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Wnt/Frizzled Signaling Controls C. elegans Gastrulation by Activating Actomyosin Contractility

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Summary

Background—Embryonic patterning mechanisms regulate the cytoskeletal machinery that drives morphogenesis, but there are few cases where links between patterning mechanisms and morphogenesis are well understood. We have used a combination of genetics, in vivo imaging, and cell manipulations to identify such links in C. elegans gastrulation. Gastrulation in C. elegans begins with the internalization of endodermal precursor cells in a process that depends on apical constriction of ingressing cells.

Results—We show that ingression of the endodermal precursor cells is regulated by pathways, including a Wnt-Frizzled signaling pathway, that specify endodermal cell fate. We find that Wnt signaling has a role in gastrulation in addition to its earlier roles in regulating endodermal cell fate and cell-cycle timing. In the absence of Wnt signaling, endodermal precursor cells polarize and enrich myosin II apically but fail to contract their apical surfaces. We show that a regulatory myosin light chain normally becomes phosphorylated on the apical side of ingressing cells at a conserved site that can lead to myosin-filament formation and contraction of actomyosin networks and that this phosphorylation depends on Wnt signaling.

Conclusions—We conclude that Wnt signaling regulates C. elegans gastrulation through regulatory myosin light-chain phosphorylation, which results in the contraction of the apical surface of ingressing cells. These findings forge new links between cell-fate specification and morphogenesis, and they represent a novel mechanism by which Wnt signaling can regulate morphogenesis.

Introduction

The morphogenetic events that shape embryonic development rely on the movements and shape changes of individual cells. Because the cellular cytoarchitecture provides the driving forces for these cellular events, one of the keys to understanding the molecular basis of morphogenetic movements is determining how well-studied developmental pathways specifying cell fate lead to modulation of the cytoskeleton in individual cells in ways that can

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produce forces capable of moving cells or deforming tissues. Toward this goal, there has been identification of many genes that function upstream of morphogenetic movements, including many essential for cell-fate specification and extracellular signaling, and there is some understanding of the cytoskeletal mechanics that drive these movements. However, there has been more limited progress in tying these two ends together to provide a coherent thread from cell fate and signaling molecules to the cytoskeletal dynamics responsible for morphogenesis [1] and [2].

One of the earliest morphogenetic events in animal development is gastrulation, the process by which the embryo reorganizes itself into three germ layers. Gastrulation in C. elegans begins at the 26-cell stage when the two endodermal founder cells, Ea and Ep, begin to migrate from the outer, ventral surface of the embryo to the embryonic interior [3] (Figure 1). The mechanisms that specify endodermal fate in these cells are well studied. SKN-1, an endomesodermal determinant, is segregated to two cells (P2 and EMS) at the four-cell stage. In P2, SKN-1 activity is repressed in the P2 cell, whereas SKN-1 activity persists in EMS to promote endomesodermal fate [4]. A Wnt interaction at the four-cell stage then specifies endodermal fate on one side of the EMS cell. Differential regulation of transcription factor activity in this cell's two daughters results in a single endodermal precursor cell (E) at the eight-cell stage [5].

After E is born, its daughter cells (Ea and Ep) ingress during a cell cycle that is extended by the introduction of a gap phase [6]. The space left on the ventral side of the embryo is filled by neighboring cells, a total of six cells from the MS, AB, and P4 lineages [7]. After ingression, Ea and Ep divide (Figure 2A). For simplicity, we use the term “gastrulation” here solely to refer to Ea-Ep ingression, the internalization of the endoderm. Gastrulation in C. elegans continues later with the internalization of other cells including mesoderm and germline progenitors [8].

