation of these proteins in other temperature-shift experiments [38, 41].

Two models could explain ZEN-4 and CYK-4's involvement in cellular polarity. One is that ZEN-4 remains at the division remnant or cell membrane after cytokinesis, where it provides a cue to orient the apicobasal axis. Interactions between adherens junctions and the mitotic apparatus have been observed previously. For example,

in *Drosophila* neuroblasts and during embryonic cellularization, adherens junctions position the mitotic spindle in a mechanism that depends on microtubules [53–55]. The converse interaction, namely that remnants of the mitotic apparatus dictate the position of adherens junctions, has not been described, although division remnants have been implicated in other examples of cellular polarity [56]. While intriguing, this marking model seems unlikely because the arcade cell epithelium can form when *zen-4* is provided postmitotically, under control of the *cdh-3* promoter. A potential caveat to this experiment is that low levels of ZEN-4 might accumulate early in *cdh-3P::zen-4* transgenic embryos and be sufficient for mutant rescue, but not for visualization. This scenario seems unlikely, however, since expression from the *cdh-3* promoter initiates long after the division remnants first form and when most are no longer detectable, even when ten times more DNA is used (1 ng/ul for visualization versus 0.1 ng/ul for rescue). Moreover, a comparison of expression from GFP reporters with their cognate, endogenous proteins by antibody staining demonstrates that GFP is an extremely sensitive means to visualize proteins and its expression corresponds well with the time those proteins are expressed and active (e.g., PHA-4 [43, 57], AJM-1 [28]). We conclude that the *cdh-3* promoter is activated after division remnants might be expected to function.

An alternative model for ZEN-4 and CYK-4 is that these proteins act within the arcade cells during epithelialization. This model proposes that the biochemical function of ZEN-4 and CYK-4 during mitosis has been coopted for epithelium formation. Two activities have been described. First, ZEN-4 and CYK-4 form the centralspindlin complex, which bundles microtubules into an antiparallel array of midzone microtubules [58, 59]. This activity implicates microtubule organization as critical for polarization. For more than a decade it has been recognized that epithelia possess a unique constellation of microtubules. This organization has been observed most dramatically in mammalian cells in which microtubules in nonpolarized cells radiate from the centrosome, whereas microtubules in epithelia are organized into three networks that are not centrosomally associated [7]. A parallel array of microtubules lies along the longitudinal axis of the cell, with the minus ends oriented toward the apical surface. Antiparallel arrays of microtubules flank the apical and basal cell surfaces. Intriguingly, these microtubule networks are formed concomitant with the onset of polarity at the plasma membrane, raising the question of whether these networks are important to polarize cells. Our studies suggest that the apical microtubules in the arcade cells may be analogous to the antiparallel microtubule network seen at the apical surface of Madin Darby Canine Kidney (MDCK) [4] and CaCo2 epithelial cells [5]; this configuration may be critical to localize components to the apical surface of the arcade cells. Consistent with this idea, Priess and coworkers previously observed that microtubule inhibitors block foregut tubulogenesis, leading to a Pun phenotype [60].

A second activity for ZEN-4 and CYK-4 during mitosis is to modulate RhoGTPase activity. CYK-4 contains a RhoGAP domain and exhibits GAP activity for *rac*, *rho*,

and *cdc42* in vitro [38]. *Drosophila* homologs of *zen-4* and *cyk-4* interact with the RhoGEF *Pebble*, suggesting this complex also activates RhoGTPases by promoting exchange of GDP for GTP [52, 61, 62]. Activation of RhoGTPases is a critical early step during epithelial polarization to recruit actin, the PAR complex, and other effectors to precise locations within the cell [63–65]. Intriguingly, CHO-1, the chicken ortholog of *zen-4*, contains an actin binding domain, and antibodies that recognize this actin binding domain disrupt cytokinesis suggesting actin organization may be an important function of these molecules [66]. We note that microtubule bundling and RhoGAP are not mutually exclusive activities, and ZEN-4 and CYK-4 may promote epithelium formation by multiple routes.

### Slow and Fast Rates of Epithelium Formation in *C. elegans*

A striking observation about *zen-4(px47)* mutants is that only the arcade cell epithelium is disrupted, while the digestive tract and epidermal epithelia remain intact. These other epithelia may form normally in *zen-4(px47)* mutants due to rescue by maternally donated *zen-4* mRNA or protein. The arcade cell epithelium, on the other hand, is the last epithelium to form in the embryo, perhaps after the maternal contribution of *zen-4* has been depleted. Alternatively, there appear to be distinct types of epithelia within the embryo, and perhaps these possess different genetic requirements. Most *C. elegans* epithelia form over a period of 30 min (Michel Labouesse, personal communication, and our unpublished data). Junctional components first appear as puncta at the cell surface and these puncta gradually coalesce and thicken into contiguous bands of adherens junctions. The arcade cell epithelium, by contrast, forms extremely rapidly, in less than 10 min [10] (our unpublished data). Intermediate stages of junctional puncta or thickening are not detected. These observations suggest that arcade cells may become polarized by a mechanism distinct from that of other epithelia and that *zen-4* may be critical specifically for this rapid mode of epithelialization.

