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Temporal regulation of epithelium formation mediated by FoxA, MKLP1, MgcRacGAP, and PAR-6

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ABSTRACT To establish the animal body plan, embryos link the external epidermis to the internal digestive tract. In Caenorhabditis elegans, this linkage is achieved by the arcade cells, which form an epithelial bridge between the foregut and epidermis, but little is known about how development of these three epithelia is coordinated temporally. The arcade cell epithelium is generated after the epidermis and digestive tract epithelia have matured, ensuring that both organs can withstand the mechanical stress of embryo elongation; mis-timing of epithelium formation leads to defects in morphogenesis. Using a combination of genetic, bioinformatic, and imaging approaches, we find that temporal regulation of the arcade cell epithelium is mediated by the pioneer transcription factor and master regulator PHA-4/FoxA, followed by the cytoskeletal regulator and kinesin ZEN-4/MKLP1 and the polarity protein PAR-6. We show that PHA-4 directly activates mRNA expression of a broad cohort of epithelial genes, including junctional factor dlgl-1. Accumulation of DLG-1 protein is delayed by ZEN-4, acting in concert with its binding partner CYK-4/MgcRacGAP. Our structure–function analysis suggests that nuclear and kinesin functions are dispensable, whereas binding to CYK-4 is essential, for ZEN-4 function in polarity. Finally, PAR-6 is necessary to localize polarity proteins such as DLG-1 within adherens junctions and at the apical surface, thereby generating arcade cell polarity. Our results reveal that the timing of a landmark event during embryonic morphogenesis is mediated by the concerted action of four proteins that delay the formation of an epithelial bridge until the appropriate time. In addition, we find that mammalian FoxA associates with many epithelial genes, suggesting that direct regulation of epithelial identity may be a conserved feature of FoxA factors and a contributor to FoxA function in development and cancer.

INTRODUCTION
Animal embryos coordinate the morphogenesis of their body and internal organs in a process that requires epithelia. Communication between mesenchyme and epithelia is necessary for morphogenesis of the mammalian kidney and lung, for example, as well as the Drosophila trachea (reviewed in Hogan and Kolodziej, 2002; Caviglia and Luschnig, 2014; Combes et al., 2015; McCulley et al., 2015). There is also synergy between different organs that helps shape the embryonic body plan. In Caenorhabditis elegans, the embryo elongates fourfold to generate a long, thin worm from an oblong ball of cells (reviewed in Chisholm and Hardin, 2005; Vuong-Brender et al., 2016). Elongation of the body is coordinated with that of the gut by physically linking the outer epidermis to the inner digestive tract. At the anterior, the link is constructed by the arcade cells, which generate an epithelial bridge between the epidermis and foregut just
before body elongation. Proper morphogenesis depends critically on this linkage. Disruption of arcade cell polarization by mutation or laser ablation blocks attachment of the gut tube to the epidermis (Heidi et al., 2001; Portereiko and Mango, 2001; Portereiko et al., 2004; Mango, 2009; Kuzmanov et al., 2014). As a consequence, the foregut fails to elongate, and affected animals cannot feed (Portereiko et al., 2004). Similarly, in sma-1 mutants, the foregut attaches to the epidermis, but the embryo body fails to extend fully, and this leads to defects in foregut morphogenesis (McKeown et al., 1998). These phenotypes indicate that proper gut morphogenesis requires attachment to the elongating epidermis. Conversely, if the arcade cells generate an epithelial bridge before epidermal matura
tion, the digestive tract distorts the morphology of the nose and produces feeding defects (Kelley et al., 2015). These observations illustrate that embryos rely on epidermis to coordinate body morphogenesis and raise the question of how cells dictate the timing of epithelium formation and attachment.

Epithelia become polarized with distinct apical and basolateral domains separated by a junctional domain (Nelson et al., 2013; Roignot et al., 2013). After cell contact–mediated cues, three major polarity complexes are recruited to the membrane of fly and vertebrate epithelium and partition the cell into apical and basolateral domains (reviewed in St Johnston and Ahringer, 2010; Roignot et al., 2013; Rodriguez-Boulan and Macara, 2014). The Crumbs complex and the partitioning-defective (PAR) complex are important for apical identity and/or junction formation (Wodarz et al., 1993, 1995; Gao et al., 2002; Hurd et al., 2003; Roh et al., 2003; Harris and Peifer, 2004; Lemmers et al., 2004; Chen and Macara, 2005; St Johnston and Ahringer, 2010; Roignot et al., 2013; Rodriguez-Boulan and Macara, 2014). The basolateral surface is marked by the Scribble complex, which contains Scribble, Discs Large (DLG), and Lethal Giant Larvae (St Johnston and Ahringer, 2010; Roignot et al., 2013). Diffusion between the apical and basolateral domains is blocked by the junctional domain, which also provides tissue integrity via cell–cell adhesion (Hartsoc and Nelson, 2008). The combined actions of these polarity modules establish and maintain polarity in a range of tissues.

In the nematode, all epithelial cells form via mesenchymal-to-
epithelial transition (MET) from unpolarized precursors (Portereiko et al., 2004; Achilleos et al., 2010). C. elegans epithelia have a single electron-dense junction, termed the C. elegans apical junction (CeAJ; McMahon et al., 2001), which has properties of both tight and adherens junctions (Pästi and Labouesse, 2014). The apicobasal distribution of factors, including those in the Crumbs, Par, and Scribble groups, is similar between worm and vertebrate epithelia (Pästi and Labouesse, 2014). The three Crumbs homologues localize to the apical surface (Bosinger et al., 2001; Segbert et al., 2004; Waaijers et al., 2015), PAR-6 localizes to the apical domain (Leung et al., 1999; McMahon et al., 2001; Nance et al., 2003), and DLG-1 is found in the basal half of the CeAJ (Bosinger et al., 2001; Firestein and Rongo, 2001; Koppen et al., 2001; McMahon et al., 2001; Segbert et al., 2004). Distinct epithelia rely on the same cohort of epithelially expressed genes, indicating that temporal control cannot be explained by distinct, paralogous factors acting in different organs. For example, par-6 and dlg-1 are critical for the integrity of adherens junctions in all tested organs, and mutants for either gene fail to elongate their body or digestive tract (Bosinger et al., 2001; Firestein and Rongo, 2001; Koppen et al., 2001; McMahon et al., 2001; Totong et al., 2007).

Transcription factors can drive both MET and the reverse process, epithelial-to-mesenchymal transition (EMT; Chaffer et al., 2007; Lamouille et al., 2014). Targets of EMT include E-cadherin and integrin (Batlle et al., 2000; Cano et al., 2000; Comijn et al., 2001; Perez-Moreno et al., 2001; Hajra et al., 2002; Bolös et al., 2003; Yang et al., 2004, 2009; Eger et al., 2005), but the direct transcription factor targets that mediate MET are largely unknown (Chaffer et al., 2007). In this study, we focus on forkhead box transcription factor subgroup A (FoxA) factors, which promote the development of several epithelial organs and are often found in tumors derived from epidermis (Friedman and Kaestner, 2006). For example, mammalian FoxA1 and FoxA2 are expressed in the lung, thyroid, kidney, and pancreas, and FoxA1 is also found in the mammary gland (Bensard et al., 2004; Friedman and Kaestner, 2006). C. elegans has one FoxA factor, pha-4, which functions in two epithelial organs, the gonad and foregut (Mango et al., 1994; Updike and Mango, 2007). In mammals, reduction of FoxA can promote EMT in lung, liver, prostate, and breast by reducing expression of Slug, E-cadherin, or MMP-9 (Tang et al., 2011; Wang et al., 2014; Zhang et al., 2015). However, homologues of Slug, E-cadherin, and metalloproteases play no known role in polarizing epithelia in worms (Costa et al., 1998; Raich et al., 1999; Fraser et al., 2000; Kamath et al., 2003; Simmer et al., 2003; Rual et al., 2004; Sönntichsen et al., 2005; Von Stetina and Mango, 2015), indicating that either FoxA factors have additional, conserved target genes or regulate distinct epithelial genes in different organs and/or species. Here we define direct targets of C. elegans FoxA and explore their orthologues in vertebrates.

In this work, we examine C. elegans epithelium formation during morphogenesis, when a ball of cells is transformed into a long, thin worm. We find that epithelia are generated just before the onset of their associated morphogenetic event. We focus on the arcade cells, which form an epithelium that bridges the epidermis and foregut during late embryogenesis. A core set of epithelial factors is activated by the pioneer factor defective pharynx development 4 (PHA-4)/FoxA, but protein accumulation and localization are delayed by zygotic enclosure defective 4 (ZEN-4)/MKLP1, cytokinesis defective 4 (CYK-4)/MgcRacGAP, and PAR-6. We extend these results to FoxA factors in mammalian tissues and determine that vertebrate FoxA factors bind many orthologous target genes. The results reveal how the exquisite timing of embryonic morphogenesis depends on temporally coordinated regulation of a common core of epithelial factors at the RNA and protein levels.

RESULTS

Overview of C. elegans epithelium formation

Timing of embryo development can be tracked by the number of E (endodermal) cells and by embryo shape (Figure 1; Sulston et al., 1983; Leung et al., 1999). The epidermis is the first epithelium to polarize, during the late 8E stage (~200 cells; Sulston et al., 1983; Podbilewicz and White, 1994; Leung et al., 1999; McMahon et al., 2001; Chisholm and Hardin, 2005). This period occurs soon after the epidermal precursors are born between the 2E and mid-8E stages and before epiboly at the bean stage (Sulston et al., 1983). Once the epithelium has formed, it migrates over the lateral and ventral surfaces of the embryo, enclosing the embryo in preparation for body elongation (Priess and Hirsh, 1988; Williams-Masson et al., 1997; Figure 1).

The digestive tract polarizes progressively, with midgut epithelialization commencing at the 8E stage and junction formation starting in the early 16E stage, whereas the foregut shows the first hallmarks of polarity at early 16E and begins to form junctions in mid-16E (Figure 1; Totong et al., 2007; Achilleos et al., 2010; Rasmussen et al., 2012). Between the comma and 1.75-fold stages, the anteriorly located arcade cells and posterior hindgut undergo
MET to generate two small epithelia that link the epidermis to the foregut and hindgut, respectively. The result is a torus with the epidermis on the outside and the digestive tract on the inside, held under tension by its distal attachments. The torus elongates fourfold to generate a worm with an extended, linear digestive tract (Chisholm and Hardin, 2005; Vuong-Brender et al., 2016). Without mechanical coupling of the gut to the epidermis, the digestive tract cannot produce its proper elongated form (Portereiko and Mango, 2001; Mango, 2009; Kelley et al., 2015).

Asynchronous accumulation of dlg-1 RNA and protein in different organs

To understand the temporal regulation of epithelium formation, we determined the onset of expression for polarity factors by surveying members of the Par (par-6) and Scribble (dlg-1) modules with single-molecule fluorescence in situ hybridization (smFISH) for RNA (Ji and van Oudenaarden, 2012). PAR-6 plays an early and key role in maturing apical junctions within the epidermis and digestive tract (Totong et al., 2007), whereas DLG-1 is one of the last components added to epithelial junctions and is essential for their maturation (Totong et al., 2007). We found that par-6 RNA was contributed maternally, as predicted from prior studies (Watts et al., 1996; Nance et al., 2003), and no increase due to zygotic par-6 RNA was detected (Supplemental Figure S1; Totong et al., 2007). dlg-1 was induced zygotically, with RNA accumulating in different organs at different times, before the generation of each epithelium (described later). We also assayed the onset of protein expression, as this demonstrates when the epithelium is in the final stages of maturation. Whereas the onset of DLG-1 protein has been documented for the epidermis (Podbielwicz and White 1994; Bossinger et al., 2001; McMahon et al., 2001; Chisholm and Hardin 2005) and midgut (Leung et al. 1999; Totong et al., 2007; Achilleos et al., 2010), it has not been described for the foregut, and is unclear how the onset of expression compares among these three epithelia.