Recent work has begun to shed light on the mechanisms required for C. elegans gastrulation [9]. One of the driving forces for Ea-Ep ingression is apical constriction, which is likely powered by an actomyosin contraction [7]. Consistent with this finding, NMY-2, a nonmuscle myosin II heavy chain, accumulates at the apical (ventral) surfaces of Ea and Ep, and this polarized accumulation requires the activity of the PAR proteins [8] and [10]. The PAR proteins were first identified as essential for polarity in the 1-cell C. elegans embryo [11] and have since been found to be required for polarity in organisms as diverse as Drosophila and humans [12]. In gastrulation-stage embryos, the PAR proteins have polarized distributions that are established by cell-cell contacts, with PAR-3, PAR-6, and PKC-3 localized apically, at contact-free areas, and PAR-1 and PAR-2 localized basolaterally where cells contact neighboring cells [8] and [10]. Depleting the embryo of specific PAR proteins just before gastrulation compromises gastrulation movements. The timing of ingression is delayed significantly compared to that in wild-type cells, but the E lineage cells still internalize [10]. This suggests that there must be additional and essential gastrulation regulators that remain to be identified.

The Wnt pathway has been implicated in cell-fate specification, cell polarization, and morphogenesis across the animal kingdom [13]. Wnt ligands or their Frizzled (Fz) receptors, or both, are required in many processes including establishment of Drosophila segment polarity [14] and [15], zebrafish and Xenopus gastrulation [16], [17] and [18], and Xenopus neural tube closure [19]. Wnt and Fz are known to act through various signal transduction pathways, generally categorized as canonical and noncanonical pathways. Canonical signaling results in translocation of β-catenin from the cytoplasm to the nucleus, where it participates in the transcriptional activation of downstream targets. In C. elegans, mom-2, the Wnt gene that functions during the four-cell stage to specify endoderm, acts in a variant of the canonical manner by activating WRM-1, a C. elegans β-catenin homolog, which results in

downregulation instead of upregulation of POP-1, a TCF/LEF transcription factor [20]. In contrast, noncanonical pathways act through cytoplasmic factors that ultimately regulate cytoskeletal components but can also act to regulate transcription independently of β-catenin [5]. Further understanding of the mechanisms by which Wnt signaling can regulate cell movements will be important for understanding morphogenesis during normal development and Wnt pathway function in tumor invasion and metastasis in human cancers [21].

We have used a candidate approach to begin to identify genes required for C. elegans gastrulation. We found that the pathways that specify the endodermal precursors also regulate ingression of these cells. We show that Wnt/Fz signaling has a role in gastrulation in addition to its function in regulating endodermal cell fate and cell-cycle timing. Although Wnt signaling functions in cell polarization in many contexts [22], Wnt signaling does not affect C. elegans gastrulation by establishing polarity in the ingressing cells or by affecting the rate of myosin accumulation at the apical cortex of these cells. Instead, we found that Wnt/Fz signaling functions in gastrulation by causing regulatory myosin light chain in the apical cortex of Ea and Ep to be phosphorylated at a contraction-activating serine residue. Our results forge new links among cell fate, cell signaling, and cell form and suggest a novel role for intercellular signaling by a Wnt protein—the regulation of morphogenetic movements through activation of myosin contractile activity.

Results

Endodermal-Fate-Specification Pathways Act Upstream of C. elegans Gastrulation

To determine whether endodermal fate specification is necessary for Ea and Ep morphogenetic behavior, we first determined whether C. elegans embryos defective in each of the endodermal-fate-specification genes also have gastrulation defects. The endodermal founder cells are specified by two intersecting pathways: A GATA-factor transcriptional cascade initially restricts mesendoderm fate to the appropriate endoderm and mesoderm precursors, and the Wnt signaling pathway then acts to repress nuclear POP-1 and thus allow endoderm development in the progeny of the E cell [4]. The endoderm-specification pathways also affect cell-cycle timing in endodermal precursors. The Ea and Ep cells are the first cells in the C. elegans embryo to introduce a gap phase, a G2 phase that results in the Ea and Ep cells dividing approximately 20 min later than MSa and MSp cells (20.2 ± 2.4 min SD; Figure 2B) [6]. This delay does not occur in endoderm-specification mutants [4]. We speculated that either endoderm specification or cell-cycle delay, or both, might be required for gastrulation. Gastrulation defects have been found in embryos defective in some of these genes, e.g., mom-2 [23], skn-1 [24], end-1 [25], and end-3 [26], but many of the relevant genetic backgrounds have not been analyzed for gastrulation movements.