In summary, we have identified two new players required for polarization and epithelium formation. Since the cellular behaviors associated with foregut tubulogenesis in *C. elegans* resemble tube formation for other organs, such as the vertebrate kidney, an exciting avenue for the future will be to determine to what degree the molecular pathways that function during *C. elegans* foregut development are relevant to tubulogenesis for other organs.

### Experimental Procedures

#### Strains and Alleles

*C. elegans* strains were obtained from the *Caenorhabditis* Genetics Center, unless stated otherwise, and maintained as described [67]. Mutations and GFP marker strains used were: SU93 [68] (*jcls1 AJM-1::GFP*) IV and SU62 [36] (*unc-44[e1260] lag-1[q385]/zen-4[w35]*) IV were gifts from the Hardin lab, CB4856 HI strain, Bristol N2, EU592 [41] *unc-8(n491) zen-4(or153ts)* IV (a gift from Bruce Bowerman's lab) and GE2636 [69] *unc-32(e189) cyk-4(t1689)/qc1 dpy-19(e1259) glp-1(q339)*, and *him-3(e1147)* IV. Other strains used in generating mapping strains include: CB3843 [70] *fem-3(e1996)/unc-24(e138)*

*dpy-20(e1282)* IV, DA740 *bli-6(sc16) unc-24(e138)* IV from Raymond Lee, and RW1324 [71] *fem-1(e1991) unc-24(e138) unc-22(s12)/stDf7* IV. New strains made in this study were SM831 *zen-4(px47) jcls1/bli-6 unc-24*, SM1077 *zen-4(px47) dpy-20(e1282)/bli-6(sc16) unc-24(e138)*, SM1272 *zen-4(px47) dpy-20/bli-6 unc-24 ex[pJP#38]* [15], SM1219 *zen-4(px47) dpy-20(e1282)/bli-6(sc16) unc-24(e138) ex[let-413::GFP]*, and SM1306 *zen-4(px47) dpy-20(e1282)/bli-6(sc16) unc-24(e138) ex[cdh-3P::zen-4]*.

#### Mapping and Transformation Rescue

Mutants were mapped by single nucleotide polymorphism mapping, comparing Bristol and Hawaiian strains of *C. elegans* or by standard three-factor mapping [72]. Injections were performed as previously described [73]. We injected the strain *px47 dpy-20(e1282)/bli-6(sc16) unc-24(e138)* with an injection mix of 0.1 ng/ul of a 7 kb PCR product from nt 7848 to nt 15234 of cosmid M03D4 and sur-5::GFP (10 ng/ul; [74]) as a marker for transformation. This 7 kb fragment contains the complete ORF of *zen-4* and was previously shown to be sufficient to rescue the allele *zen-4(w35)* [36]. PCR primers used to generate the PCR product were: *zen-4* left, GTG GCTTTTATAGTTAGAGAAATGGGG, and *zen-4* right, CGGACTCTG ATCTAGCCACTGATACAT. Six rescued lines were established by picking *Dpy-20* animals. In one *px47* homozygous rescued line, 27 of 30 embryos that expressed a SUR-5::GFP coinjection marker were viable.

#### Immunostaining

In situ antibody staining was done as described [75] with the following changes: embryos were fixed with 2% paraformaldehyde, followed by room-temperature methanol for 10 min after permeabilization by freeze crack. The mounting medium was made of 50% glycerol in PBS with 1 × DAPI and p-phenylenediamine to prevent fading. Immunostaining with MH27 [48], monoclonal anti-tubulin (clone DM1- $\alpha$ ; Sigma), or UNC-70 (generously provided by Vann Bennet) antibodies, was performed as described [41, 45]. PAR-3 and PKC-3 antisera were provided by Ken Kemphues [76]. AIR-2 antibody was the kind gift of Jill Schumacher [77]. Samples were examined with a Zeiss confocal microscope equipped with LSM software.

#### Temperature-Sensitive Shifts

Two-cell embryos were harvested from homozygous mothers (*cyk-4[t1689ts]* or *zen-4[or153ts]*) grown at permissive temperature. The embryos were incubated in a PCR machine for 30 min at 15°C, 210 min, 195 min or 120 min at 18°C, and then 195 min at 26°C (nonpermissive temperature). Embryos were then examined with differential interference contrast optics for ventral enclosure defects, undivided cells, and pharyngeal attachment.

#### Supplemental Data

Supplemental Data including Experimental Procedures and two figures are available at <http://www.current-biology.com/cgi/content/full/14/11/932/DC1/>.

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