The epidermis was the first epithelium to express dlg-1 mRNA. It was initially detected at the late 4E stage but with no detectable DLG-1 protein (Figures 1 and 2A). The level of dlg-1 mRNA increased during the 8E stage (Figure 2B) and was maintained throughout the 16E and elongation stages (comma, 1.5-fold; Figure 2, C–F). DLG-1 protein was first observed during the late 8E stage, with puncta of protein visible on the membrane of nascent epidermal cells (Figure 2B). These puncta began to coalesce as the early 16E stage (Figure 2C) and formed a continuous, circumferential junction by the mid-16E stage (Figure 2D). The level of DLG-1 increased during the elongation stages (comma, 1.5-fold; Figure 2, E and F), as the cells changed shape to convert the embryo from a ball into a vermiform.

The digestive tract began to express dlg-1 mRNA at the 8E stage (Figure 2, H and N), and, similar to the epidermis, the levels of RNA increased throughout the 8E and 16E stages (Figure 2, I, J, and O–P). DLG-1 protein was first observed in midgut precursors at the early 16E stage (Figure 2I), where puncta of protein appeared at the lateral surface and rapidly coalesced at the apical surface, in agreement with previous studies (Supplemental Figure S2; Leung et al., 1999; Totong et al., 2007; Achilleos et al., 2010). By the mid-16E stage (~20–40 min later), the puncta of DLG-1 had banded together to form cell junctions (Figure 2J), which continued to expand and mature as the embryo elongated (comma, 1.5-fold stages; Figures 1 and 2, K and L). The RNA remained expressed in the intestine throughout all of these stages (Figure 2, I–L).

In the foregut, DLG-1 protein was first detectable by the mid-16E stage (Figure 2P), suggesting that translation of dlg-1 mRNA was delayed in this tissue by ~20–40 min. We observed membrane-associated DLG-1 puncta on cell surfaces throughout the foregut at the 16E stage (Supplemental Figure S2). These spots accumulated at the nascent apical surface by the bean stage (Supplemental Figure S2), where they joined together to form connected junctions by the comma stage (Figures 1 and 2Q). The RNA remained expressed throughout these stages (Figure 2, O–R). Together, these data show that the digestive tract forms in a piecemeal manner, with the midgut polarizing and maturing before the foregut, and both after the epidermis.
By the end of the comma stage, the epithelial cells of the foregut and midgut were linked together via adherens junctions, yet the digestive tract remained isolated from the epidermis. The digestive tract became attached to the epidermis at the anterior via epithelialization of the arcade cells (Figure 1; Portereiko and Mango, 2001). The arcade cells are born during the mid-16E stage, starting ∼290 min after the first division (Sulston et al., 1983). The majority of these cells are anterior to the foregut primordium and express dlg-1 mRNA from birth (Figure 2O). DLG-1 protein accumulated ∼100 min later in the arcades (Figure 2R), after the epidermis and foregut had both formed epithelia and soon before the arcade cells became an epithelium (i.e., between the comma and 1.25-fold stage, ∼390–400 min after the first division; Portereiko and Mango, 2001; Portereiko et al., 2004). The presence of RNA but lack of protein was detectable by the 16E stage (Supplemental Figure S2C) but was clearest at the comma stage, when the arcade cells clustered together as a group anterior to the foregut epithelium (Figure 2Q). Thus there was a delay in protein accumulation, suggesting that either the RNA was translationally repressed or protein was made but degraded immediately. These results reveal that dlg-1 RNA and protein are uncoupled in different organs, with the protein appearing just before polarity establishment. Figure 1 summarizes the onset of RNA and protein expression presented here.

**PHA-4 activates dlg-1 in the foregut and arcade cells**

The onset of dlg-1 RNA expression suggested a transcriptional component to temporal regulation of epithelium formation. One central regulator of the digestive tract is PHA-4, a pioneer transcription factor that is also the master regulator for the C. elegans foregut (Horner et al., 1998; Gaudet and Mango, 2002; Mango, 2009; Hsu et al., 2015). To determine whether PHA-4 was important for dlg-1 expression, we performed RNA interference (RNAi) against pha-4 and

**FIGURE 2**: Onset of dlg-1 RNA and protein expression in epithelia. dlg-1 RNA is pseudocolored magenta (top); DLG-1 protein is labeled in white (bottom). RNA and protein data are from different embryos. Nuclei of the epidermis (A–F) and 4E midgut (G) are visualized by DAPI (blue). PHA-4::GFP (green) marks the midgut (H–L), foregut (M–R), and arcade cells (white bars in Q and R). (A–F) dlg-1 RNA is first detected in the 4E epidermis (A), whereas the onset of protein expression is at the 8E stage (B). RNA and protein are continuously expressed throughout the 16E and morphogenetic stages (C–F). Note that the protein first forms spot junctions (B, C), which resolve into continuous bands (D–F). (G–L) The midgut first expresses RNA at the 8E stage and protein at the early 16E. The protein first forms spot junctions (I), which quickly resolve into continuous bands (J). The RNA and protein are also detected during morphogenesis (K, L). (M–R) Like the midgut, RNA expression in the foregut begins at the 8E stage (N). Unlike the midgut, protein expression is first visible as spots by the mid rather than the early 16E stage (compare I vs. O and P). Foregut cells have formed continuous junctions by the comma stage (Q). The arcade cells (between the white bars) have RNA but no protein at the comma stage (Q), and between the comma and 1.5-fold, the arcade cells express protein and form continuous junctions (R). All images are maximum intensity projections. Anterior is to the left. Scale bars, 5 μm. Embryos were fixed and either hybridized with probes to detect RNA expression or stained with antibodies to detect protein. The midgut and foregut/arcade cell images are from the same embryo; the epidermis images at the same stage are from different embryos. smFISH data were imported into Imaris 3D software (Materials and Methods).
observed reduced DLG-1 in the foregut and arcade cells but normal expression in the midgut and epidermis (Figure 3A). Antibody staining against endogenous PHA-4 confirmed that RNAi was effective (Figure 3B). Apical factors PAR-3 and ezrin/radixin/moesin homologue 1 (ERM-1) were similarly disrupted by loss of pha-4 (Figure 3, C–F), suggesting widespread disruption of epithelial factors.

Next we determined whether regulation of dlg-1 transcription by PHA-4 was direct. PHA-4 binding has been mapped by chromatin immunoprecipitation sequencing (ChIP-seq; Zhong et al., 2010). Examination of ChIP-seq data demonstrated two binding sites for PHA-4, one near the upstream dlg-1 transcription start site (TSS; Figure 4A; Saito et al., 2013) and a second site just before the ATG start and within the first intron (Figure 4A). To test whether these sites contributed to dlg-1 expression, we used a DLG-1::green fluorescent protein (GFP) reporter, P7 dlg-1, with 7 kb of upstream sequences, which was able to rescue loss of endogenous dlg-1 (McMahon et al., 2001). The reporter expressed DLG-1 appropriately: endogenous dlg-1 and transgenic gfp RNA were both detectable in the foregut and midgut at the 8E stage (i.e., at the onset of endogenous gene expression; Figure 2H and Supplemental Figure S3) and abundant in these tissues by the 16E stage (Figure 4B).

Live imaging demonstrated that DLG-1::GFP protein was enriched at the midline of the midgut and clustered into junctions at the 16E stage (Figure 4C). In the mid-16E, DLG-1::GFP was detectable as spots spread throughout the foregut, similar to the endogenous protein (compare Figures 4C and 2P). At the comma stage, both endogenous and transgenic RNAs were detectable throughout the digestive tract (including the arcade cells; Figure 4K), whereas DLG-1::GFP protein, like endogenous DLG-1, was visible only in the foregut and midgut (Figure 4L). By the 1.5-fold stage, DLG-1::GFP was also visible in the arcade cells, as expected (Figure 4U). Thus the P7 dlg-1 transgene contained all the information necessary for appropriate temporal expression of RNA and protein in the digestive tract and epidermis (Figure 4, D, M, and V).

We performed promoter deletion analysis to determine whether PHA-4–bound sites contributed to dlgs-1 activation. We generated worms expressing two different DLG-1::GFP constructs: one that contained all PHA-4–binding sites (P4.9 dlg-1 for 4.9 kb) and one that carried the site near the ATG start but lacked the major binding site at the upstream TSS (P4.3 dlg-1 for 4.3 kb;

**FIGURE 3:** PHA-4/FoxA is required for polarity protein expression in the foregut. The arcade cell epithelium lies between red arrowheads, the foregut epithelium between blue arrowheads, and the midgut epithelium between purple arrowheads, and hindgut cells are marked by a green arrowhead. (A, C, E) In control RNAi embryos, PHA-4 (white) is strongly expressed in arcade, foregut, and hindgut cells and dimly expressed in the midgut. Markers of polarity (green), such as DLG-1 (A), PAR-3 (C), and ERM-1 (E), are detected at the apical or junctional domains of the digestive tract. (B, D, F) After pha-4 RNAi, PHA-4 is undetectable in the arcade, foregut, or midgut cells. The hindgut is resistant to pha-4(RNAi) and serves as an internal control for antibody staining. In pha-4(RNAi) embryos, polarity protein expression is ablated in arcade cells (red arrowhead) and decreased (DLG-1, PAR-3) or absent (ERM-1) in the foregut. If protein remains, it is detected only in the foregut remnant (blue arrowhead). Expression in the midgut is unchanged (purple arrowheads). All images are maximum intensity projections through the digestive tract. Anterior is to the left. Scale bars, 5 μm.
Figure 4A. \(P_4^+\)\(\text{dlg-1}\) faithfully recapitulated the onset of expression in the epidermis (Figure 4, G, P, and Y; unpublished data) and midgut (Figure 4, E, F, N, O, W, and X, and Supplemental Figure S3), similar to endogenous \(\text{dlg-1}\). However, \(P_2\)\(\text{dlg-1}\) was silent at the 8E (Supplemental Figure S3) and 16E stages in the foregut and arcades, when endogenous \(\text{dlg-1}\) was abundant (Figure 4E; compare gfp RNA [green] to \(\text{dlg-1}\) RNA [orange]); \(\text{dlg-1}\) was undetectable (Figure 4F). Expression in the foregut of \(P_4\)\(\text{dlg-1}\) transgenic embryos commenced slowly, progressing from posterior to anterior. Specifically, by the comma stage, the foregut (but not the arcade cells) had abundant gfp RNA (Figure 4N), whereas protein was most abundant in the posterior foregut (Figure 4O). At the 1.5-fold stage, transgenic mRNA and protein were detected throughout the digestive tract, although gfp expression was very dim in the arcade cells in most embryos (Figure 4, W and X). GFP was expressed at normal levels in the arcade cells by the twofold stage (unpublished data). Adding back 600 base pairs of sequence (\(P_4\)\(\text{dlg-1}\)), including the upstream PHA-4–binding site, restored wild-type expression in the foregut (Figure 4, H, I, Q, R, Z, and AA, and Supplemental Figure S3). These data suggest that the PHA-4–binding site near the upstream TSS is critical for onset of expression in the foregut, whereas other PHA-4–binding sites and sites for other factors mediate late foregut expression (or maintenance of expression) and expression in the midgut and epidermis.