Mutant embryos were imaged by 4D time-lapse videomicroscopy and were subsequently analyzed for gastrulation movements. For the experiments described in this paper, we defined successful gastrulation as the ingression of Ea and Ep to the point where they are completely surrounded by their neighbors before they divide (Figure 2A). We found that nearly all of the endoderm-specification mutants were also gastrulation defective, with rates of failure reaching as high as 100% in mom-4, lit-1, and end-3 embryos (Table 1). The only endoderm-specification-pathway gene not required for gastrulation was elt-2, a downstream-acting transcription factor whose expression is not detected until just after Ea-Ep ingression [27].

In an attempt to further identify potential links between patterning pathways and gastrulation, we determined whether ectopic endodermal cells in a mutant undergo ectopic gastrulation. PIE-1 is a CCCH zinc-finger protein that becomes segregated to posterior cells, where it specifies cell fates in part by repressing the function of the endomesoderm-specifying gene skn-1. Embryos from pie-1 loss-of-function mothers produce ectopic endoderm from E's

posterior neighbor, P3, a cell that normally produces muscle and germline founder cells [25]. Time-lapse recordings of pie-1 mutant embryos showed ectopic gastrulation: The two daughters of P3 invariably ingressed soon after Ea-Ep (17.5 ± 3.3 min SD; Table 1). Because loss of function of endoderm-specifying genes interferes with gastrulation and loss of function of pie-1, a gene that prevents ectopic endoderm from forming, results in ectopic gastrulation, we conclude that the genes that specify endodermal cell fate in Ea and Ep function upstream of the cytoskeletal mechanisms that drive gastrulation. The most downstream player we found, the transcriptional activator END-1 [4], suggests that some molecular player(s) in gastrulation may be regulated transcriptionally, consistent with a known requirement for transcription in gastrulation [9].

Wnt/Frizzled Signaling Functions in Gastrulation as Well as Endoderm Specification

Next, we asked whether any of these endoderm-specification genes played a direct role in determining gastrulation behavior. Wnt ligands, Frizzled receptors, and Frizzled's downstream effectors function upstream of morphogenetic events in diverse organisms [15], [16], [17], [18] and [28]. We asked whether Wnt/Fz signaling functions during C. elegans gastrulation independently of its role in specifying endoderm by using rhabditin granules as a terminal-differentiation marker for endoderm development (see the Supplemental Experimental Procedures available with this article online). If Wnt/Fz signaling affects gastrulation solely through its role in endoderm specification, then mom-2/Wnt or mom-5/Fz embryos that produce endoderm should gastrulate and those embryos that fail to make endoderm should fail to gastrulate. As expected, we found that all mom-2 deletion allele embryos that failed to produce endoderm also failed to gastrulate (n = 33); however, we were surprised to find that most of the escapers—the endoderm-producing mom-2 embryos—also failed to gastrulate (82%; n = 17) (Figure 2B). Similarly, most mom-5 embryos that produced endoderm failed to gastrulate (85%; n = 34) (Figure 2B). We also examined the expression of two endoderm-specific molecular markers, END-1, which is expressed during gastrulation, and ELT-2, which is expressed after gastrulation. mom-5(RNAi) embryos exhibited similar gastrulation defects to mom-5(zu193) embryos, and the END-1 and ELT-2 expression was comparable to that in wild-type cells in all embryos examined (n = 17 for END-1 and n = 9 for ELT-2; Movies S1 and S2 and Figure S1). Because Ea and Ep in mom-5 embryos generally fail to gastrulate despite producing END-1 and producing ELT-2 and rhabditin granules later, we propose that Wnt/Fz signaling has two functions: regulating endoderm specification and also, at least partially independently, regulating gastrulation.

