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Current Biology

MRCK-1 Drives Apical Constriction in *C. elegans* by Linking Developmental Patterning to Force Generation

Highlights

- MRCK-1 is a key regulator of apical constriction during *C. elegans* gastrulation
- It functions by activating myosin and cortical tension to drive apical constriction
- CDC-42 signaling and developmental patterning direct MRCK-1 localization
- MRCK-1 and myosin recruit adherens junction proteins in the constricting cells

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In Brief

Marston et al. show that the myosin kinase MRCK-1 is a key regulator of cortical tension driving apical constriction in *C. elegans* gastrulation. MRCK-1 links cell and developmental patterning to local activation of force-generating motors, and MRCK-1-regulated myosin activation also results in a local recruitment of important adhesion proteins.

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Article

MRCK-1 Drives Apical Constriction in *C. elegans* by Linking Developmental Patterning to Force Generation

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SUMMARY

Apical constriction is a change in cell shape that drives key morphogenetic events including gastrulation and neural tube formation. Apical force-producing actomyosin networks drive apical constriction by contracting while connected to cell-cell junctions. The mechanisms by which developmental patterning regulates these actomyosin networks and associated junctions with spatial precision are not fully understood. Here we identify a myosin light-chain kinase MRCK-1 as a key regulator of *C. elegans* gastrulation that integrates spatial and developmental patterning information. We show that MRCK-1 is required for activation of contractile actomyosin dynamics and elevated cortical tension in the apical cell cortex of endoderm precursor cells. MRCK-1 is apically localized by active Cdc42 at the external, cell-cell contact-free surfaces of apically constricting cells, downstream of cell fate determination mechanisms. We establish that the junctional components α-catenin, β-catenin, and cadherin become highly enriched at the apical junctions of apically constricting cells and that MRCK-1 and myosin activity are required in vivo for this enrichment. Taken together, our results define mechanisms that position a myosin activator to a specific cell surface where it both locally increases cortical tension and locally enriches junctional components to facilitate apical constriction. These results reveal crucial links that can tie spatial information to local force generation to drive morphogenesis.

INTRODUCTION

Morphogenesis is driven by forces produced within individual cells [1]. The molecular machines that produce these forces must be localized precisely within cells. Understanding the links between developmental biology and cell biology that can determine exactly where force-producing mechanisms are positioned is fundamental to understanding how complex morphologies form.

Apical constriction, the shrinking of apical cell surfaces, is a cell shape change that drives diverse tissue shape changes, including gastrulation in many systems and neural tube formation in vertebrates [2]. Apical constriction is driven by contraction of networks composed of actin filaments and non-muscle myosin II that are localized near apical cell surfaces and that connect to adhesive, apical cell-cell junctions [3]. In cells undergoing apical constriction, these networks can be organized in at least two types of structures: junctional belts that are found at cell-cell junctions and that contract via a purse-string mechanism [3, 4], and medio-apical networks that crisscross the entire apical cortex [5]. Recent experiments in diverse animal systems demonstrate that medio-apical networks are under tension and contribute forces that drive cell shape change [6–8]. To understand apical constriction mechanisms, we are investigating how these medio-apical networks, and the junctions to which they connect, are deployed and maintained with spatial and temporal precision by developmental patterning mechanisms.

The gastrulation movements in the early *Caenorhabditis elegans* embryo are a valuable system to address these questions. The internalization of the endoderm precursor cells (EPCs) occurs through contraction of apical actomyosin networks [9, 10]. There exists a strong understanding of how embryonic cell fates are specified in *C. elegans* [11], as well as an understanding of how the embryonic cells become polarized along their apicobasal axis [12]. Apicobasal polarization in the early embryo is regulated by a system that distinguishes apical cell surfaces, which are free of contacts with other cells, from basolateral surfaces, which make contact with other embryonic cells. The current model for apicobasal polarization involves classical cadherins recruited basolaterally—to sites of cell-cell contact—through homotypic binding of cadherin ectodomains. Cadherin cytoplasmic tails then sequentially recruit p120-catenin, the coiled-coil protein PICC-1, and finally the Rho family GTPase-activating protein (RhoGAP) PAC-1, which locally inactivates CDC-42 at sites of cell-cell contact [13]. In a set of elegant experiments, it was shown that

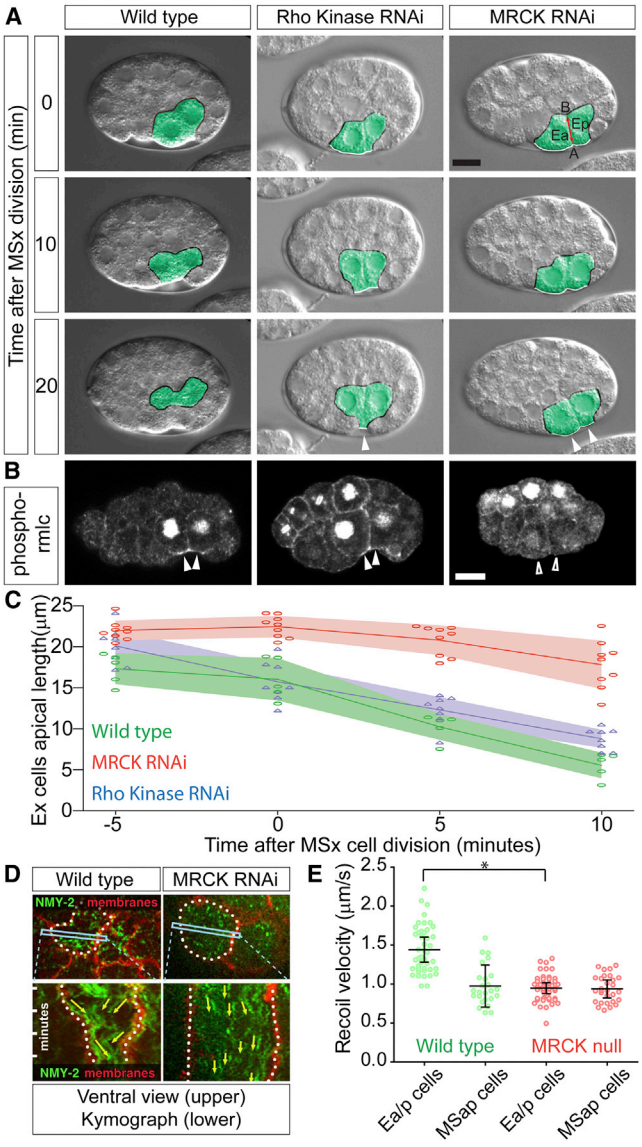

Figure 1. MRCK-1 and Rho Kinase Are Required for Early Gastrulation Movements in *C. elegans*, and MRCK-1 Regulates Apical Myosin

(A) Internalization of the two EPCs (Ea and Ep, pseudocolored green). The exposed surfaces of these cells (marked with white border, changed to black when covered) become completely covered by the neighboring cells within 20 min of MSx division. In Rho Kinase (*let-502*) and *mrck-1* RNAi embryos, there were still exposed surfaces at the 20-min time point (arrowheads). Each panel is a single midsagittal optical section from a differential interference contrast (DIC) time-lapse movie, with time since MSx division noted. In this and all figures, embryos are oriented with anterior to the left and dorsal up. In the top right panel, Ea and Ep are labeled, and the apical (A)-basal (B) axis of the EPCs is denoted by a red line. Scale bar represents 10 μm.