In summary, three lines of evidence suggest that PHA-4 contributes to the proper onset of \(\text{dlg-1}\) expression: first, DLG-1 expressing DLG-1:GFP under the \(P_{2,3}\)\(\text{dlg-1}\) promoter have transgenic gfp mRNA that matches endogenous \(\text{dlg-1}\) mRNA in the epidermis and midgut. Expression in the foregut is delayed (white arrowhead). In the 16E stage (E), gfp mRNA is nearly absent in the foregut. By the comma stage (N), most of the foregut has gfp mRNA, but the arcade cells have no expression. Transgenic mRNA expression in the arcade cells and anterior foregut is seen by the 1.5-fold stage (W). Adding back 600 base pairs of sequence (\(P_4\)\(\text{dlg-1}\)) restores wild-type expression in the foregut (unpublished data). For every row, DLG-1::GFP images are two focal planes from the same embryo. Note that the RNA and protein data are from different embryos. Areas corresponding to the arcade cells are labeled with a red line, foregut in blue, and midgut in purple. (B, K, T) Embryos expressing DLG-1:GFP under the \(P_{2,3}\)\(\text{dlg-1}\) promoter have transgenic gfp mRNA expression that matches endogenous \(\text{dlg-1}\) mRNA (compare orange and green mRNA profiles). DLG-1::GFP protein (C, D, L, M, U, V) is similar to endogenous (compare to Figure 2). (E, N, W) Embryos expressing DLG-1:GFP under the \(P_4\)\(\text{dlg-1}\) promoter have transgenic gfp mRNA and protein expression profiles that match their endogenous counterparts in all tissues. All images are maximum intensity projections. Anterior is to the left. Scale bars, 5 \(\mu\)m.
We examined other genes that have important functions in epithelial morphogenesis but a broad expression pattern within the embryo. We found that 20 of 30 genes had PHA-4 peaks associated with cis-regulatory sequences, three of 30 had PHA-4 associated with the TSS of the polarity gene and the TSS of a neighboring gene on the opposite strand in a potentially bidirectional promoter, and seven of 30 had no associated PHA-4 peak (Table 1). On the other hand, we rarely observed PHA-4 associated with genes that are expressed in other epithelia but not in foregut or arcade cells (only one of 12). For example, ifb-2, which codes for an apically localized intermediate filament protein expressed in the midgut (Bossinger et al., 2004), lacked PHA-4 bound to its regulatory sequences (Figure 5 and Supplemental Table S1). These findings suggest that PHA-4 regulates a broad cohort of epithelial factors within the foregut and arcade cells.

FoxA factors may regulate a conserved epithelial program

In mammals, FoxA factors promote epithelial organ development, and their loss often correlates with EMT of tumors. We asked whether FoxA factors target core epithelial factors in mammals as they do in C. elegans. We queried existing FoxA ChIP-seq data sets for mouse cells (Soccio et al., 2011) and human cells (Motallebipour et al., 2009; Hurtado et al., 2011; Gosalia et al., 2015; Wang et al., 2015) to determine whether FoxA factors bound to the regulatory elements of epithelial factors, examining 1000 base pairs up and

PH4 activates an epithelial program during foregut polarization

To extend our observations beyond dlg-1, we examined PHA-4 association with genes encoding other polarity proteins, relying again on the modENCODE PHA-4 ChIP-seq data set (Zhong et al., 2010). Strikingly, genes for every epithelial polarity protein annotated as expressed in the embryonic foregut (27 of 27) had an associated PHA-4 peak, many close to the TSS or within the first large intron, which are common sites for bona fide PHA-4 targets (Gaudet and Mango, 2002; Gaudet et al., 2004; Figure 5 and Table 1). For example, par-3 had two PHA-4 ChIP peaks within the large, first intron (which is a promoter for the smaller, epithelial-specific isoform; Achilleos et al., 2010), erm-1 had an upstream peak and one in a large intron, and the α-catenin hmp-1 contained one peak at the TSS. In support of PHA-4 regulating these epithelial-specific genes, we found reduced levels of ERM-1 and PAR-3 in par-4(RNAi) mutant foreguts (Figure 3, C–F; antibodies for HMP-1 were not available for testing).
| Gene | PHA-4 bound? | Location of peak(s) | Expression pattern |
|------|--------------|---------------------|--------------------|
| afd-1 | Yes | Upstream, at TSS, and/or intronic, depending on isoform | Epithelial |
| ajm-1 | Yes | Intronic | Epithelial |
| cdh-4 | Yes | Intronic | Epithelial |
| crb-1 | Yes | Intronic | Epithelial |
| crb-3 | Yes | Upstream | Epithelial |
| dlg-1 | Yes | Upstream at TSS, intronic | Epithelial |
| eat-20 | Yes | Upstream, at TSS, intronic | Epithelial |
| erm-1 | Yes | Upstream, intronic | Epithelial |
| hmp-1 | Yes | Upstream at TSS | Epithelial |
| hmp-2 | Yes (operon) | Upstream at TSS of operon | Epithelial |
| hmr-1 | Yes | Upstream, at TSS, intronic | Epithelial |
| jac-1 | Yes | Upstream, intronic | Epithelial |
| lam-1 | Yes | Upstream at TSS | Epithelial |
| lam-2 | Yes | Upstream at TSS | Epithelial |
| lam-3 | Yes | Upstream | Epithelial |
| let-413 | Yes | Upstream at TSS | Epithelial |
| let-502 | Yes | Upstream at TSS | Epithelial |
| let-805 | Yes | Upstream and within gene body | Epithelial |
| lgl-1 | Yes | Upstream at TSS | Epithelial |
| magi-1 | Yes | Upstream near TSS, intronic | Epithelial |
| mel-11 | Yes | Upstream TSS | Epithelial |
| par-3 | Yes | Upstream and at TSS of smaller, epithelial isoform | Epithelial |
| par-6 | Yes (operon) | Upstream at TSS of operon | Epithelial |
| pat-3 | Yes | Upstream at TSS | Epithelial |
| pkc-3 | Yes | Upstream at TSS | Epithelial |
| sax-7 | Yes | Upstream, intronic | Epithelial |
| vab-9 | Yes | Upstream, at TSS | Epithelial |
| cdc-42 | Yes | Upstream at TSS | Broad |
| die-1 | Yes | Upstream, at TSS, intronic | Broad |
| ifa-1 | Yes | Upstream at TSS and intronic | Broad |
| ifb-1 | Yes | Intronic | Broad |
| let-858 | Yes | Upstream at TSS | Broad |
| mig-10 | Yes | Upstream/intronic, depending on isoform | Broad |
| pix-1 | Yes | Upstream at TSS, intronic | Broad |
| srgp-1 | Yes | Upstream at TSS | Broad |
| unc-34 | Yes | Within first intron; also one several kilobases upstream | Broad |
| unc-94 | Yes | Within large introns 1 and 3 (or upstream of smaller isoform at TSS) | Broad |
| vab-10 | Yes | Upstream, at TSS, intronic | Broad |
| vab-19 | Yes | Upstream | Broad |
| wsp-1 | Yes | Upstream, intronic | Broad |
| eps-8 | Yes | Upstream, at TSS, intronic | Broad |
| cyk-4 | Yes | Upstream at TSS | Ubiquitous |
| gex-2 | Yes | Upstream at TSS (potentially at bidirectional promoter) | Ubiquitous |
| gex-3 | Yes | Upstream at TSS | Ubiquitous |
| rho-1 | Yes | Upstream at TSS | Ubiquitous |

**TABLE 1:** Analysis of foregut epithelial genes for PHA-4 binding.

(Continues)
downstream of the gene body, as well as the gene body itself. We did not analyze enhancers, as they are often difficult to assign to a particular gene.

We examined homologues of the 50 epithelial-specific factors that were bound by PHA-4 in C. elegans (Table 1). We found that 27 of 50 mouse epithelial homologues had an associated FoxA2 peak in primary liver cells (Table 2). Caco2 cells are derived from colorectal cancer but mimic normal polarized enterocytes when cultured to confluency (Fogh et al., 1977; Alvarez-Hernandez et al., 1991; Meunier et al., 1995). Analysis of FoxA2 ChIP-seq data revealed FoxA2 peaks near the human homologues of 33 of 50 C. elegans epithelial genes (Gosalia et al., 2015). Intriguingly, PARD3 and PARD3B—homologues of PAR-3, a master regulator of epithelial polarity—had 11 and 13 peaks, respectively, throughout the gene body, mostly in introns, suggesting potential regulation by FoxA2. PARD3 and PARD3B were also bound by FoxA1 and FoxA2 in human embryonic stem cell–derived gut tube (GT) and foregut (FG) cells (Wang et al., 2015). In GT and FG cells, respectively, FoxA1 bound 33 and 32 of 50 worm epithelial genes, whereas FoxA2 bound 43 and 45 of 50 genes (Figure 6 and Table 2). In addition to the par-3 homologues listed here, mouse and human homologues of dlg-1 and erm-1 were also bound by FoxA1/2 in all data sets analyzed.

We also analyzed two data sets from human cancer cells derived from epithelial organs. HepG2 cells are derived from a hepatocellular cancer (Knowles et al., 1980). FoxA1 binding was detected in 28 of 50 human homologues of nematode genes (Motallebipour et al., 2009; Table 2). Of these, 16 were shared between mouse liver and human liver cancer cells. We also examined MCF7 breast cancer cells and observed that FoxA1 bound 42 of 50 homologues of nematode epithelial genes (Hurtado et al., 2011; Table 2). In MCF7 cells, members of all three polarity groups were bound (CRB1-2, PARD3/6, DLG1-3), as were mediators of cell–cell (e.g., CDH1) and cell–matrix (e.g., ITGB1-8) interactions.

In sum, of 50 gene families surveyed, FoxA1 or FoxA2 was observed in at least seven of eight data sets for 26 of the gene families (Table 2 and Supplemental Table S2). Included in this list are genes essential for proper epithelial polarity in mammalian cells (E-cadherin, β-integrin, Discs Large), as well as mediators of tight and adherens junction formation and function (Par3, ZO1, MAGI2, α-catenin, p120-catenin, Rho kinase). These results implicate mammalian FoxA for regulation of a common set of epithelial factors in many tissue types and suggest that FoxA factors may function as master regulators of epithelial identity in multiple species.

**TABLE 1: Analysis of foregut epithelial genes for PHA-4 binding. Continued**

| Gene | PHA-4 bound? | Location of peak(s) | Expression pattern |
|------|--------------|---------------------|-------------------|
| spc-1 | Yes | Upstream at TSS | Ubiquitous |
| toca-1 | Yes | Upstream, at TSS, intronic | Ubiquitous |
| unc-70 | Yes | Upstream at TSS, intronic | Ubiquitous |
| wve-1 | Yes | Upstream at TSS (potentially at bidirectional promoter) | Ubiquitous |
| zen-4 | Yes | Upstream at TSS | Ubiquitous |
| abi-1 | Yes | Upstream at TSS (potentially at bidirectional promoter) | Unknown |
| apr-1 | No | n/a | Ubiquitous |
| ced-10 | No | n/a | Ubiquitous |
| ect-2 | No | n/a | Ubiquitous |
| mig-2 | No | n/a | Ubiquitous |
| toca-2 | No | n/a | Ubiquitous |
| clc-1 | No | n/a | Unknown |
| rac-2 | No | n/a | Unknown |

Fifty-seven C. elegans genes that have roles and/or are expressed in epithelial cells, including those of the foregut. Of these, 50 have an associated PHA-4 ChIP peak. The location of the ChIP peaks (the majority of which are at the TSS of the bound genes) and expression pattern of the genes are also shown.