The multidomain protein Dishevelled (Dsh) functions downstream of the Frizzled receptor in several systems [29]. C. elegans has three Dsh homologs: DSH-1, DSH-2, and MIG-5. Although null alleles of Dsh homologs affect endoderm specification, RNA interference (RNAi) of dsh-1, dsh-2, and mig-5 individually or together has not [30]. This facilitated the determination of whether Dsh-mediated signaling is required for C. elegans gastrulation independently of a potential role in endoderm specification. We carried out RNAi of these three genes individually and in combination. With the exception of dsh-1 RNAi, all of these treatments resulted in some embryos that failed to gastrulate despite producing endoderm, and RNAi of all three genes simultaneously produced a more penetrant gastrulation-defective phenotype than RNAi of any single gene alone (Figure 2 and Figure S2), suggesting that multiple Dsh proteins act redundantly to regulate gastrulation (we refer to these three proteins collectively as Dsh below). Our attempts to determine whether canonical or noncanonical signaling is involved in gastrulation downstream of Dsh have not yet resolved this issue. We conclude that MOM-2/Wnt, MOM-5/Fz, and multiple Dishevelled homologs function in the ingression of endodermal precursors in addition to their roles in endoderm specification. Because a mom-2 null allele does not abolish gastrulation movements completely (Table 1),

we conclude from these data that Wnt signaling acts partially redundantly as direct or indirect regulators of gastrulation with one or more additional pathways.

Wnt/Frizzled Signaling Affects Gastrulation Independently of Control of Ea-Ep Cell-Cycle Length

Previous work suggested that the G2 phase introduced in the Ea-Ep cell cycle near the time of gastrulation might be important for gastrulation: In gad-1 (gastrulation defective) mutant embryos, endodermal cell fate is properly specified, but the Ea and Ep cells do not have a G2 phase nor do they ingress [31]. The division delay in Ea and Ep compared to their cousins MSa and MSp has been considered an aspect of endodermal cell fate because it is absent in many endoderm-deficient mutants [4]. We confirmed that the Ea-Ep division delay in mom-2 embryos is generally shorter than that in wild-type embryos (Figure 2B). Very few mom-2 embryos gastrulated (6%; n = 50), and the few embryos that did gastrulate had somewhat longer cell cycles than the average for mom-2 embryos (8.5, 13, and 16.5 min compared with an average of 6.2 min). Similar results were seen in mom-5 embryos and Dsh RNAi embryos (Figure 2B and Figure S2). These results demonstrate that embryos can gastrulate even when the Ea-Ep cell cycle is shorter than that in wild-type embryos, but they raised the possibility that more precocious cell division might prevent gastrulation movements, perhaps by reorganizing the actomyosin network for cell division in a way that precludes apical constriction from occurring at the same time.

We therefore conducted two types of experiments to test more directly whether Wnt signaling functions in gastrulation independently of its effect on cell-cycle timing. First, we lengthened the cell cycles of Ea and Ep in the absence of Wnt signaling. Ea and Ep cell cycles can be artificially lengthened by brief irradiation of Ea and Ep nuclei with a laser (referred to here as laser-delay; see Supplemental Experimental Procedures). This treatment did not interfere with ingression in wild-type embryos (9/9 cases; Figure 3A). In gad-1 embryos, laser-delaying Ea and Ep cell division rescued ingression in approximately half of the cases (9/19 cases; Figure 3A), and control laser-delay of cell pairs in AB or P2 lineages in gad-1 embryos did not cause ingression (data not shown). The rescue of ingression by laser-delay of Ea and Ep suggests that gad-1 functions in gastrulation only indirectly, through regulating Ea and Ep division timing, and also indicates that laser-delay can be used to rescue ingression in a mutant that would otherwise have premature Ea-Ep division.

In contrast to the gad-1 experiments, irradiation of Ea and Ep nuclei to delay Ea and Ep cell division completely failed to rescue gastrulation movements in mom-2 embryos (0/14 cases; Figure 3A). Because neither long Ea-Ep cell cycles in untreated mom-5 embryos nor artificially extended Ea-Ep cell cycles in mom-2 embryos are sufficient for gastrulation to occur, and yet many such embryos would normally produce endoderm (Figure 3 legend), we propose that endoderm specification and long Ea-Ep cell cycles may not be sufficient for gastrulation to occur in the absence of this Wnt signaling pathway, and that Wnt signaling has additional functions in gastrulation.