(B) Phosphorylated regulatory myosin light chain (rmlc) localization. Activated myosin was detectable at the apical cortex of EPCs in embryos with a temperature-sensitive mutation in Rho kinase at the non-permissive temperature (arrowheads), but it was absent from *mrck-1* RNAi embryos (empty arrowheads). The antibody used also strongly marks nuclei prior to nuclear envelope breakdown and during cell division, which we consider as background staining because it is not eliminated by RNAi targeting rmlc [23]. Scale bar represents 10 μm. Quantification from multiple embryos is found in Figure S1A.

(C) Quantification of the length of the apical (exposed) surfaces of the EPCs in multiple embryos. Each point represents an individual embryo, and the lines represent the mean of several embryos (*mrck-1* RNAi n = 9, Rho kinase n = 8, and wild-type n = 5). Shading represents 95% confidence interval (95% CI).

(D) Myosin (NMY-2, green) and membranes (marked using the PH domain from PLCδ, red, marked with dotted white line) were visualized in ventral mounts. A kymograph derived from a line across an EPC (box) showed that, as the endoderm cell apical surfaces contracted, myosin foci moved toward the center of the apical cortex in a wild-type, but not in an *mrck-1* RNAi, embryo.

(E) Recoil velocity of myosin particles in embryos that have been cut by UV laser incision, as a measure of relative cortical tension (Movie S1 [7]). Each point represents a single particle measured. The average recoil velocity was calculated in each embryo, and bars represent the mean of multiple embryos (wild-type Ea/p n = 9 embryos, MSap n = 5, *mrck-1*-null Ea/p n = 10, and MSap n = 6; *p = 0.029). Error bars in all graphs are 95% CI.

See also Figure S1 and Movie S1.

generating ectopic cell contacts can change in predictable ways the localization of cadherin, PAC-1, and other polarity proteins in *C. elegans* embryos, confirming that this system relies on positional information defined by sites of cell-cell contact [13, 14]. Many of these proteins show conserved interactions in mammalian cells [15, 16], but how these apicobasal polarization mechanisms deploy force-producing mechanisms to specific parts of cells is not well understood in any system.

For actomyosin-based contractile forces to drive changes in tissue shape, the forces must be mechanically propagated to neighboring cells. The cadherin-catenin complex (CCC) has been shown to be a force-bearing link between the actomyosin cortices of adjacent cells [17, 18]. Interestingly, actomyosin dynamics, regulated by Rho family small GTPases, have been shown to have significant effects on the behavior of cadherin-catenin-based adherens junctions. However, the nature of these effects can vary from system to system. For example, actomyosin-based contractility can enhance junctional stability in some systems [19] and promote junctional turnover in other systems [20]. During *Drosophila melanogaster* gastrulation, there is an apical enrichment of adherens junctions, which is lost if apical constriction is inhibited [19, 21], and during *Xenopus* gastrulation, myosin activity leads to altered C-cadherin dynamics [22]. These studies raise the interesting possibility that modulation of actomyosin networks might result in enrichment of junctional complexes at network connection sites.

Here we have identified molecular links between developmental patterning mechanisms and cytoskeletal force-producing mechanisms as a step toward understanding how developmental patterning can accurately position both force-producing and force-transmitting systems. We report the identity of a myosin activator, MRCK-1, that is required for activation of myosin in the apical cortex of the gastrulating cells and for apical constriction in vivo. We further show that MRCK-1-regulated myosin contractility is a key regulator of cell-cell adhesion components during these cell movements.

RESULTS

MRCK-1 Is Required for Myosin Activation, Cortical Tension, Apical Constriction, and Early Gastrulation in *C. elegans*

In wild-type embryos, two EPCs, called Ea and Ep, move from the embryo's surface to its interior starting at the 26- to 28-cell stage (Figure 1A). Other cells move in later, but, for convenience,

we refer here to the internalization of the endoderm precursors as gastrulation. We have shown previously that myosin is activated apically in these endoderm precursors through phosphorylation of myosin regulatory light chain [23]. We therefore searched for molecular links between developmental patterning mechanisms and cytoskeletal force-producing mechanisms by seeking to identify myosin activators required for *C. elegans* gastrulation. The myosin light-chain kinases (MLCKs) LET-502 (Rho-associated kinase) and MRCK-1 (myotonic dystrophy kinase-related Cdc42-binding kinase) are known to activate myosin and are essential for normal *C. elegans* development [24, 25]. LET-502 is required for early cytokinesis, while both LET-502 and MRCK-1 have roles in embryonic elongation. Unlike traditional MLCKs, MRCK family kinases and Rho-associated kinases are not regulated by calcium binding. Instead, their activities are modulated by interaction with the Rho family small GTPases and other binding partners [26, 27].

We used double-stranded RNA (dsRNA) injection in adult *C. elegans* to disrupt either *let-502* or *mrck-1* gene functions in their progeny, and we used live-cell time-lapse microscopy to look for gastrulation defects. In embryos in which we targeted *mrck-1* by RNAi, the EPCs consistently failed to move inward (Figure 1A) and instead divided on the embryo surface (16/16 embryos). In these embryos, the apical surfaces of the endoderm cells constricted significantly more slowly than in wild-type embryos (Figure 1C). Failure of the EPCs to internalize also was seen in embryos from adults homozygous for an *mrck-1*-null mutant, *mrck-1*(*ok586*) (embryos born from heterozygotes are rescued by maternal contributions but their progeny fail to develop to adults). Endoderm precursors left on the embryo surface do move in just prior to the next cell division (the division of four E lineage cells to eight cells), suggesting that a redundant mechanism can rescue the cell internalization defect.

It is impossible to produce embryos completely deficient for LET-502/Rho kinase because LET-502 functions in cytokinesis, and null embryos fail to develop [25]. Therefore, we performed partial LET-502 knockdown by imaging embryos 18 hr post-injection, when there were still embryos developing without early cytokinesis defects. In these embryos, we saw a low penetrance defect (3/13) in which the EPCs moved inward but incompletely, leaving one or both of the EPCs exposed to the embryonic exterior after division (Figure 1A). Similar results were seen using a temperature-sensitive allele of LET-502 (*let-502*(*sb118ts*)) shifted to the non-permissive temperature prior to dissection (4/12 embryos with gastrulation defects compared with 0/8 for wild-type embryos similarly shifted). Quantification of the apical membrane length of the dsRNA-treated embryos over time showed that the apical membranes contracted at a rate similar to that in wild-type embryos (Figure 1C).

We next examined myosin activation in these kinase-deficient embryos. In contrast to wild-type embryos, *mrck-1*(*RNAi*) embryos contained little or no detectable phosphorylated regulatory myosin light chain in the apical domain of the EPCs, suggesting that MRCK-1 is required for the activation of apical myosin in these cells (Figure 1B; Figure S1A). We found that the pattern of activated regulatory myosin light chain in the *let-502*(*sb118ts*) embryos at the non-permissive temperature was similar to that of wild-type embryos (Figure 1B; Figure S1A). We conclude that MRCK-1 is required for the activation of apical myosin during these cell movements and that LET-502 is likely active at this time and makes at least a minor contribution to gastrulation.