**ZEN-4 is required for protein accumulation in the arcade cell epithelium**

Once transcription is activated throughout the digestive tract, the arcade cells delay accumulation of DLG-1 protein until polarity onset (Figure 2, comma stage). The ZEN-4 kinesin is essential for arcade cell polarity (Portereiko et al., 2004), and we tested whether zen-4 had a role in dgl-1 accumulation. zen-4 mutants had abundant dgl-1 mRNA at the comma/1.25-fold stages (when the arcade cells normally polarize), similar to wild-type siblings (Figure 7, A and B). However, zen-4 mutants lacked DLG-1 protein at the 1.25-fold stage (compare Figure 7, G to H). Maternally supplied par-6 and erm-1 gave similar results to dgl-1. Levels of par-6 and erm-1 RNA were virtually identical in zen-4 mutant arcade cells versus control arcade cells (Figure 7, D and F), but there was no detectable protein (Figure 7, J and L). Protein was also missing for apical (PKC-3, PAR-3) and junctional (AJM-1, HMR-1) markers, as determined previously (Portereiko et al., 2004). Using an overexpressed AJM-1::GFP reporter that increased levels in both wild-type and mutant embryos, we estimate that zen-4 mutants have 20-fold lower AJM-1 than the wild type (Supplemental Figure S4). We conclude that, normally, ZEN-4 promotes accumulation of polarity proteins to form the arcade cell epithelium.

The effect of zen-4 on accumulation of polarity proteins suggested that ZEN-4 would function early, before polarity onset. To test this idea, we performed temperature-shift experiments to pinpoint when zen-4 activity was required (Figure 8). We used a temperature-sensitive allele of zen-4, or153ts (Severson et al., 2000), which abrogates binding to its obligate partner in cytokinesis, cyk-4, when shifted to the restrictive temperature (Pavicic-Kaltenbrunner...
| Description                                      | C. elegans PHA-4 FoxA2 bound\(^a\) | Mouse liver FoxA2 bound\(^b\) | HepG2 cells FoxA1 bound\(^c\) | MCF7 cells FoxA1 bound\(^d\) | Caco2 cells FoxA2 bound\(^e\) | GT cells FoxA1 bound\(^f\) | FG cells FoxA1 bound\(^f\) | GT cells FoxA2 bound\(^f\) | FG cells FoxA2 bound\(^e\) |
|-------------------------------------------------|-----------------------------------|--------------------------------|--------------------------------|--------------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Fat cadherin                                    | cdh-4                             |                                |                                |                                |                             |                             |                             |                             |                             |
| Disks Large                                     | dlg-1                             |                                |                                |                                |                             |                             |                             |                             |                             |
| Epidermal growth factor receptor kinase substrate 8 | eps-8                             |                                |                                |                                |                             |                             |                             |                             |                             |
| Ezrin/radixin/moesin                            | erm-1                             |                                |                                |                                |                             |                             |                             |                             |                             |
| p120/β-catenin                                   | jac-1                             |                                |                                |                                |                             |                             |                             |                             |                             |
| β-Laminin                                       | lam-1                             |                                |                                |                                |                             |                             |                             |                             |                             |
| Scribble                                        | let-413                            |                                |                                |                                |                             |                             |                             |                             |                             |
| Rho kinase                                      | let-502                            |                                |                                |                                |                             |                             |                             |                             |                             |
| Membrane-associated guanylate kinase inverted (MAGI) | magi-1                            |                                |                                |                                |                             |                             |                             |                             |                             |
| Smooth muscle myosin–associated phosphatase regulatory subunit | mel-11                            |                                |                                |                                |                             |                             |                             |                             |                             |
| Rac GTPase                                      | mig-10                             |                                |                                |                                |                             |                             |                             |                             |                             |
| PAR-3/PDZ                                       | par-3                             |                                |                                |                                |                             |                             |                             |                             |                             |
| β-Integrin                                      | pat-3                             |                                |                                |                                |                             |                             |                             |                             |                             |
| L1CAM                                           | sax-7                             |                                |                                |                                |                             |                             |                             |                             |                             |
| Transducer of Cdc42-dependent actin assembly     | toca-1                             |                                |                                |                                |                             |                             |                             |                             |                             |
| βG-Spectrin                                     | unc-70                             |                                |                                |                                |                             |                             |                             |                             |                             |
| Spectraplakin                                    | vab-10                             |                                |                                |                                |                             |                             |                             |                             |                             |
| Zona occludens 1 (ZO1)                           | zoo-1                             |                                |                                |                                |                             |                             |                             |                             |                             |
| Crumbs-like                                     | eat-20                             |                                |                                |                                |                             |                             |                             |                             |                             |
| E-cadherin                                      | hmr-1                             |                                |                                |                                |                             |                             |                             |                             |                             |
| γ-Laminin                                       | lam-2                             |                                |                                |                                |                             |                             |                             |                             |                             |
| Kank ankyrin repeat protein                     | vab-19                             |                                |                                |                                |                             |                             |                             |                             |                             |
| p140/Sra-1                                      | gex-2                             |                                |                                |                                |                             |                             |                             |                             |                             |
| α-Catenin                                       | hmp-1                             |                                |                                |                                |                             |                             |                             |                             |                             |
| α-Laminin                                       | lam-3                             |                                |                                |                                |                             |                             |                             |                             |                             |
| Atypical protein kinase C (aPKC)                | pkc-3                             |                                |                                |                                |                             |                             |                             |                             |                             |
| Abelson kinase–interacting protein              | abi-1                             |                                |                                |                                |                             |                             |                             |                             |                             |
| Crumbs                                          | crb-1                             |                                |                                |                                |                             |                             |                             |                             |                             |
| Nck-associated protein                           | gex-3                             |                                |                                |                                |                             |                             |                             |                             |                             |
| GTPase                                          | cyk-4                             |                                |                                |                                |                             |                             |                             |                             |                             |
| Enabled/VASP                                     | unc-34                             |                                |                                |                                |                             |                             |                             |                             |                             |
| β-Catenin                                       | hmp-2                             |                                |                                |                                |                             |                             |                             |                             |                             |
| Slit-Robo GTPase-activating protein              | srgp-1                             |                                |                                |                                |                             |                             |                             |                             |                             |
| Afadin                                          | afd-1                             |                                |                                |                                |                             |                             |                             |                             |                             |
| PAR-6/PDZ                                       | par-6                             |                                |                                |                                |                             |                             |                             |                             |                             |
| Tropomodulin                                    | unc-94                             |                                |                                |                                |                             |                             |                             |                             |                             |
| Myotactin                                       | let-805                            |                                |                                |                                |                             |                             |                             |                             |                             |
| p21-activated kinase–interacting exchange factor | pix-1                             |                                |                                |                                |                             |                             |                             |                             |                             |
| α-Spectrin                                      | spc-1                             |                                |                                |                                |                             |                             |                             |                             |                             |

**TABLE 2:** List of conserved epithelial factors with bound mammalian FoxA.
TABLE 2: List of conserved epithelial factors with bound mammalian FoxA. Continued

| Description                        | C. elegans PHA-A bounda | Mouse liver FoxA2 boundb | HepG2 cells FoxA1 boundc | MCF7 cells FoxA1 boundd | Caco2 cells FoxA2 bounde | GT cells FoxA1 boundf | FG cells FoxA1 boundf | GT cells FoxA2 boundf | FG cells FoxA2 boundf |
|------------------------------------|-------------------------|--------------------------|--------------------------|--------------------------|--------------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| Cdc42 GTPase                        | cdc-42                  |                          |                          |                          |                          |                       |                       |                       |                       |
| Intermediate filament proteins     | ifa-1                   |                          |                          |                          |                          |                       |                       |                       |                       |
|                                    | ifb-2                   |                          |                          |                          |                          |                       |                       |                       |                       |
| Nucampholin                        | let-858                 |                          |                          |                          |                          |                       |                       |                       |                       |
| Rho GTPase                         | rho-1                   |                          |                          |                          |                          |                       |                       |                       |                       |
| Mitotic kinesin-like protein 1     | zen-4                   |                          |                          |                          |                          |                       |                       |                       |                       |
| (MKLP1)                            |                         |                          |                          |                          |                          |                       |                       |                       |                       |
| Brain cell membrane protein 1      | vab-9                   |                          |                          |                          |                          |                       |                       |                       |                       |
| (BCMP1) claudin                    |                         |                          |                          |                          |                          |                       |                       |                       |                       |
| WASP                               | wsp-1                   |                          |                          |                          |                          |                       |                       |                       |                       |
| WAVE                               | wve-1                   |                          |                          |                          |                          |                       |                       |                       |                       |
| Coiled coil                        | ajm-1                   |                          |                          |                          |                          |                       |                       |                       |                       |
| Crumbs                             | crb-3                   |                          |                          |                          |                          |                       |                       |                       |                       |
| Zinc finger transcription factor   | die-1                   |                          |                          |                          |                          |                       |                       |                       |                       |

Green indicates that the PHA-4 or FOXA homologue binds to regulatory regions in the listed worm gene and its homologues in the listed mammalian ChIP-seq data sets. Gray boxes denote no binding for any homologues. For a complete list of the bound homologues, see Supplemental Table S2.

et al., 2007). We inactivated zen-4 during three time periods: close to the time of arcade cell birth (early); during arcade cell polarization (late); and between the early and late time points (middle). Embryos were shifted to restrictive temperature for a 70-min window, followed by incubation at permissive temperature to the 1.5-fold stage, when the arcade cells have formed an epithelium in the wild type. We confirmed rapid inactivation of zen-4 at these late stages by testing for cytokinesis defects (Supplemental Figure S5), which is a canonical phenotype of or153ts (Severson et al., 2000).

The majority of embryos shifted to nonpermissive temperature at the time of arcade cell birth had defects in polarization (84%; 27 of 32; Figure 8). On the other hand, almost all embryos shifted during the middle and late periods appeared wild type (35 of 37 and 55 of 56 for the middle and late windows, respectively; Figure 8). These data suggest that ZEN-4 is required around the time of arcade cell birth rather than at the time of polarization, consistent with the idea that ZEN-4 acts early to produce polarity proteins, which are later assembled into apical and basolateral domains. As a control, inactivation of zen-4(ts) at the birth of the epidermal cells did not disrupt polarity in the epidermis, indicating that zen-4 is required specifically in the arcade cells and that blocked cytokinesis is not sufficient to disrupt epithelial polarity (Figure 8).

Structure–function analysis of ZEN-4 reveals a requirement for binding to CYK-4/GAP

ZEN-4/MKLP relies on four protein domains to fulfill its roles in cytokinesis and neurite outgrowth (White and Glotzer, 2012; del Castillo et al., 2015). We performed structure–function analysis to gain insight into which domains were important for arcade cell polarization (Figure 9A). We assayed both rescue of the zen-4(px47) mutant phenotype (see Materials and Methods) and localization of a GFP-tagged reporter. To monitor px47 rescue, we examined progeny from heterozygous zen-4(px47) mothers. Normally 25% offspring are zen-4(px47) homozygotes, and of these, half or more arrest with unpolarized arcade cells (Table 3 and Supplemental Table S3). A wild-type ZEN-4::GFP transgene rescued homozygotes, producing ~0% embryonic lethality from heterozygous mothers (Figure 9A, Table 3, and Supplemental Table S3). These animals displayed a dynamic localization pattern for ZEN-4::GFP that mimicked endogenous ZEN-4 and its homologues (Figure 9B; Powers et al., 1998; Raich et al., 1998; Deavours and Walker, 1999; Chen et al., 2002; Minestrini et al., 2002, 2003; Verbrugghe and White, 2007). ZEN-4::GFP was nuclear in interphase cells, localized to the ingressing furrow and cortex during mitosis, and remained at the division remnant after abscission.

ZEN-4(NeckΔ)::GFP lacks five residues around residue D520 within the neck domain. This construct was unable to rescue zen-4(px47): zero of two transgenic ZEN-4(NeckΔ)::GFP lines rescued zen-4(px47) mutants, like the no-transgene control (Figure 9A, Table 3, and Supplemental Table S3). The neck domain serves two purposes. It is essential to bind CYK-4/MgcRacGAP, a GTPase-activating protein that controls ZEN-4 kinesin activity (Mishima et al., 2002; Pavicic-Kaltenbrunner et al., 2007; White et al., 2013). It is also important to localize ZEN-4 to the cell cortex and division remnant (Figure 9F). To determine whether ZEN-4 requires CYK-4 activity or division remnant localization, we tethered ZEN-4(NeckΔ) to CYK-4 using a flexible linker (see Materials and Methods; Neuhold and Wold, 1993; Castanon et al., 2001). As a positive control, we linked CYK-4 to wild-type ZEN-4. As shown in Figure 10A, Table 4, and Supplemental Table S4, both wild-type and mutant fusions
were sufficient for rescue, indicating that association with CYK-4 is critical for ZEN-4 function and disrupted by the NeckΔ mutation.