Second, if Wnt signaling acts once to specify endoderm and cell-cycle timing and again later during gastrulation, then manipulating the presence of a Wnt signal after induction of endodermal fate and cell-cycle timing should determine whether gastrulation occurs. At the four-cell stage, P2 induces endoderm in the neighboring EMS cell via Wnt signaling [23], [32] and [33]. The timing of this induction is well characterized and can be manipulated in an in vitro blastomere culture system [34]. We used these cell manipulations to present a Wnt signaling cell to specify E cell fate, and we then replaced it with either Wnt-minus or Wnt-plus signaling cells to look for specific effects on gastrulation. First, a number of control experiments were performed. When we separated wild-type P2 from wild-type EMS cells and recombined these cells with wild-type partners that were at the same developmental time point,

most recombinants exhibited gastrulation movements (75%, n = 16; Figure 3B). In contrast, mom-2 P2 and mom-2 EMS negative control recombinations resulted in little or no movement (Figure S3), as seen previously in related experiments with mom-2 [7]. We then confirmed that gastrulation defects could be rescued by recombining mom-2 EMS cells with wild-type P2 cells (70%, n = 20; Figure 3B). Next, we asked whether a Wnt signaling cell during endoderm specification was sufficient for gastrulation movements by replacing a Wnt-plus P2 cell with Wnt-minus cells after endoderm induction. We found that after endoderm induction had occurred, a Wnt-plus P2 cell is able to rescue gastrulation movements (70%, n = 20) significantly more effectively than a Wnt-minus signaling cell (36%, n = 14; Figure 3B), suggesting that the P2 cell or its descendants, or both, are likely to be a source of Wnt signaling for gastrulation.

Together, these results suggest that although Wnt signaling can affect gastrulation indirectly by regulating endodermal cell fate and division timing, Wnt signaling also has a second role during gastrulation. This could be a second, independent Wnt-Fz interaction, or possibly a higher threshold response to the interaction that establishes endoderm cell fate. These results prompted us to look for more direct cellular effects of Wnt/Fz signaling during gastrulation by examining the effect of Wnt/Fz signaling on the polarization and cytoskeletal motility of ingressing cells. Below, we show that in the absence of Wnt signaling, some cell biological events implicated in ingression occur normally in Ea and Ep, but others do not.

Ea and Ep Apicobasal Polarization Proceeds Independently of Wnt/Frizzled Signaling

Because Wnt and Fz-dependent signaling are known to affect cell polarity in several systems [35], we examined whether Ea-Ep apicobasal polarity was disrupted in the absence of Wnt/Fz signaling. During gastrulation, the PAR proteins are localized in apicobasally polarized patterns and are required for apical myosin enrichment and efficient ingression movements [8] and [10]. We examined wild-type embryos expressing PAR-2::GFP and compared them to PAR-2::GFP embryos from mothers that were either fed with a bacterial strain expressing mom-2 dsRNA or injected with mom-5 dsRNA. PAR-2::GFP;mom-2(RNAi) embryos and PAR-2::GFP;mom-5(RNAi) embryos exhibited similar gastrulation defects to mom-2(or309) and mom-5(zu193) embryos respectively (see Supplemental Experimental Procedures). We confirmed that GFP is detected in PAR-2::GFP embryos in a basolateral pattern in Ea-Ep (11/11 embryos; Figure 4A). We found that PAR-2::GFP;mom-2(RNAi) embryos also display PAR-2::GFP in a basolateral pattern (16/16 embryos; Figure 4B) as do PAR-2::GFP;mom-5 (RNAi) embryos (8/8 embryos; Figure 4C), indicating that Wnt-Fz signaling does not regulate PAR-2 basolateral distribution at this stage.

A second step in cell polarization occurs when NMY-2, a nonmuscle myosin II heavy chain, accumulates at the apical surfaces of Ea and Ep, where it contributes to gastrulation movements [7] and [8]. It is possible that Wnt/Fz signaling may effect gastrulation by acting in parallel with PAR proteins to enrich NMY-2 apically. The majority of cortical NMY-2 accumulation in Ea and Ep occurs during the G2 phase of the Ea and Ep cells [8]. We used mom-5 mutants as a source of Wnt signaling-deficient embryos because Ea-Ep division timing in mom-5 mutant embryos more closely resembles that in wild-type embryos than it does in mom-2 mutants (Figure 2B). We found that NMY-2::GFP accumulated in the apical cortex of Ea and Ep in mom-5 embryos as much as in wild-type embryos and did not accumulate apically in other cells at this stage (Figure 5A). Quantification of cortical to cytoplasmic NMY-2::GFP ratios confirmed this: Wild-type and mom-5 Ea-Ep cells accumulated NMY-2::GFP at similar rates, and both accumulated significantly more NMY-2::GFP than did cells not of E lineage in either background (Figure 5B). It is therefore unlikely that Wnt/Fz signaling affects gastrulation by regulating the accumulation of NMY-2. These results suggest that Wnt/Fz