To determine whether MRCK-1 upregulates myosin-based cortical tension in the EPCs, we measured myosin movements and cortical tension in *mrck-1*(*RNAi*) or *mrck-1*-null mutant embryos (see Movie S1 for an example). Centripetal movements of myosin from the edges toward the center of apical cell cortexes, which reflect contractile behavior of the actomyosin cortex [7], are almost completely absent in the EPCs of *mrck-1*(*RNAi*) embryos (Figure 1D).

We used laser-cutting experiments to estimate tension in the apical cortex of gastrulating cells. As we had shown previously [7], the EPCs showed significantly higher cortical tension than their neighboring mesoderm precursor cells (Figure 1E). In *mrck-1*-null mutant embryos, the apical cortex of EPCs had lower tension, indistinguishable from that of neighboring cells (Figure 1E). Therefore, we conclude that MRCK-1 is required during gastrulation of the EPCs to activate myosin and thereby increase actomyosin contractility and cortical tension.

We examined whether MRCK-1 also is required for later cell internalization events by imaging embryos through the stage at which mesoderm precursor cells of the MS lineage internalize. In 4/8 *mrck-1*(*ok586*) embryos, internalization of MS-derived cells occurred normally, while in the remaining cases, MS descendants failed to internalize correctly and in some cases divided aberrantly on the surface of the embryo (Figure S1B). Because we cannot determine whether the failure of MS cells to internalize is a secondary consequence of mis-positioning of EPCs, we conclude that MRCK-1 may play either a direct or indirect role in the internalization of mesodermal precursor cells. Therefore, we focused on the EPCs in our further experiments to understand MRCK-1 regulation and function.

MRCK-1 Localizes to the Apical Domains of Cells Undergoing Apical Constriction

Because we found that MRCK-1 is required to activate myosin and increase cortical tension specifically in the apical cortex of internalizing endoderm precursors, we hypothesized that MRCK-1 might be localized to the apical cell cortex of EPCs. Alternatively, MRCK-1 could be distributed more widely, and other mechanisms could limit where myosin can be activated by MRCK-1. To study MRCK-1 localization at its endogenous levels, we used CRISPR-Cas9-triggered homologous recombination [28] to tag the endogenous *mrck-1* locus with the yellow fluorescent protein YPet, chosen because of its high brightness and the lower auto-fluorescence in embryos excited at longer wavelengths (Figure S2). Our approach offers the following three distinct advantages over other tagging methods that have been used commonly in developmental model systems: (1) since endogenous loci are tagged, most if not all native transcriptional regulatory elements are preserved; (2) 100% of the protein of interest is fluorescently labeled (i.e., there is no unlabeled population); and (3) since the genes tagged here are all essential, the viability of homozygous animals carrying the tagged genes can be used to determine whether the tagged proteins are functional. The outcrossed MRCK-1::YPet line showed wild-type levels of embryonic viability (Figure S2) and had no discernible gastrulation phenotype, suggesting that the tagged protein functioned normally.

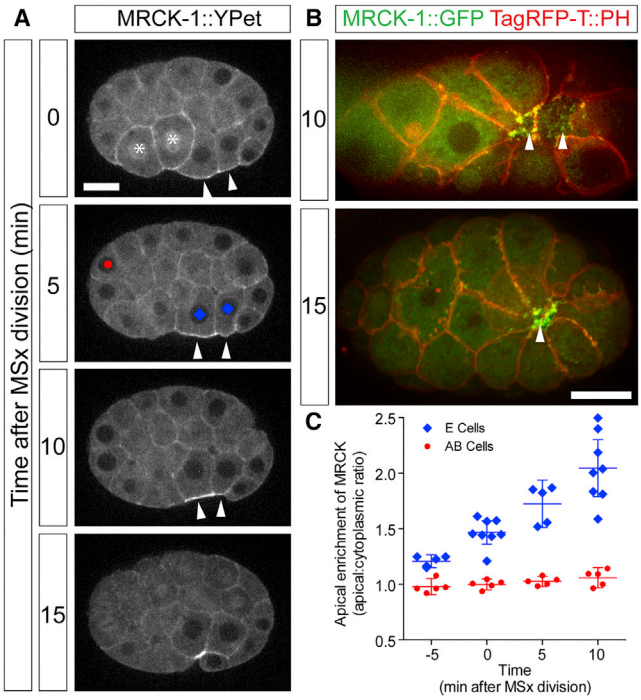

Figure 2. MRCK-1 Accumulates Apically in EPCs during Gastrulation

(A) MRCK-1::YPet accumulated at the apical surface (arrowheads) of the EPCs (labeled with blue diamonds) during gastrulation (Movie S2). Cells marked with asterisk are dividing. Scale bar represents 10 μm.

(B) Ventral views. MRCK-1::GFP (green) decorated the exposed surfaces of the EPCs. Cell membranes were marked with the PH domain of PLCδ tagged with TagRFP-T (red).

(C) MRCK-1::YPet accumulated apically specifically in the EPCs. Each point represents average apical enrichment in denoted cell type in a single embryo (symbols match those drawn at 5-min time point in A). Bars represent means of multiple embryos ±95% CI.

See also Figure S2 and Movie S2.

We found that MRCK-1::YPet was enriched in the apical cortex specifically in the gastrulating EPCs (Figure 2A; Movie S2). A GFP-tagged strain constructed similarly showed similar localization and allowed us to co-visualize MRCK-1 and an mCherry-tagged PH domain to show membranes (Figure 2B), revealing in ventral views that MRCK-1::GFP localized to puncta across the medio-apical surfaces of EPCs as well as to apical cell-cell borders (Figure 2B). Fluorescently tagged MRCK-1 was not enriched in the apical domain of other early embryonic interphase cells, although some enrichment could be seen in the cortex of dividing cells (Figure 2A). In the gastrulating EPCs, enrichment was detected starting from 5 min prior to MSx division (MSx indicating the two daughters of the MS cell), and within 15 min it had reached 2-fold enrichment over cytoplasmic levels (Figure 2C). This was significantly different from non-dividing, non-internalizing AB cells, where little enrichment over cytoplasmic levels was detected in the same time frame. Comparison of the time course of apical constriction and MRCK-1 localization (Figures 1 and 2) shows that apical enrichment of MRCK-1::YPet is concurrent with or even precedes apical constriction and continues throughout the gastrulation movements of the EPCs, consistent with a role for MRCK-1 regulating myosin function during EPC cell movements.

Endoderm Cell Fate Specification Is Necessary and Sufficient for MRCK-1 Localization in the Appropriate Cells

Because apical enrichment of MRCK-1 was only seen in the EPCs, we tested whether cell fate specification contributes to the localization of MRCK-1. In *C. elegans*, endoderm specification is regulated by two partially redundant GATA transcription factors, END-1 and END-3. We crossed MRCK-1::YPet into an *end-3*-null mutant, *end-3*(*ok1448*), and then we targeted *end-1* by RNAi. We saw complete failure of gastrulation in these embryos, as seen previously for a chromosomal deficiency that deletes both genes [23, 29]. Furthermore, MRCK-1::YPet failed to become apically enriched in EPCs (Figure 3; Movie S3, quantified in Figure S3, $p < 0.001$).