The tethered dimer localized ZEN-4::CYK-4 to the division remnant (Figure 10, B and C). To determine whether localization was sufficient for rescue, we tethered ZEN-4 to the Aurora kinase AIR-2/AuroraB, which is enriched at the division remnant partially overlapping with CYK-4 (Schumacher et al., 1998) and is required to localize ZEN-4 normally (Severson et al., 2000). A wild-type ZEN-4::AIR-2 fusion served as a control. AIR-2 was only weakly able to rescue ZEN-4(NeckΔ) despite complete localization to the division remnant (Figure 10, Table 4, and Supplemental Table S4). Embryos bearing ZEN-4(NeckΔ)::AIR-2 had 18% lethality (range 17–19%), compared with ~23% lethality for no-transgene control embryos (range 17–27%). The wild-type ZEN-4::AIR-2 control was fully functional (0–4% lethality for array+ embryos), demonstrating that AIR-2 did not interfere with ZEN-4 function. We next queried whether the function of ZEN-4 was simply to ensure that CYK-4 traffics to the division remnant. To test this possibility, we tethered CYK-4 to AIR-2. Although

FIGURE 6: FOXA2 binds to promoter and introns of human epithelial genes. Genome browser displays of FOXA2-binding peaks for selected genes from the ChIP-seq data set generated by Wang et al. (2015) from foregut-induced human embryonic stem cells. Significant FOXA2 peaks (MACS2; see Materials and Methods) are highlighted in gray. Only a single representative gene model is shown, although several have alternative variants.
the CYK-4::AIR-2 tether was indeed localized to the division remnant (Figure 10F), it was unable to rescue zen-4(px47) mutants (20% of both transgene+ and no-transgene control embryos displayed polarity defects; Table 4 and Supplemental Table S4). We conclude that accumulation at the division remnant is not sufficient for rescue and that both CYK-4 and ZEN-4 are needed for polarization.

CYK-4 activates the ATPase and kinesin activities of ZEN-4 on microtubules (Mishima et al., 2002; White et al., 2013; Tao et al., 2016). To determine whether microtubules were critical for ZEN-4 function in polarity, we mutated the P-loop and loop 12 within the kinesin domain (Figure 9A). The P-loop contains the ATPase activity of the kinesin (Saraste et al., 1990; Nakata and Hirokawa, 1995), and mutations in this domain in ZEN-4 homologues result in a motor-dead protein that can still bind microtubules (Chen et al., 2002; Matulienė and Kuriyama, 2002). Loop 12 of kinesins is important for binding to microtubules (Woehlke et al., 1997; Raich et al., 1998). These mutations had a mild defect on ZEN-4 localization and did not affect rescue (Figure 9, C and D, Table 3, and Supplemental Table S3). Thus we have no evidence that microtubule activities are important for ZEN-4 during polarity. Finally, we note that ZEN-4 normally localizes to nuclei during interphase. Disruption of two predicted nuclear localization signals (NLSs) strongly reduced nuclear accumulation but had no observable effect on rescue (Figure 9E, Table 4, and Supplemental Table S4). Similarly, mutation of either NLS1 or NLS2 alone did not affect ZEN-4 activity (Figure 9A, Table 3, and Supplemental Table S4). Similarly, mutation of either NLS1 or NLS2 alone did not affect ZEN-4 activity (Figure 9A, Table 3, and Supplemental Table S4). These data suggest that neither the kinesin function nor nuclear localization is necessary for polarity, although we cannot rule out that residual ZEN-4 in the nucleus or movement on microtubules is sufficient for rescue. In addition, nuclear localization is unperturbed in the NeckΔ mutant, which suggests that nuclear localization may not be sufficient for polarity.

CYK-4 can modulate actin dynamics by way of its GTPase-activating domain (Jenkins et al., 2006; Canman et al., 2008; Loria et al., 2012; Zhang and Glotzer, 2015; Zhuravlev et al., 2017). We previously determined that actin is severely disrupted in zen-4(px47) mutant arcade cells (Portereiko et al., 2004). Therefore we determined whether a gain-of-function allele of the RhoGEF ect-2, which suppresses the actomyosin-driven cytokinetic furrow ingestion defect of cyk-4 mutants (Zhang and Glotzer, 2015), could also rescue the polarity defect of zen-4 mutant arcade cells. We observed no suppression, as ect-2(xs110); zen-4(px47) double mutants had a similar percentage of embryos with unpolarized arcade cells as the zen-4 control (17 and 16%, respectively; Supplemental Table S5). These data do not rule out a role for actin dynamics, however, as the ect-2(gf) allele may not be strong enough to overcome the zen-4 polarity defect. In summary, ZEN-4 relies on its interaction with CYK-4 to induce polarity. On the other hand, the microtubule-related ATPase and kinesin activities of ZEN-4 are dispensable for epithelium formation.

PAR-6 is required to position DLG-1 within nascent arcade cell epithelia

The data suggest that PHA-4 and ZEN-4 control RNA and protein production of polarity factors in the arcade cells. Once produced, mislocalized (N). In the other 60% (21 of 35), DLG-1 is properly localized but fails to form continuous junctions, similar to the adjacent foregut (blue arrowhead; O). All images are maximum intensity projections. Anterior is to the left. Scale bars, 3 μm (A–F), 5 μm (G–L), 2 μm (M–O).
standard polarity pathways likely organize the apical, junctional, and basolateral domains within the arcade cells (Rodriguez-Boulan and Macara, 2014). We tested this idea by examining par-6 mutants. Because par-6 is important for the first embryonic cell divisions, we removed PAR-6 protein at the gastrula stage, using an elegant ZF1 system (par-6m+z-; Nance et al., 2003; Totong et al., 2007). par-6 mutants produced DLG-1 protein (Figure 7, N and O) but often failed to localize it properly to the junctional domains. Instead, we detected DLG-1 in the cytoplasm of par-6(m+z-) mutants (Figure 7N). These data reveal that temporal regulation of the arcade cell epithelium depends on three levels of tissue-specific control: first, at the transcriptional level; second, at the level of protein expression; and third, at the level of protein localization to nascent adherens junctions.

**DISCUSSION**

The past three decades have seen major advances in our understanding of cell polarization during MET (Chaffer et al., 2007). Little is known, however, about coordination between distinct epithelia to produce the mature body plan. Our data show that *C. elegans* epithelia polarize just before the morphogenetic event in which that epithelium is involved (e.g., epiboly, torus formation). In particular, the arcade cells delay polarization until after the epidermis and gut epithelia have matured sufficiently to withstand the pulling forces of embryo elongation. In support of this notion, if the arcade cells polarize before the epidermis is fully mature (e.g., as in fbn-1/fibrillin mutants), the epidermis is pulled into the embryo by pulling forces from the digestive tract, likely due to resistance of the foregut to stretching or lengthening (Kelley et al., 2015). We discovered four regulators that ensure delayed timing in arcade cells: PHA-4/FoxA initiates expression of polarity genes within the arcade cells, ZEN-4/MKLP1 and CYK-4/MgcRacGAP dictate the onset of protein accumulation, and PAR-6 controls subcellular localization within the nascent epithelium.

**FIGURE 8:** ZEN-4 activity is required at arcade cell birth. (A) The temperature-shift strategy. Rectangles denote developmental time (scaled to 20°C). Blue represents permissive temperature (15°C), and red denotes restrictive temperature (26°C). The control sample was incubated at permissive temperature only. The early shift started ∼50 min before arcade cell birth, the late shift started ∼50 min before arcade cell polarization, and the mid shift is in between. Representative DIC (B–E) and DLG-1 (F–I) images of embryos after the foregoing incubations. The foregut-associated basement membrane (BM) is highlighted by a red dotted line. The control, mid, and late embryos all have a polarized arcade cell epithelium spanning the epidermis and foregut. Note that the BM surrounds an elongated foregut. The early shift results in an unattached foregut because the arcade cells (arrowhead) failed to polarize (C, G). Note that the BM completely surrounds the foregut primordium, which is unattached to the anterior epidermis (C), and that DLG-1 is not expressed in the arcade cells (G). (J) The epidermal temperature-shift strategy. The epidermal cells are born over a longer period of time than the arcade cells (gray), and this period encompasses the time at the restrictive temperature (red). Embryos were shifted back to permissive temperature before the onset of epidermal polarization. (K, L) Representative maximum intensity projections through the epidermis of embryos at the end of the shift. In the control embryo (K), DLG-1 (white) is found at the cell junctions of epidermal cells that have formed two rows on the dorsal surface. These cells are mononucleate. In the shifted embryo (L), the epidermal cells generate and localize DLG-1 properly, even though many cells are obviously multinucleate (arrowhead) due to cytokinesis defects. Anterior is to the left. Scale bars, 5 μm.

**PHA-4/FoxA regulates a complete epithelial program**

The FoxA pioneer transcription factor PHA-4 promotes the onset of dlg-1 expression within the foregut and arcade cells but not other cell types. In addition to dlg-1, the majority of apical, junctional, and basolateral genes had an associated PHA-4 ChIP peak (Figure 5 and Table 1); most associated either with the TSS or within the first intron,
which are hallmarks of bona fide PHA-4 target genes (Gaudet and Mango, 2002; Gaudet et al., 2004). All of these genes are also expressed in other epithelia (or other cell types). We confirmed that expression for two of these factors (PAR-3, ERM-1) was selectively reduced in the foregut and arcade cells (Figure 3). This rules out the possibility of a single epithelial program regulated by a common set of transcription factors in all epithelial organs and suggests that the mechanism of timing is controlled at the tissue and organ levels by tissue/organ-specific transcription factors such as PHA-4.

The P₄dlg-1 construct was able to recapitulate the endogenous expression of DLG-1 protein in the foregut/arcade cells and the epidermis (Figure 4). A study of the C. elegans transcriptional start site and enhancer landscape identified a putative enhancer for dlg-1 at ~6 kb upstream of the ATG start (Chen et al., 2013). Our analysis revealed that this enhancer was not necessary for normal dlg-1 expression in the foregut/arcade cells, at least in the context of a complex multicopy array. It is possible that this region functions as a shadow enhancer that promotes robust dlg-1 expression but is not essential (Hong et al., 2008; Perry et al., 2010). In addition, the P₄dlg-1 construct carried a heterologous 3′ untranslated region (UTR), suggesting that the endogenous 3′UTR is not necessary for proper expression in the foregut and arcade cells. Perhaps regulation occurs through the 5′UTR, as suggested for some posttranscriptional events in C. elegans (Lee and Schedl, 2004; Jungkamp et al., 2011; Vora et al., 2013). Note that in the midgut, some cells expressed P₄dlg-1 RNA one cell cycle earlier (unpublished data), suggesting additional cis-regulatory sites for this tissue. The appropriate expression in the foregut and arcade cells. Perhaps regulation occurs through the 5′UTR, as suggested for some posttranscriptional events in C. elegans (Lee and Schedl, 2004; Jungkamp et al., 2011; Vora et al., 2013). Note that in the midgut, some cells expressed P₄dlg-1 RNA one cell cycle earlier (unpublished data), suggesting additional cis-regulatory sites for this tissue.
An appealing hypothesis is that the arcade cells depend on an extra protein, whereas other epithelia displayed no or a much shorter delay. (Portereiko is disrupted in lectivity of Snyder, 2002; Slaughter serves as a marker for the next division (reviewed in Casamayor and Pohl, 2014; Dionne the dorsoventral axis in C. elegans, 2009; Bi, 2017), and therefore they could be functioning in a similar manner in polarity. Another possibility is that ZEN-4 and CYK-4 act as a scaffold to promote accumulation of polarity proteins at the appropriate time.