To determine whether endodermal cell fate is not only necessary but also sufficient for apical MRCK-1 localization in these embryonic cells, we targeted the *pie-1* gene by RNAi. *pie-1* encodes a CCCH zinc-finger protein that represses RNA polymerase II-dependent gene expression in germline lineage cells. In its absence, germline lineage cells transform into somatic lineages, producing multiple cell types ectopically, including endodermal precursors in an *end-1*- and *end-3*-dependent manner [29, 30]. These additional endoderm precursors previously have been shown to undergo cell internalization shortly after the normal EPCs internalize [23]. In *pie-1*(*RNAi*) MRCK-1::YPet embryos, MRCK-1 was apically localized in the normal EPCs (Figure 3; Movie S3) and later also in the additional, ectopic EPCs during their internalization (Figure 3; Movie S3, quantified in Figure S3). We conclude that endoderm cell fate specification is necessary and sufficient for MRCK-1 to localize apically in the appropriate cells and that endodermal GATA factors play yet-to-be-characterized direct or indirect roles in this process.

Active Cdc42 Recruits MRCK-1 to the Apical Cell Cortex

MRCK-1 contains a conserved Cdc42- and Rac-interactive binding (CRIB) motif at its C terminus that is likely to mediate interactions with active CDC-42 [31]. To determine whether MRCK-1 is localized by active CDC-42, we inserted YPet into MRCK-1 directly upstream of the CRIB motif, adding two stop codons to YPet to produce a version of MRCK-1::YPet lacking a CRIB domain. We were unable to recover a CRISPR line with this construct; therefore, we used a fosmid-based transgene to express this fusion protein. A wild-type fosmid with an identical YPet insertion to the CRISPR line showed the same expression pattern of MRCK-1 as the CRISPR line (Figure S4A), and it was able to rescue an MRCK-1-null strain (*mrck-1*(*ok586*)). We found that the MRCK-1 ΔCRIB::YPet, which could not rescue the null strain, was no longer recruited to the cortex and instead appeared to be completely cytoplasmic (Figure 4; Movie S4; Figure S4B).

To further test whether MRCK-1 localization depends on recruitment to active CDC-42, we knocked down PAC-1, a GAP for CDC-42 that acts to exclude active CDC-42 from sites of cell-cell contact in the early *C. elegans* embryo [13, 14]. We predicted that if MRCK-1 was recruited to the apical cortex by active CDC-42, then targeting *pac-1* with dsRNA would result

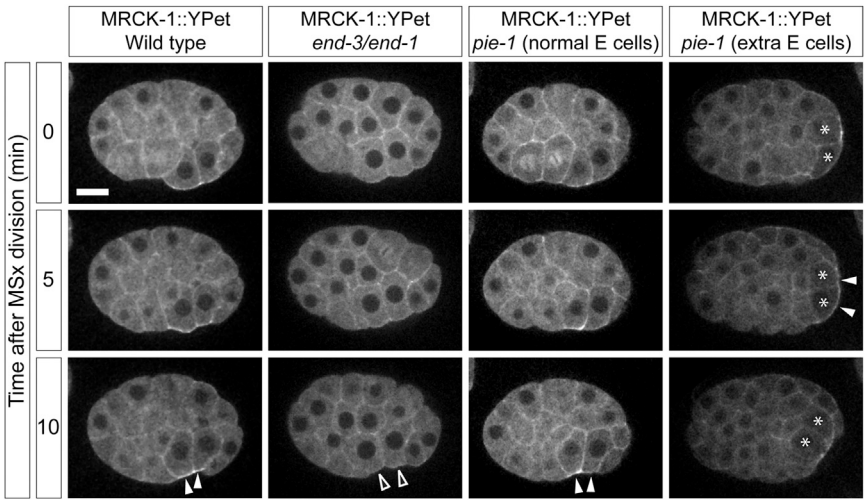

Figure 3. Cell Fate Specification Mechanisms Regulate MRCK-1 Accumulation

MRCK-1::YPet localization in wild-type embryos and embryos with altered cell fates (Movie S3). *end-3/end-1* refers to *end-3*(*ok1448*) treated with *end-1*(*RNAi*) and causes a loss of endoderm cell fate. *pie-1* refers to *pie-1*(*RNAi*), which causes additional cells to transform to the endodermal cell fate (extra E cells marked with asterisks). Accumulation is marked with closed arrowheads and failure to accumulate is marked with open arrowheads. Scale bar represents 10 μm. Quantification from multiple embryos is found in Figure S3. See also Figure S3 and Movie S3.

in ectopic localization of MRCK-1 to sites of cell-cell contact. Consistent with this, *pac-1*(*RNAi*) embryos showed normal enrichment of MRCK-1::YPet to the apical cortex of EPCs (Figure 4; Movie S4) and significant ectopic MRCK-1 recruitment to the site of contact between the two EPCs (Figure 4, graph), which was dependent on the CRIB domain of MRCK-1 (Figure S4C). MRCK-1 might activate myosin and cortical tension at this ectopic site, because we sometimes saw an atypical indentation between the Ea and Ep apical surfaces (3/8 *pac-1* (*RNAi*) embryos), suggesting that the Ea/Ep border was shortening (Figure 4; Movie S4). Finally, embryos depleted of CDC-42 at gastrulation stage by tagging CDC-42 with a ZF-1 domain, depleted this way to allow it to function earlier when it is needed for polarization of the zygote [14], showed a similar gastrulation defect to the *mrck*-1 mutant embryos (Figure S4D, 12/13 embryos). Examination of myosin localization in embryos where CDC-42 was partially depleted by RNAi showed that CDC-42 is required to recruit myosin to the apical membranes of the EPCs (Figure S4E). Taken together, the requirement of CDC-42 for gastrulation movements and myosin recruitment, the requirement for MRCK-1's CRIB domain for cortical localization, and the ectopic localization of tagged MRCK-1 in *pac-1* mutant embryos suggest that MRCK-1 is recruited to the apical cortex in the EPCs by active CDC-42.

MOM-5/Frizzled Contributes to MRCK-1 Localization

We previously identified an additional developmental input to myosin regulation during *C. elegans* gastrulation. We showed that myosin activation was dependent upon Wnt-Frizzled signaling [23]. To determine whether Wnt-Frizzled signaling also affects MRCK-1 localization, we targeted the Wnt receptor Frizzled (MOM-5) by RNAi in the MRCK-1::YPet strain, and we quantified the MRCK-1 apical recruitment in these embryos (Figure 4; Movie S4). Knockdown of MOM-5 by RNAi results in a partially penetrant gastrulation-defective phenotype and also has effects on cell-cycle length in EPCs [23]. To avoid the confounding effects of cell-cycle length alteration, we scored *mom-5*(*RNAi*) embryos that had gastrulation defects and normal cell-cycle timing in the EPCs. In these embryos, MRCK-1 recruitment to the apical cell cortex was still detected but was significantly reduced compared to the wild-type levels (Figure 4; Movie S4), suggesting that Frizzled signaling contributes to *C. elegans* gastrulation and myosin activation at least in part by contributing to apical MRCK-1 recruitment.