Tethering of mutant ZEN-4 to the division remnant gave either full (CYK-4) or partial (AIR-2) rescue (Figure 10, Table 4, and Supplemental Table S3). This result raises the intriguing possibility that failure in epidermal enclosure and subsequently elongation. To assess for rescue, compare the percentage unpolarized between the transgene-negative (control) and transgene-positive columns for a given transgenic line. For example, pxEx390 displays 15% unpolarized arcades for the transgene-negative embryos, whereas only 0.7% of the transgene-positive embryos have unpolarized arcades and thus are considered to rescue the phenotype. By these criteria, only the NeckΔ construct fails to rescue the arcade cell polarity defect of zen-4(px47) mutants (compare red text to black text in the same column). The n for a given phenotype in a given transgenic line is in parentheses.

**TABLE 3: ZEN-4 structure–function analysis.**

| Construct                      | Array | Transgene negative, percentage unpolarized (n) | Transgene positive, percentage unpolarized (n) |
|-------------------------------|-------|------------------------------------------------|-----------------------------------------------|
| No-transgene control (SM1052) | n/a   | 20 (91/448)                                     | n/a                                           |
| Wild type                     | pxEx390 | 15 (17/115)                                     | 0.7 (1/150)                                   |
|                               | pxEx392 | 15 (8/54)                                       | 0 (0/92)                                      |
|                               | pxEx393 | 12 (8/67)                                       | 0 (0/136)                                     |
|                               | pxEx396 | 16 (18/115)                                     | 0 (0/128)                                     |
| P-loopΔ                       | pxEx395 | 20 (12/60)                                      | 0 (0/52)                                      |
|                               | pxEx399 | 12 (22/181)                                     | 2 (5/246)                                     |
|                               | pxEx402 | 15 (16/107)                                     | 0 (0/89)                                      |
|                               | pxEx486 | 17 (14/83)                                      | 1 (1/82)                                      |
|                               | pxEx488 | 13 (4/29)                                       | 1 (1/72)                                      |
| Loop12Δ                       | pxEx422 | 18 (25/136)                                     | 1.5 (2/129)                                   |
|                               | pxEx424 | 17 (30/181)                                     | 0 (0/363)                                     |
|                               | pxEx489 | 14 (7/51)                                       | 0 (0/157)                                     |
| NLS1Δ                         | pxEx397 | 18 (8/44)                                       | 2 (2/82)                                      |
|                               | pxEx423 | 16 (16/101)                                     | 0 (0/67)                                      |
|                               | pxEx425 | 16 (6/41)                                       | 5 (2/43)                                      |
| NLS2Δ                         | pxEx398 | 18 (58/324)                                     | 0.6 (2/327)                                   |
|                               | pxEx420 | 19 (13/67)                                      | 0 (0/94)                                      |
| NLS1/2Δ                       | pxEx455 | 16 (15/91)                                      | 1.5 (2/134)                                   |
|                               | pxEx456 | 11 (12/110)                                     | 0 (01/132)                                    |
| NeckΔ                         | pxEx391 | 17 (32/189)                                     | 20 (44/222)                                   |
|                               | pxEx444 | 16 (39/119)                                     | 19 (47/249)                                   |

The left column displays the ZEN-4 structure/function construct expressed in the listed transgenic lines. SM1052 is the reference strain with genotype zen-4 dpy-20/ bli-6 unc-24, maintained as a heterozygote. The progeny, by Mendelian genetics, should be 75% wild type and 25% mutant, of which the majority displays the unpolarized arcade cell phenotype (assessed by Nomarski optics, looking for foregut attachment, which occurs via arcade cell polarization), whereas a subset display a failure in epidermal enclosure and subsequently elongation. To assess for rescue, compare the percentage unpolarized between the transgene-negative (control) and transgene-positive columns for a given transgenic line. For example, pxEx390 displays 15% unpolarized arcades for the transgene-negative embryos, whereas only 0.7% of the transgene-positive embryos have unpolarized arcades and thus are considered to rescue the phenotype. By these criteria, only the NeckΔ construct fails to rescue the arcade cell polarity defect of zen-4(px47) mutants (compare red text to black text in the same column). The n for a given phenotype in a given transgenic line is in parentheses.

Data. Previous studies revealed that ZEN-4 and CYK-4 can modulate actin dynamics by regulating RhoA and Rac activity (Jenkins et al., 2006; Canman et al., 2008; Loria et al., 2012; Zhang and Glotzer, 2015; Zhuravlev et al., 2017), and therefore they could be functioning in a similar manner in polarity. Another possibility is that ZEN-4 and CYK-4 act as a scaffold to promote accumulation of polarity proteins at the appropriate time.

Tethering of mutant ZEN-4 to the division remnant gave either full (CYK-4) or partial (AIR-2) rescue (Figure 10, Table 4, and Supplemental Table S3). This result raises the intriguing possibility that localization to the remnant may contribute to ZEN-4 function. The division remnant has been studied most extensively in yeast, where the bud scar remains to be seen whether ZEN-4 function is conserved.

Once DLG-1 and other polarity proteins are produced, they are rapidly localized within the cell. We found that par-6 was necessary for localization of DLG-1 within the arcade cells in ~50% of tested embryos (Figure 7). These data extend our previous observations that in half of the tested embryos, several markers of epithelial polarity (actin, HMR-1/cadherin, ERM-1, AJM-1) failed to localize properly within the arcade cells in par-6 mutants (von Stetina and Mango, 2015). In the other half, protein is localized properly (like DLG-1) but fails to form continuous junctions between other arcade cells and between tissues (i.e., foregut and arcades). In other epithelia, this failure to mature junctions is the most severe par-6
A. Tethered-Dimer Analysis

| Construct | # lines | Rescue? |
|-----------|---------|---------|
| ZEN-4     | 4       | +       |
| ZEN-4:CYK-4 | 3      | +       |
| ZEN-4(Neck Δ) | 2      | -       |
| ZEN-4(Neck Δ):CYK-4 | 2 | + |
| ZEN-4:AIR-2   | 3       | +       |
| ZEN-4(Neck Δ):AIR-2 | 3 | +/- |
| CYK-4:AIR-2   | 1       | -       |

FIGURE 10: ZEN-4 tethered dimer analysis demonstrates the importance of the ZEN-4/CYK-4 interaction. (A) Experimental design. Wild-type ZEN-4 or ZEN-4(NeckΔ) were tethered to either GFP-tagged CYK-4 or AIR-2 (AuroraB kinase) via a flexible linker (black line). CYK-4 was tethered to GFP-tagged AIR-2 (bottom construct); note that additional lines were generated, and these did not express or rescue; unpublished data). The number of lines and ability to rescue the zen-4(px47) arcade cell polarization defect are shown. The untethered ZEN-4 or the ZEN-4(NeckΔ) data from Figure 9 are shown for reference. See Table 4 and Supplemental Table S4 for more details. (B–F) Live imaging of GFP-tagged ZEN-4 tether constructs at the 16E stage. Single focal plane through the digestive tract. Top, a merge of DIC and tether::GFP (green), which is also displayed alone in the middle row. Most signal (green) is found at division remnants (white arrowheads, bottom) by colocalization with known division remnant markers UNC-59/septin (Nguyen et al., 2000) or AIR-2 (Schumacher et al., 1998) (both white). UNC-59 is enriched at the division remnant after furrow ingress. Anterior is left. Scale bars, 5 μm (whole embryo), 2 μm (close-ups).

MATERIALS AND METHODS

Strains and maintenance

Nematodes were grown at 20°C using standard conditions (Brenner, 1974), unless otherwise stated. Some strains were provided by the Caenorhabditis Genetics Center. Strains used: N2 (SM1880); SM469 [pxIs6 (pha-4::GFP::Histone)] (Portereiko and Mango, 2001); SM1052 [zen-4 (px47) dpy-20(e1282)/bli-6(sc16) unc-24(e138) IV] (Portereiko et al., 2004); SM1271 [zen-4 (px47) jcs1(AJM-1::GFP, pRF4)/bli-6(sc16) unc-24(e138) IV] (Portereiko et al., 2004); SU93 [jcs1(AJM-1::GFP, pRF4)] (McK oppen et al., 2001); F2223 [mclsl204 (p(dlg-t::DLG-1::GFP, pRF4))] (McMahon et al., 2001); J1743 [par-6(tm1425)/him-1(unc-54(h1040) I; him-8(e1489) IV] (Totong et al., 2007); FT36 [unc-101(m1) par-6(e170)]; zils43 (pie-1::GFP::PAR-6::ZF1 + unc-119(+))] (Totong et al., 2007); and EU554 [zen-4(or153) IV] (Severson et al., 2000). For a description of the par-6MZ genetic strategy, see Von Stetina and Mango (2015). Strains generated for this study are listed in Supplemental Table S6.

Temperature-shift experiments

zen-4(or153ts) was maintained at 15°C. To perform arcade cell temperature shifts on staged embryos, gravid adults were transferred to a precooled (4°C) dissection chamber with 50 μl of ice-cold M9 Buffer (22 mM KH2PO4, 22 mM Na2HPO4, 85 mM NaCl, 1 mM MgSO4). Embryos were released by dissecting the adults using 26-gauge needles. Two-cell embryos were quickly collected and transferred via mouth pipette to a 0.2-ml PCR tube or to a polylysine-coated slide on ice. Once 10–30 two-cell embryos were collected, the tube was placed in a Bio-Rad PCR machine precooled to 15°C or the slide was placed into a humidified chamber at 15°C. Embryos were incubated at 15°C until the appropriate stage was reached and then shifted to 26°C to inactivate zen-4. After the inactivation period was over, embryos were shifted back to 15°C until the 1.5- to 2-fold stage, when embryos from tubes were transferred to a 4% agar pad on a glass slide to assess foregut attachment by differential interference contrast (DIC) microscopy or the slides were processed for immunostaining (see later description). Incubation times were determined based on Sulston’s embryonic lineage timing at 20°C and adjusted accordingly; 15°C was considered 1.5 times slower than 20°C, whereas 26°C was considered 1.2 times faster. Timing for specific stages for N2 and zen-4(or153ts) was determined with test incubations (unpublished data). The specific programs for each shift were as follows:

Early shift (circa arcade cell birth): 360 min at 15°C (equivalent of 240 min at 20°C), 70 min at 26°C (equivalent of 84 min at 20°C), and 270 min at 15°C (equivalent to 180 min at 20°C).

Mid shift (between birth and polarization): 480 min at 15°C (equivalent of 320 min at 20°C), 41 min at 26°C (equivalent of...
49.2 min at 20°C), and 140 min at 15°C (equivalent to 93 min at 20°C).

Late shift (circum polarization): 540 min at 15°C (equivalent of 360 min at 20°C), 70 min at 26°C (equivalent of 84 min at 20°C), and 60 min at 15°C (equivalent to 50 min at 20°C).

Temperature shifts for epidermal cells were performed in a similar manner, in which dissected two-cell embryos were transferred to a polylysine-coated slide and incubated in a humidified chamber at the appropriate temperature. Control embryos were incubated at 15°C for 525 min (equivalent of 350 min at 20°C). Shifted embryos were incubated for 195 min at 15°C (equivalent of 130 min at 20°C), 70 min at 26°C (equivalent of 84 min at 20°C), and 205 min at 15°C (equivalent to 137 min at 20°C). The slides were then processed for immunostaining.