Taken together, our results suggest that multiple mechanisms contribute to the spatial specificity of MRCK-1 localization. Cell fate specification mechanisms contribute by determining in which cells MRCK-1 becomes recruited to the cell cortex. MRCK-1 is then recruited to the contact-free cell surfaces via its CRIB domain binding to the active CDC-42-decorated, apical cell cortex. Once at this site, MRCK-1 is required for myosin activation and contractile dynamics of actomyosin in the cell cortex, which increase cortical tension and drive the cell movements.

The CCC Enriches at Apical Junctions in Internalizing Cells

For MRCK-1-mediated myosin activation to drive apical constriction, actomyosin networks must connect to junctional complexes at the edges of the apical cell cortex. Cortical tension has been proposed to play important roles in adherens junction complex formation and stabilization in purified proteins and in cultured cells [19, 21, 32]. Cadherin complex proteins are known to function in *C. elegans* gastrulation, albeit redundantly, by promoting the linking of actomyosin networks to membranes at apical cell-cell contact zones [7, 33], but little is known about how these junctional proteins become localized. Therefore, we sought to examine the localization of the cadherin complex proteins and then to determine whether MRCK-1 and myosin activity contribute to junctional complex localization and/or stabilization.

To track the in vivo localization of junctional components, we used CRISPR to make GFP insertions into the endogenous loci encoding the three essential proteins of the CCC (Figure S2A). We were able to produce viable homozygous strains for HMR-1/cadherin-GFP, HMP-1/α-catenin-GFP, and GFP-HMP-2/β-catenin. β-catenin was particularly sensitive to the GFP insertion site as multiple insertion sites abrogated gene function (Figure S2B). Our tagging results suggest that prior transgene studies may have used GFP fusions in which tagged proteins were not fully functional [34]. We used strains in which tagging endogenous loci resulted in 99%–100% homozygote viability (Figure S2B) for further experiments.

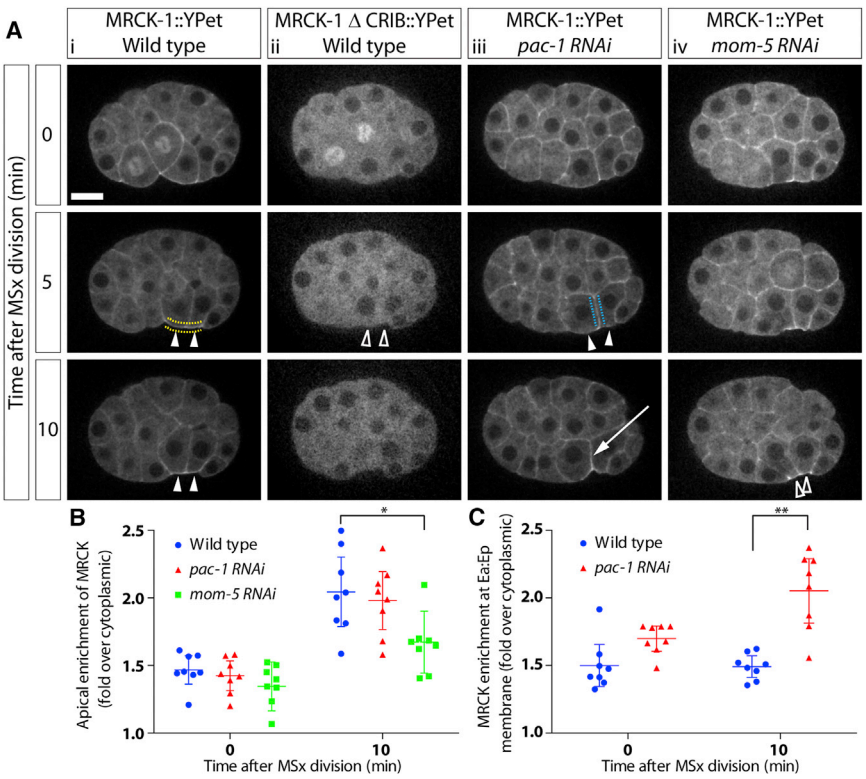

Figure 4. MRCK-1 Localization Is Regulated by Rho Family GTPases and Wnt Signaling

(A) (i) MRCK-1::YPet localization in wild-type embryos is shown. (ii) MRCK-1 ΔCRIB::YPet localization in wild-type embryos is shown; accumulation is quantified in Figure S4. (iii) MRCK-1::YPet localization in *pac-1*(*RNAi*) embryos is shown. (iv) MRCK-1::YPet localization in *mom-5*(*RNAi*) embryos is shown. Apical accumulation is marked with closed arrowheads, failure to accumulate is marked with open arrowheads, and accumulation at basolateral membranes is marked with an arrow (Movie S4) Scale bar represents 10 μm.

(B) Quantification of accumulation at the apical membrane from multiple embryos (dotted yellow line shows quantified membranes). Each point represents average enrichment at denoted membrane in a single embryo. Bars represent mean of multiple embryos ±95% CI (wild-type n = 8, *pac-1*(*RNAi*) n = 8, and *mom-5*(*RNAi*) n = 8; *p = 0.0209).

(C) Quantification of accumulation at the membrane between the two EPCs from multiple embryos (dotted blue line) is shown (wild-type n = 8 and *pac-1*(*RNAi*) n = 8; **p = 0.006).

See also Figure S4 and Movie S4.

Consistent with previous immunostaining studies [35, 36], endogenously tagged CCC components localized to sites of cell-cell contact in early embryos (Figures S5A, S5C, and S5E; Movie S5). The distribution of HMR-1/cadherin-GFP along these contacts was non-uniform, with enrichment often seen at apicolateral junctions: at sites of cell-cell contact close to the apical cell surface. Previous electron microscopy studies of gastrulation-stage embryos have not identified electron-dense adherens junction-like structures [9]. However, for convenience, we refer to these structures as adherens junctions based on the apicolateral enrichment of CCC components at cell-cell contacts. While several cell-cell contacts displayed some degree of this CCC polarization (Figures S5A–S5D), this pattern was most striking at the contact between the apically constricting EPCs, Ea and Ep (Figures S5E and S5F).

To examine whether junctions matured during apical constriction, we quantified the apicobasal polarization of the three CCC proteins along the Ea/Ep border during the period of apical constriction (Figures 5A–5F). We found that all three CCC proteins were enriched at the apical-most portion of cell-cell contacts between Ea and Ep throughout this period, with the enrichment being strongest late in apical constriction (Figures 5A–5F). These data together suggest that adherens junctions formed and/or were stabilized near the border of Ea and Ep, with specific enrichment proximal to the apical surface.

Although the enrichment of CCC proteins was the most obvious at the Ea/Ep apical contact, we also observed CCC enrichment at apical contacts between the EPCs and their neighbors. We sought to quantify in detail which junctions bordering the E cells accumulated cadherin over time. We measured the levels of cadherin-GFP at the cell-cell contact between the two EPCs and between the EPCs two neighboring cells, MSap and P_4 (Figures 5G and 5H). The intensity of HMR-1/cadherin-GFP displayed the largest fold increase at the apical contact between Ea and Ep, but it also displayed a significant increase between Ea and MSap. The level of HMR-1/cadherin-GFP did not display a significant increase at the contact between Ep and P_4. Taken together, these results reveal detailed dynamics of CCC components imaged in vivo using fluorescent tags inserted into native loci. The enrichment of CCC components to the edges of apical cortexes under high tension [7] suggested to us that tension might upregulate CCC enrichment, leading us to test whether connections to force-producing components are required for CCC enrichment in vivo in this system.