Embryos for arcade cell shifts were scored for unattached foreguts using DIC optics on a Zeiss AxioImager M2 with Apotome. Alternatively, embryos from the arcade cell shifts were fixed and immunostained for DLG-1 expression and imaged using a Zeiss LSM880 confocal microscope. To determine whether the canonical phenotype associated with zen-4ts was obtained, embryos were processed and incubated using the early shift settings but removed from the protocol 10 min after returning to the permissive temperature. These embryos were adhered to polylysine slides, fixed, and immunostained for ERM-1 expression and imaged via structured-illumination on a Zeiss AxioImager M2 with Apotome. Embryos for epidermal experiments were fixed, immunostained to detect DLG-1, and assessed for multicell cell and polarity using a Zeiss LSM880 confocal microscope.

Molecular biology

Supplemental Table S7 lists the primers used. For all PCR reactions, either AccuStart HiFi Taq Polymerase (QuantaBio) or PrimeStar Taq Polymerase (Takara) was used. pML902 (Pdlg::DLG-1::GFP::unc-54 3’UTR; McMahon et al., 2001) was used as the template to generate P4I::dlg::DLG-1::GFP::unc-54 3’UTR (Supplemental Figure S6), P4I::dlg::DLG-1::GFP::unc-54 3’UTR, and P4I::dlg::DLG-1::GFP::unc-54 3’UTR. After amplification, a Qiagen PCR purification kit was used to clean up the reaction. The PCR products were subsequently used for injection to generate transgenics (see later description).

ZEN-4::AIR-2 (bsem1129) was generated by swapping out GFP for yellow fluorescent protein (YFP) from bsem1105 (ZEN-4::YFP) using Ncol and BamHI. bsem1129 contains genomic DNA from 3 kb upstream of the predicted start site, the entire coding region up to the stop codon, fused with GFP and the unc-54 3’UTR from pPDF5.77 (Addgene plasmid 1495, a gift from Andy Fire, Stanford University).

ZEN-4 deletion constructs were generated by a variation of overlap PCR (Hobert, 2002), where the overlap primers contained sequence adjacent to the region to be removed and replaced with a Ksr restriction site. Supplemental Table S7 lists the specific nucleotides (and the corresponding amino acids) removed in each construct. Nuclear localization sites were predicted by PSORTII (http://psort.bio.nig.ac.jp/form2.html); Nakai and Horton, 1999). bsem1129 was used as the template to generate each 5’ and 3’ fragment, which were cleaned using the Qiagen PCR purification kit. We used 10 ng of each 5’ and 3’ fragment for the overlap reaction. Overlap products were gel-purified (Qiagen) and cloned into pGEM-T (Promega) or pCR4Blunt-TOPO (Invitrogen). The NLS1/NLS2 double deletion was generated by subcloning a Bcl-generated fragment from bsem1231 (ZEN-4::NLS1::GFP) into the Bcl site of bsem1210 (ZEN-4::NLS2::GFP). Constructs were verified to have the expected nucleotides removed, and no deleterious mutations, by Sanger sequencing.

CYK-4::GFP (bsem1289) was generated via overlap PCR by amplifying the Caenorhabditis briggsae CYK-4 genomic coding sequence (up to the stop codon) from bsem1093 and fusing it to GFP::unc-54 3’UTR from pPDF5.77. AIR-2::GFP (bsem1129) was generated via overlap PCR by amplifying the AIR-2 genomic coding sequence (up to the stop codon) from N2 genomic DNA.

The left column displays the tether construct in the listed transgenic lines (Array). SM1052 is the reference strain with genotype zen-4 dpy-20 bli-6 unc-24, maintained as a heterozygote. The progeny should be 75% wild type and 25% mutant, of which the majority displays the unpolarized arcade cell phenotype. To assess for rescue, compare the percentage unpolarized between the transgene-negative (control) and transgene-positive columns for a given transgenic line. A comparison of transgene-negative to transgene-positive progeny reveals that restoring CYK-4 binding to ZEN-4(NeckΔ) is able to rescue the arcade cell polarity defect of zen-4(ts47) mutants, whereas binding to AIR-2 may partially rescue. Restoring CYK-4 to the division remnant in the absence of ZEN-4 via tethering to AIR-2 is unable to rescue, demonstrated by the quantified line and the 13 additional lines containing either wild-type or kinase-dead AIR-2 (unpublished data). Polarity defects were scored by Nomarski optics, assessing whether the foregut had attached to the epidermis, which occurs via arcade cell polarization. Red lettering is indicative of a failure to fully rescue (compare to black text within the same column).

TABLE 4: Tethered dimer analysis.

| Construct          | Array | Transgene negative, percentage unpolarized (n) | Transgene positive, percentage unpolarized (n) |
|--------------------|-------|-----------------------------------------------|-----------------------------------------------|
| No-transgene control (SM1052) | n/a   | 20 (91/448)                                   | n/a                                           |
| ZEN-4::CYK-4       | pxEx518| 10 (9/96)                                      | 0.6 (1/170)                                   |
|                    | pxEx519| 16 (13/80)                                     | 3.6 (5/137)                                   |
|                    | pxEx541| 13 (22/171)                                    | 0.5 (1/206)                                   |
| ZEN-4(NECKΔ)::CYK-4 | pxEx516| 11 (12/109)                                    | 0.1 (1/74)                                    |
|                    | pxEx517| 12 (46/398)                                    | 0.1 (3/285)                                   |
| ZEN-4::AIR-2       | pxEx532| 15 (34/228)                                    | 0.7 (1/145)                                   |
|                    | pxEx543| 18 (6/33)                                      | 0 (0/135)                                     |
|                    | pxEx544| 22 (14/63)                                     | 4 (3/82)                                      |
| ZEN-4(NECKΔ)::AIR-2 | pxEx523| 20 (26/128)                                    | 13 (25/194)                                   |
|                    | pxEx524| 12 (17/142)                                    | 13 (20/155)                                   |
|                    | pxEx556| 22 (22/101)                                    | 15 (25/172)                                   |
| CYK-4::AIR-2       | pxEx635| 18 (13/186)                                    | 20 (63/314)                                   |
and fusing it to GFP::unc-54 3'UTR from pPD95.77. The resulting overlap PCR products were subcloned into pCR4-TOPOBlunt and sequence verified. Each half of the ZEN-4 tether constructs was generated by PCR, with the added tether sequence as an overhang. The flexible tether sequence chosen has successfully generated forced dimers in Drosophila (Neuhold and Wold, 1993; Castanon et al., 2001).

To generate the CYK-4::AIR-2 tether, CYK-4 with a modified tether in the 3' primer (second codon changed to generate a KpnI site; see Supplemental Table S7 for more details) was amplified from bsem1093 and subcloned into pCR4-TOPOBlunt to create bsem1480. The tether that precedes AIR-2::GFP was also modified to the KpnI version using overlap PCR and subcloned into pCR4-TOPOBlunt to create bsem1336. The KpnI/Sph fragments from bsem1480 and bsem1336 were ligated together to create bsem1481 (Pcyk-4::CbCYK-4::Kpn tether::AIR-2::GFP TOPO). Overlap PCR was used to remove K59 (Bishop and Schumacher, 2002; Rogers et al., 2002) and four additional amino acids to generate a kinase-dead version of AIR-2::GFP, which was subcloned into pCR4-TOPOBlunt to create bsem1483. The KpnI/Sph fragments bsem1481 and bsem1483 were ligated together to create bsem1484 (Pcyk-4::CbCYK-4::Kpn tether::AIR-2(KD)::GFP TOPO). Overlap PCR was used to merge the zen-4 promoter from bsem1336 and CYK-4::Kpn tether from bsem1484, and the final product was subcloned into pCR4-TOPOBlunt to create bsem1485 (Pzen-4::CbCYK-4::Kpn tether TOPO). The KpnI/Sph fragments from bsem1336 and bsem1485 were ligated together to create bsem1486 (Pzen-4::CYK-4::tether::AIR-2::GFP). The KpnI/Sph fragments from bsem1484 and bsem1485 were ligated together to create bsem1487 (Pzen-4::CYK-4: tether::AIR-2(KD)::GFP). We used both kinase-intact and kinase-dead versions of AIR-2, and observed no rescue with either tether.

Pelt-7::mCherry_pGEM-T (bsem1146), Pdp-13::mCherry_pGEM-T (bsem1178), and Pelt-7::mCherry::HIS-58/H2B_pGEM-T (bsem1177) were generated by overlap PCR. The 2.9-kb elt-7 promoter was amplified from wild-type genomic DNA and fused with either mCherry::unc-54 3'UTR from pCFJ90 (Addgene plasmid 19327; a gift from Christian Frokjaer-Jansen and Erik Jorgensen, University of Utah; Frokjaer-Jensen et al., 2008) or with mCherry::H2B::unc-54 3'UTR from bsem1145. bsem1145 was generated by releasing mCherry::HIS-58/H2B from pie-1::mCherry::HIS-58/H2B (a gift from Jon Audhya, University of Wisconsin–Madison; McNally et al., 2006) using BamHI (NEB) and subcloning this fragment into the BamHI site of bsem1176 (unc-54 3'UTR TOPO). The 260-base pair dpy-13 promoter was amplified from wild-type genomic DNA using primers with attB1 and attB2 overhangs. The resulting product was recombined into pDONR221 using BP Clonase II (Invitrogen) to create bsem1184. The dpy-13 promoter was then amplified from bsem1184 and fused with mCherry::H2B::unc-54 3'UTR amplified from bsem1145. The overlap products were then subcloned into pGEM-T (Promega).

**Microinjection/transgenic generation**

We injected 2 ng of BamHI-linearized ZEN-4::GFP or of the deletion constructs described earlier (as purified overlap PCR products or linearized plasmids digested with either SapI or SphiI) into SM1052 (zen-4(pkx47) dpy-20(e1282)/ blades-6(sce16) unc-24(e138)) along with 5–10 ng of linearized bsem1146 (Ncol), bsem1177 (Ncol), and/or bsem1178 (SapI; fluorescent coselectable markers), 55 ng of EcoRI-linearized pRF4 (rol-b(d) coselectable marker), and salmon testes DNA (amount necessary to bring the total DNA in the injection mix to 100 ng; D1626; Sigma-Alrich) to generate complex array-containing Roller animals.

ZEN-4 tether constructs were generated by in vivo recombination in the nematode gonad. Each half of the tether was generated by PCR and injected in equimolar amounts along with linearized bsem1177, bsem1178, pRF4, and salmon testes DNA.

We injected 2 ng of SphiI-linearized bsem1486 into SM1052, along with 10 ng of Ncol-linearized bsem1177, 55 ng of EcoRI-linearized pRF4, and 33 ng of salmon testes DNA (D1626; Sigma-Alrich) to generate a CYK-4::AIR-2 expressing transgenic line.

We injected 1 ng/μl Pdlg-1–containing purified PCR product (see earlier description), 55 ng/μl linearized pRF4 (rol-b(d) coselectable marker), and 44 ng/μl Salmon Testes DNA (D1626; Sigma-Alrich) into N2 (SM1880) to generate complex array-containing Roller animals.

**RNAi**

RNAi was performed as described (Timmons et al., 2001), with minor modifications. A single bacterial colony (either mCherry as a negative control or pha-4) was picked to grow in 6 ml of Luria broth plus antibiotic (25 μg/ml kanamycin) for 8 h at 37°C. Bacteria were pelleted and resuspended in an isopropyl-β- thiolgalactoside (IPTG)/antibiotic mixture (8 mM IPTG, 25 μg/ml kanamycin), and 40 μl was spread onto a 35-mm Nematode Growth Medium (NGM; Sternagel, 2006) plate. The plates were covered with foil and incubated at room temperature for 48 h before use. N2 gravid adults were bleached to release eggs, which were hatched overnight in M9 without food to synchronize the population. Starved L1s were transferred to OP50-containing NGM plates and grown to the L4 stage at 20°C. L4s were transferred to a conical tube and washed in M9 two or three times to remove all traces of OP50 bacteria. Approximately 80 L4s were dropped onto an mCherry or a pha-4 RNAi plate and incubated at 25°C for 24 h. The now adults were 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 fixed with paraformaldehyde (PFA) immediately for immunostaining.