Cadherin Requires α- and β-Catenin for Apical Junction Enrichment

To test whether tension and connections are necessary, we next sought to determine whether HMR-1/cadherin-GFP required β-catenin and α-catenin (Figure S2) to become enriched apically. We used RNAi to knock down the transcripts encoding these proteins and observed the localization of HMR-1/cadherin-GFP. In an independent set of experiments, we assessed quantitatively the extent of HMP-1 and HMP-2 protein knockdown by RNAi. We did this by first mounting three sets of embryos side by side: embryos expressing the fluorescent tags (GFP-HMP-2/β-catenin or HMP-1-GFP/α-catenin), wild-type unlabeled embryos, and embryos expressing the fluorescent tags but with the tagged components targeted using dsRNA. This method makes it possible to quantitatively assess knockdown in individual embryos and at localization sites of interest, for example, at cell-cell junctions. In all cases, the level of fluorescence in the

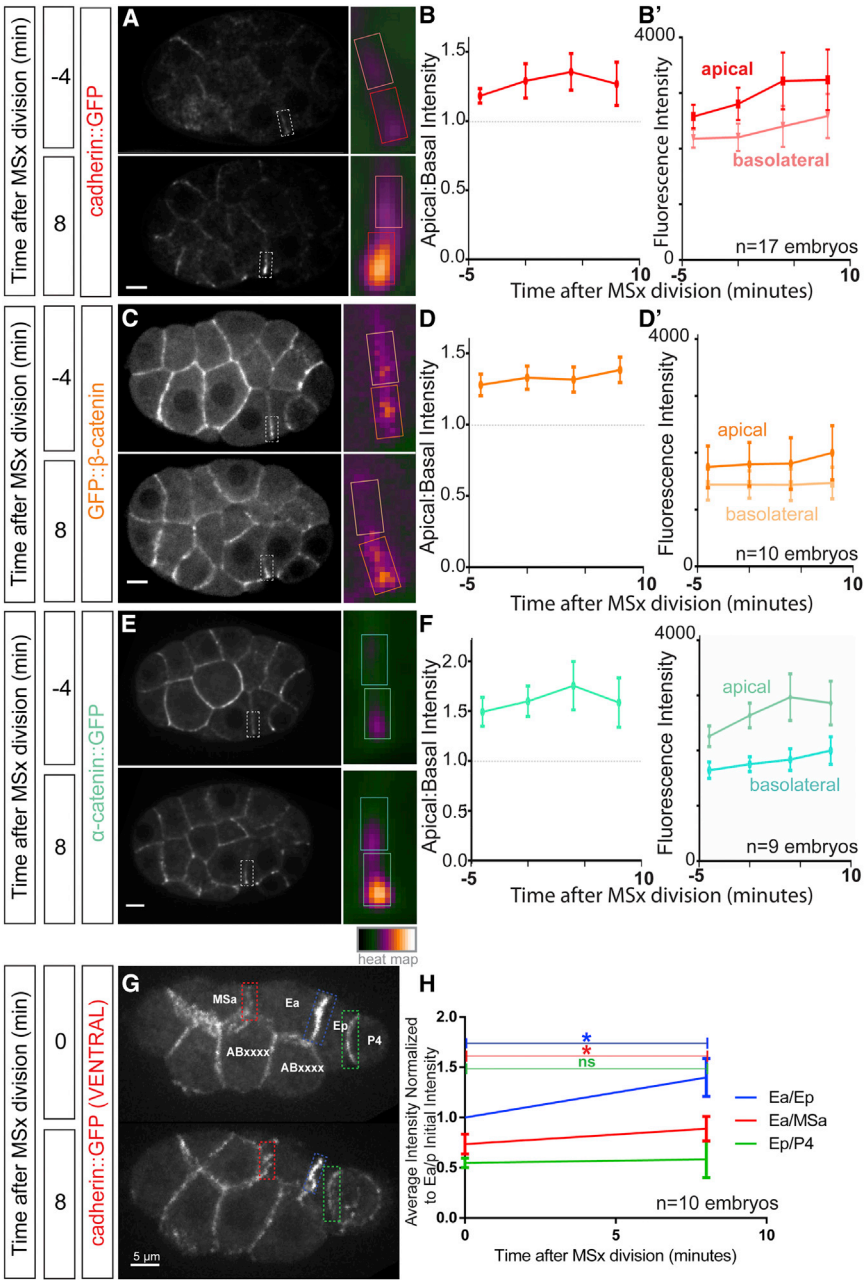

Figure 5. The CCC Polarizes along the Apical-Basal Axis of Junctions of Apically Constricting Cells

(A, C, and E) Spinning disk confocal fluorescence images of cadherin-catenin complex (CCC) components tagged with GFP. The border between the apically constricting E cells is highlighted with a dotted white box, which is enlarged and pseudo-colored in the inset (right). Areas used for quantification of apical and basolateral intensities in (A), (C), and (E) are circumscribed with colored boxes. (B, D, and F) Plots depicting apical-to-basal intensity ratio of fluorescence of CCC components. Apical (dark colors) and basolateral (light colors) fluorescence intensity values over time for HMR-1/cadherin-GFP, GFP-HMP-2/β-catenin, and HMP-1/α-catenin-GFP are plotted in (B'), (D'), and (F'), respectively.

(G and H) Spinning disk confocal fluorescence images of HMR-1/cadherin-GFP-expressing, ventrally mounted embryos at 0 and 8 min following MSa/p cell division initiation. Cell identities are labeled, and the borders quantified in (G) are highlighted with colored dotted boxes corresponding to the symbols, lines, and error bars depicted in (H). Fluorescence intensity was normalized to the brightness of Ea/Ep at the time of MSa/p cell division initiation. Significant increases in HMR-1/cadherin accumulation are observed over time at Ea/Ep and Ea/MSa borders, but not at Ep/P_4 (n = 10 embryos). Error bars represent 95% CI (*$p < 0.05$).

See also Figure S5 and Movie S5.

tagged, knockdown embryos was indistinguishable from that in wild-type unlabeled embryos, indicating that RNAi was highly effective (Figure S6). We found that both β-catenin and α-catenin knockdown resulted in a reduction or complete loss of polarization of HMR-1/cadherin-GFP along the apical-basal axis (Figures 6B, 6C, and 6F), suggesting that cadherin requires β-catenin and α-catenin in order to localize properly in this system.

Actomyosin Contractility Regulates CCC Distribution in Apically Constricting Cells

Actomyosin-generated tension is predicted to be highest at the cell-cell contact between Ea and Ep, as both cells are actively generating high tension [7]. Given that this cell-cell contact was the site of the greatest CCC polarization along the apicobasal axis and that cadherin localization depended on β-catenin and α-catenin, which can link cadherin directly to actomyosin networks [17, 18], we hypothesized that apical actomyosin activity might be required to enrich the CCC specifically at apical cell-cell contacts. To test this, we disrupted actomyosin contraction in two ways: first by preventing myosin activation by knocking down MRCK-1, and second using a temperature-sensitive allele of myosin. In *mrck-1*(*RNAi*) embryos, the apicobasal polarization of HMR-1/cadherin-GFP was severely disrupted compared to wild-type (Figures 6D and 6F). The accumulation of HMR-1/cadherin-GFP at the apical junction between the Ea and Ep cells was significantly weakened, and instead cadherin-GFP accumulated along the lateral contact between Ea and Ep. Thus, MRCK-1 activity is required for proper apicobasal polarization of cadherin-GFP localization. Consistent with expectations, *pac-1*(*RNAi*), which disrupts MRCK-1 localization, resulted in reduced apical HMR-1/cadherin-GFP accumulation (Figure S7).

To directly test a role for myosin, we used a temperature-sensitive allele, *ne3409*, of the essential *C. elegans* non-muscle myosin II homolog *nmy-2* to disrupt its function specifically

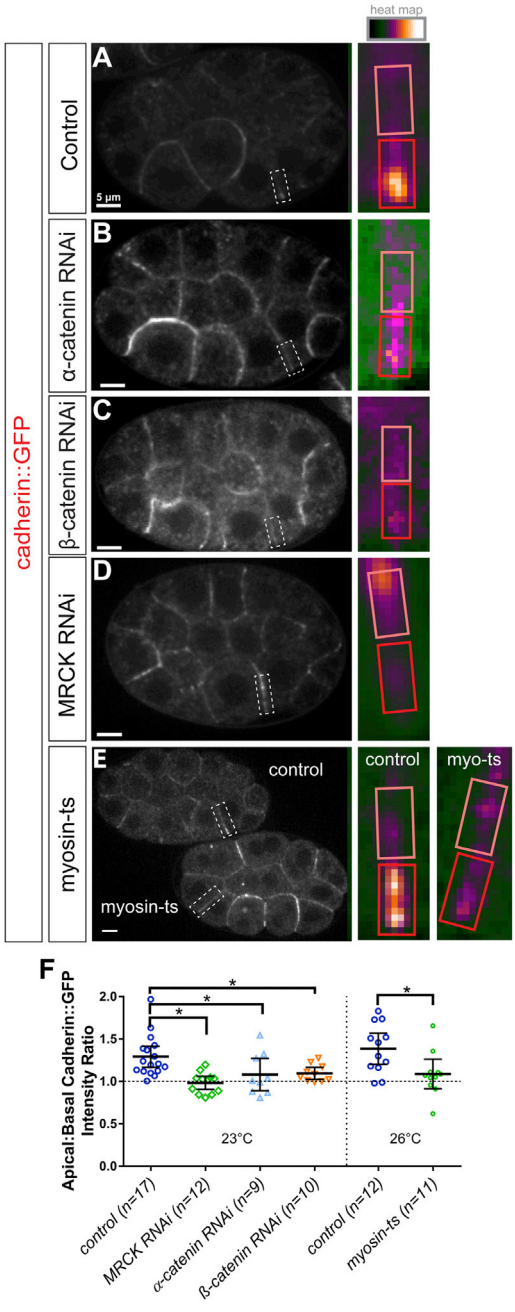

Figure 6. Myosin II Activity Is Required for Apical Enrichment of HMR-1/Cadherin-GFP in Apically Constricting Cells

(A–E) Spinning disk confocal fluorescence images of laterally mounted, gastrulation-stage HMR-1/cadherin-GFP-expressing embryos. Genotypes are listed in the boxes to the left. The border between the apically constricting E cells is highlighted with a dotted white box, which is enlarged and pseudo-colored in the inset (right).

(F) Plot depicting HMR-1/cadherin-GFP apical-to-basal fluorescence intensity ratio for all above genotypes. Error bars represent 95% CI, and n values describe the number of embryos (*$p < 0.05$).

See also Figures S6 and S7.

during EPC internalization. Previous studies revealed that this allele results in myosin loss of function within 20 s of shifting from the permissive temperature (15°C) to the restrictive temperature (26°C) [37]. We mounted four-cell-stage HMR-1/cadherin-GFP; *nmy-2*(*ne3409*) and HMR-1/cadherin-GFP control embryos side by side at 15°C–17°C, and we returned them to 15°C for ~2 hr until they approached gastrulation stage. Then, we shifted the embryos to the restrictive temperature (26°C) and imaged HMR-1/cadherin-GFP localization (Figures 6E and 6F). At the restrictive temperature in *nmy-2*(*ne3409*) embryos, HMR-1/cadherin-GFP was no longer enriched at the apical junction between the Ea and Ep cells, phenocopying the pattern observed in *mrck-1*(*RNAi*) embryos (Figure 6D). HMR-1/cadherin-GFP control embryos displayed normal cadherin-GFP enrichment under these conditions. We conclude that MRCK-1 can polarize HMR-1/cadherin-GFP distribution along the apical-basal axis through its myosin-activating activity.

DISCUSSION

Here we have described how *C. elegans* early gastrulation movements are regulated by the myosin kinase MRCK-1. We have shown how this kinase is controlled both within the animal, by transcription factors that specify endodermal fate, and subcellularly, through apicobasal patterning of Cdc42 activation. Together, these signals specify in which cells and also at which subcellular site constriction will occur. We further show that myosin activation is required to stabilize the force-transmitting cadherin-catenin adherens junctions at the sites of cell-cell contacts between the internalizing cells and their neighbors, presumably leading to a strengthening of adhesion at sites where adhesion is required. This work describes both molecular and mechanistic insights into how developmental patterning mechanisms can result in apical constriction and morphogenesis, summarized by the model in Figure 7.

The mechanisms we identify here show both significant similarities and differences with two other examples of apical constriction that are known to drive morphogenetic movements. In all these systems, including *C. elegans*, polarized activation of myosin is a key component driving the cell movements [38, 39]. During *Drosophila* gastrulation and vertebrate neural tube formation, polarized activation of RhoA and/or ROCK leads to myosin activation [40, 41]. In *C. elegans*, polarized inactivation of Cdc42 leads to polarized activation of myosin at contact-free external surfaces [14]. Although we see a weak effect on gastrulation in LET-502- (ROCK-)depleted embryos, suggesting a possible role, and we previously have seen inhibition of gastrulation using MLCK inhibitors (albeit at high enough concentrations to cause non-specific effects [10, 42]), our evidence would suggest that MRCK-1 is the primary activator of myosin during these movements. This is the first example of MRCK-1 being utilized to drive apical constriction, and it demonstrates that diverse myosin regulators can drive apical constriction in different animal model systems.