**Live imaging**

Embryos were placed on a 4% agar pad (Difco Noble Agar) in 10 mM levamisole. A #1.5 coverslip (Corning) was added, and nail polish was added at the corners to prevent the coverslip from moving. Embryos were scored using Nomarski optics for foregut attachment defects under a Zeiss AxioImager M2 running AxioVision or ZEN2 software. For live imaging of GFP fluorescence, embryos were imaged using a Zeiss LSM710, LSM780, or LSM880 confocal microscope. For AJM-1::GFP quantification in SU93 and SM1271 animals, defects under a Zeiss AxioImager M2 with Apotome or Apotome.2 software. For live imaging of GFP fluorescence, embryos were imaged using either an AxioImager M2 with Apotome or Apotome.2 to remove out-of-focus light or the Zeiss LSM710, LSM780, or LSM880 confocal microscope. For AJM-1::GFP quantification in SU93 and SM1271 animals, z-stacks through the foregut/arcade cells were taken using the same gain and laser power for each embryo. The z-stacks were rotated in Zen Blue software (Zeiss) and culled of any noninformative slices. These modified stacks were imported into Fiji software (Schindelin et al., 2012). The region of interest containing the arcade cells was cropped out of original image and changed to 16 bit, and the stack was z-projected using the Sum Slices setting and then measured to obtain the mean gray value and area. To control for differences in area between samples, the average intensity per pixel was calculated by dividing the mean gray value by the area.

**Immunostaining**

Embryos were placed on poly-L-lysine (P-8920; Sigma-Aldrich)–coated slides in 50 μl of water and processed using the freeze-crack method (Duerr, 2006; Shakes et al., 2012). After cracking, slides
were incubated in ice-cold MeOH for 8 min. The exception was for pha-4(RNAi)-processed embryos, for which the water was removed and replaced by PFA fix (2% PFA diluted from a 16% methanol-free stock [Alfa Aesar] in 1x phosphate-buffered saline [PBS]) and then incubated for 3 min in ice-cold MeOH after cracking. Slides were blocked in TNB buffer (100 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1% bovine serum albumin) with 10% normal goat serum (NGS; 102643-580; VWR). All antibodies were also diluted using TNB plus 0.1% NGS. The following antibodies were used: mouse anti–ERM-1 (1:10; Developmental Studies Hybridoma Bank [DSHB]; Hadwiger et al., 2010); mouse anti–PAR-3 (1:10; P4A1; DSHB; Nance et al., 2003); rabbit anti–PHA-4 (1:1000; Kaltenbach et al., 2005); mouse anti–PSD95, which detects DLG-1 (MA1-045; 1:250; Pierce; Firestein and Rongo, 2001); rabbit anti–PAR-6 (1:1000, from Tony Hyman, Max Planck Institute; Hoege et al., 2010); rabbit anti–AIR-2 (1:200; from Jill Schumacher, MD Anderson Cancer Center; Schumacher et al., 1998); rabbit anti–UNC-59 (1:440; from Amy Maddox, University of North Carolina at Chapel Hill; Maddox et al., 2005); mouse anti–GFP (1:500; MAB3580; EMD/Millipore); and chicken anti–GFP (1:200; AB16901; EMD/Millipore). As noted, ERM-1 and PAR-3 antibodies were obtained from the DSHB, which was developed under the auspices of the National Institute of Child Health and Human Development and maintained by the Department of Biology, University of Iowa (Iowa City, IA). Secondary antibodies conjugated to Alexa 488, Alexa 568, or Alexa 647 were obtained from Invitrogen. Images were obtained on a Zeiss LSM780 or 880 confocal microscope. Images were exported from ZEN Blue (Zeiss), and maximum intensity projections were generated in ZEN Black (Zeiss) and rotated, resized, and cropped in Adobe Photoshop.

**smFISH procedure and data processing**

smFISH was performed mostly as described (Raj and Tyagi, 2010) and is summarized here with some modifications. Custom Stellaris FISH Probes were designed against dlg-1, erm-1, par-6, and gfp by using the Stellaris FISH Probe Designer (Biosearch Technologies, Petaluma, CA; www.biosearchtech.com/stellarisdesigner). Exact probe sequences are given in Supplemental Table S7. dlg-1, erm-1, and par-6 probes were labeled with Quasar 570 dye, pha-4 probes were labeled with CalFluorRed610 or CalFluorOrange635, and gfp was labeled with Quasar 670. Probes were resuspended in 400 μl of TE (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0), aliquoted, and stored at –20°C. Probes in use were stored at 4°C. One to five 60-μm NGM plates with gravid adults and many laid embryos were washed with water to remove adults and larvae into a 15-ml conical tube. To release the laid embryos, additional water was added to the plate and lightly rubbed with a gloved fingertip. After one or two washes in double-distilled H2O (ddH2O), pelleted embryos and worms were transferred to a 1.5-ml centrifuge tube and pelleted. To release the young embryos from the gravid adults, 100–200 μl of bleach solution (240 μl of bleach, 50 μl of 5 M NaOH, and 710 μl of ddH2O) was added to the pellet and incubated for 5 min at room temperature using a thermomixer (Eppendorf) to mix the sample every 30 s. The embryos were quickly washed three times using M9 buffer. The pelleted embryos were then fixed in 100–200 μl of 3.7% formaldehyde solution (100 μl of 37% formaldehyde, 100 μl of 10x PBS, 800 μl of ddH2O) for 15 min at room temperature using a thermomixer to mix the sample every 3 min. The tube was flash-frozen in liquid nitrogen for 1 min to freeze-crack the eggshells and then either stored at –80°C or processed immediately. The frozen sample was thawed at room temperature and then washed twice with 200 μl of 1x PBS. The pelleted embryos were resuspended in 200 μl of 70% EtOH and stored overnight at 4°C. The next day, the EtOH was exchanged for wash buffer (0.5 ml formamide, 0.5 ml 20x saline–sodium citrate [SSC; Invitrogen], 4 ml of ddH2O), and then the sample was pelleted. A 50-μl amount of hybridization buffer (100 μl of denoized formamide [Calbiochem Omnipur/EMD Millipore], 0.1 g of dextran sulfate [Acros], 100 μl of 20x SSC, 800 μl of ddH2O) containing smFISH probes was added to the embryos (0.75 μl for dlg-1 and pha-4 probes, 1 μl for erm-1 and par-6 probes, and 2 μl for gfp probes) and incubated for 4 h at 37°C in a thermomixer, with mixing every 15 min. The embryos were rinsed once quickly using wash buffer and then washed for 1 h at 37°C in a thermomixer (mixing every 5 min). Pelleted embryos were resuspended in 30–50 μl of ddH2O and then added to polylysine-coated slides and allowed to settle. The ddH2O was removed, and 7 μl of SlotFade with 4’,6-di-amidino-2-phenylindole (DAPI) was added. A #1.5 coverslip was added, excess mounting material was wicked away with a Kimwipe, and it was sealed with clear nail polish. Slides were imaged immediately or stored at –20°C until ready to image. Images were obtained on a Zeiss LSM880 confocal microscope with a Plan-Apochromat 63×/1.4 oil objective. For imaging dlg-1 and gfp transcripts, 568- and 633-nm laser lines were used. To spectrally distinguish Quasar 570 and CalFluorRed610 or CalFluorOrange635 (e.g., imaging dlg-1 and pha-4 in same embryo), we excited Quasar570 with a 514-nm laser and adjusted the GAASP detector range on the LSM880 to acquire emission wavelengths between 561 and 596 nm; we excited CalFluorRed610 or CalFluorOrange635 with a 594-nm laser and acquired emission wavelengths between 623 and 694 nm. GFP protein expression survived the fixation method and could be visualized with a 488-nm laser. Selected z-stacks that encompassed the digestive tract but excluded the epidermis were exported from ZEN Blue (Zeiss) and imported into Imaris 3D software (www.bitplane.com) for converting the raw smFISH signal into painted spheres. For quantitation of transcripts in the arcade cells, zen-4 dpy-20/mls11 (SM2300) embryos were processed as described and hybridized with pha-4 probes plus dlg-1, par-6, or erm-1 probes. The zen-4/+ versus zen-4 mutant embryos were determined by the presence or absence, respectively, of GFP expression from the balancing transgene. Embryos betweencomma and 1.25-fold (stages before or after arcade cell polarization) were imaged using a Zeiss LSM880 confocal. Z-stacks encompassing the arcade cells (identified as the cells anterior to the foregut that expressed pha-4 RNA) were selected and imported into Imaris for quantitation. Only the region corresponding to the arcade cells was quantified. To determine statistical significance, the Wilcoxon rank-sum test was used in GraphPad Prism. In all scatter plots, the mean is shown by the long horizontal line and the 95% confidence intervals by the shorter horizontal lines. For dlg-1 and associated pha-4 quantitation, p = 0.9312 and 0.1636, respectively. For par-6 and associated pha-4 quantitation, p = 0.0862 and 0.1171, respectively. For erm-1 and associated pha-4 quantitation, p = 0.9551 and 0.1453, respectively.

**PHA-4 and FoxA ChIP-seq analysis**

For the C. elegans analysis, bed files from the early and late embryo PHA-4 ChIP-seq data sets (as well as the MACS2 peak analysis bed file; Zhong et al., 2010) and the Ahringer and Meyer TSS data sets (Chen et al., 2013; Kruesi et al., 2010) and the Ahringer and Meyer TSS data sets were downloaded and visualized in the Broad Institute Integrative Genomics Viewer (Robinson et al., 2011; Thorvaldsdottir et al., 2013). Genes known to be expressed in epithelial tissue (those listed in Table 1 and Supplemental Table S1) were queried, and a gene was annotated as PHA-4 bound if a MACS2-called PHA-4 peak was found upstream, at the TSS, or within the first intron.
For the mammalian analysis, FoxA1/FoxA2 ChIP-seq data were downloaded as peak calls for the following data sets: FoxA2 ChIP-seq from mouse liver (Soccio et al., 2011); FoxA1 ChIP-seq from HEPG2 cells (Motallebipour et al., 2009); FoxA2 ChIP-seq from Caco2 cells (Gosalia et al., 2015); and FoxA1 and FoxA2 from human embryonic stem cells differentiated into gut tube or foregut cells (Wang et al., 2015). For the Caco2 data set, two replicates were downloaded, and the intersection was used for subsequent analysis. For FoxA1 ChIP-seq from MCF7 cells (Hurtado et al., 2011), two replicates in FASTQ format were obtained and then processed to call peaks. Quality trim of reads was performed using Trimmomatic (Bolger et al., 2014), mapping of treatment replicates and control input to genome was performed using Bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml; Langmead and Salzberg, 2012), and peaks were called using MACS2 (https://pypi.python.org/pypi/MACS2; Zhang et al., 2008). The intersection of the replicate peaks was generated and used for subsequent analysis.

Where needed, the input files were lifted to either mouse mm10 (GRCm38) or human hg38 (GRCh38) genomes using the UCSC LiftOver program (http://genome.ucsc.edu/cgi-bin/hgLiftOver). Mouse and human gene annotation, as well as mouse and human orthologues of C. elegans genes, were downloaded from Ensembl before release 80. FoxA peaks that were up to 1000 base pairs upstream, up to 1000 base pairs downstream, or within the gene body of mouse and human genes were identified, and the final output was made up of homologues of the 50 C. elegans PHA-4-bound, epithelial-specific genes (Table 1) that had FoxA binding as per these parameters. A manual inspection was performed to determine the percentage of genes that had FoxA binding upstream or within the first or second intron. An affirmative call did not exclude binding in other areas beyond upstream or introns 1 and 2.

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