Upstream of RhoA and Cdc42, there are more similarities and differences. In *Drosophila* as in *C. elegans*, correct patterning is required. In *Drosophila*, a pair of transcription factors, Snail and Twist, cause autocrine activation of G protein-coupled receptors on the apical surface of the invaginating cells, leading to RhoA

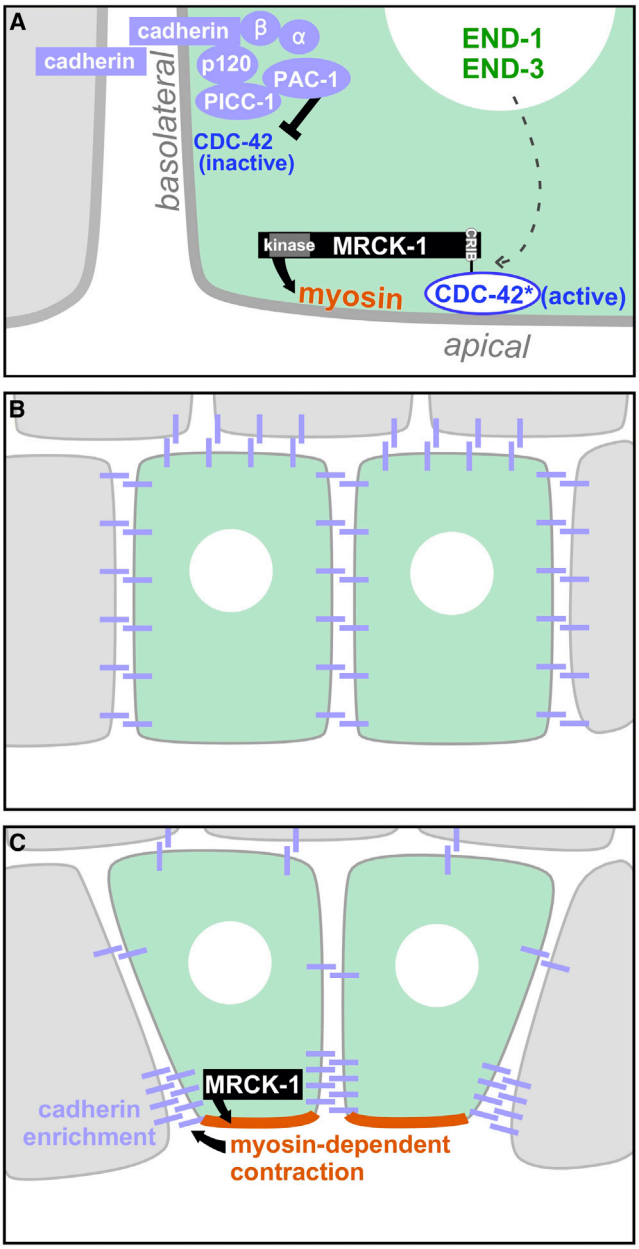

Figure 7. Model: An Outline of How the Force-Producing Mechanisms that Drive Apical Constriction In Vivo Are Spatially Regulated

(A) Active CDC-42, restricted to apical membranes by basolateral inhibition (black inhibitory arrow), recruits MRCK-1 apically via MRCK-1's CRIB domain. MRCK-1 activates myosin (black arrow), as evidenced by regulatory light chain phosphorylation, and increases tension in the apical cortex. MRCK-1 apical enrichment occurs specifically in the two EPCs, dependent (through unknown mechanisms) on the EPC-specific END-1/END-3 transcription factors (dotted arrow).

(B and C) MRCK-1-dependent myosin activity and the actomyosin-cadherin linkers α-catenin and β-catenin contribute through as-yet unexplored mechanisms to junctional cadherin enrichment.

activation [43, 44]. During neural tube closure, the protein Shroom is recruited to apical junctions where it recruits Rho-associated kinase ROCK [45, 46]. RhoA activation downstream of planar cell polarity components [47] is thought to act with Shroom to coordinate ROCK and myosin activation [46, 48]. It remains to be determined whether events analogous to the G-protein coupled receptor (GPCR) activation or Shroom localization of other systems are required for specific localization or activation of MRCK-1 in the internalizing EPCs in *C. elegans*. Vertebrate MRCK can be activated by regulators binding to its C1 domain [49], suggesting that there could be other regulation in addition to Cdc42 binding. The fact that END-1/3 and MOM-5 regulate MRCK-1 localization during this process suggests further molecular mechanisms to identify.

Previous studies from epithelial cell culture have revealed myosin-dependent enrichment of the CCC at cell-cell junctions [50], and in vivo studies from *Drosophila* have shown that signaling upstream of myosin activation (i.e., Fog pathway and Rho pathway) is required for proper localization of CCC components during apical constriction in ventral furrow cells [19, 21, 38]. We show that perturbing myosin activity, either indirectly by depleting a kinase required for its activity (MRCK-1) or more directly by a temperature-sensitive mutation in the gene encoding an essential myosin II heavy chain, causes a dramatic rearrangement of cell-cell adhesion components, suggesting that tension-dependent regulation of cadherin enrichment may be evolutionarily widespread. Unexpectedly, we also observed ectopic enrichment of cadherin-GFP to the basolateral cell-cell contact upon myosin disruption, suggesting that the relationship between cadherin recruitment and cortical tension is context dependent. Recent in vitro results identifying a force-dependent catch bond between α-catenin and F-actin suggest that myosin-based contractile force improves binding of the CCC to the F-actin cytoskeleton [32]. This improved binding could, in principle, promote adherens junction recruitment and/or stabilization. Our results showing myosin-dependent recruitment of adherens junctions during apical constriction provide in vivo data that are consistent with this model.

We previously reported that apical actomyosin contractions precede apical constriction movements, suggesting that apical constriction is triggered by temporally regulated attachments between the actomyosin cortex and apical junctions [7]. We observed a subtle increase in the concentration of CCC components between the early (2–6 min after MSx cell division) and late (8–11 min after MSx cell division) phases of apical constriction, which might feasibly contribute coupling between cytoskeletal and junctional movements. However, the apical enrichment of CCC components largely precedes the early, uncoupled phase of apical constriction. Thus, while it remains possible that the CCC accumulation we describe here contributes to an improvement in coupling between cytoskeletal and junctional movements, it is likely not sufficient to account for the behavior described previously.

The HMR-1/cadherin complex has been shown in previous studies to play an instructive role in the establishment of apicobasal polarization in early embryonic cells by recruiting the Cdc42 GAP PAC-1 to sites of cell-cell contact through the linker protein PICC-1 [13]. This leads to a decrease in Cdc42 activity along cell-cell contacts relative to the exposed surfaces. We show that the myosin kinase MRCK-1 is downstream of Cdc42 and that its myosin-activating activity positions HMR-1/cadherin to sites of apical cell-cell contact in apically constricting cells.

Therefore, HMR-1/Cadherin contributes to its own relocalization: basolateral HMR-1/Cadherin leads indirectly to apical myosin activation, which results in HMR-1/Cadherin relocalization to rings encircling the apical surface, forming the adherens junctions on which actomyosin contractions pull [3]. It will be interesting to see the extent to which this kind of feedback loop between adhesions and myosin contractility might occur in other systems.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and five movies and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2016.06.010.

AUTHOR CONTRIBUTIONS

Conceptualization, D.J.M., C.D.H., and B.G.; Investigation, D.J.M., C.D.H., K.A.P., T.D.C., D.J.D., A.M.P., R.P.M., and A.H.C.; Writing – Original Draft, D.J.M., C.D.H., and B.G.; Writing – Review & Editing, D.J.M., C.D.H., K.A.P., T.D.C., D.J.D., D.P.K., and B.G.; Funding Acquisition, D.P.K. and B.G.; Resources, D.P.K. and B.G.; Supervision, D.J.M., D.P.K., and B.G.

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