signaling does not appear to affect gastrulation through regulation of apicobasal polarity in the ingressing cells.

Frizzled Signaling Functions Upstream of Apical Constriction

Because myosin II heavy chain becomes enriched on the apical side of the ingressing cells at a normal rate but ingression does not occur in Wnt-pathway mutants, we speculated that Wnt signaling might instead regulate contraction of the apical actomyosin network. To test this, we first measured the rate of apical constriction during gastrulation in wild-type and mom-5 (RNAi) embryos. We generated 4D movies of gastrulating embryos and marked the sites of contact between Ea and Ep and the surrounding cells at their apical surfaces. We then calculated the distances between these sites of cell contact. We considered Ea-Ep apical-surface measurements for only the first 10 min after MSa-MSp division so that the premature Ea-Ep division that occurs in some mom-5 embryos could not affect the results. During this time in wild-type embryos, the length of the apical domain decreased at a rate of 7.1 ± 1.3 nm/s (Figure 6). In contrast, the length of the apical domain of the Ea and Ep cells in mom-5 embryos that produced endoderm but failed to internalize Ea and Ep decreased significantly more slowly, at a rate of 2.3 ± 3.6 nm/s ($p < 0.05$). These data suggest that Wnt signaling regulates constriction of the apical domains of the Ea and Ep cells.

Myosin II Becomes Activated in the Apical Cortex of the Ea and Ep Cells Near the Time of Ingression

The failure of apical constriction in the absence of Wnt signaling suggested that apical myosin II might normally become activated to contract at the time of gastrulation and that Wnt signaling might function in this activation. Myosin II has two heavy chains, two essential light chains and two regulatory light chains, and phosphorylation of the two regulatory light chains (referred to here as rMLC) at serine 19 is required for formation of active myosin filaments that can drive contraction in various systems [36]. We used an antibody that recognizes this phosphoepitope, which we refer to as p-rMLC, to determine the localization of activated myosin before and during gastrulation. We found first that commonly used methods for immunostaining C. elegans embryos with methanol failed to preserve the p-rMLC signal, but the signal could be detected reliably with a formaldehyde fix designed to best preserve actin filaments [37] (see Supplemental Experimental Procedures). One-cell embryos stained in accordance with this protocol showed cortical staining consistent with published results [38] and [39], and cortical staining was abolished by RNAi to the rMLC-encoding gene mlc-4 (16/16 embryos; Figures S4 and S5). Furthermore, the cortical staining colocalized with NMY-2, the myosin heavy chain (Figure S6), suggesting that the antibody we used can recognize p-rMLC at the cell cortex. During gastrulation, we detected a striking enrichment of p-rMLC at the apical surfaces of Ea and Ep (Figure 7). p-rMLC staining appears enriched apically in the ingressing Ea and Ep cells specifically starting at the 26-cell stage (after MSa-MSp division) and until the Ea and Ep cells are fully ingressed, and it is not enriched apically in any other nondividing cells during these stages. We conclude that apically localized regulatory myosin light chain is activated in Ea and Ep near the time of ingression, by an as yet unidentified kinase.

Frizzled Signaling Activates Myosin II at the Ea and Ep Apical Cortex

Next, we asked whether Wnt-Fz signaling functions upstream of rMLC phosphorylation by immunostaining mom-5 embryos with anti-p-rMLC. In light of the fact that Ea and Ep cells can divide earlier in mom-5 embryos than in wild-type, we were careful to only compare wild-type and mom-5 embryos of similar stages (Figure 8 and Supplemental Experimental Procedures). In contrast with wild-type embryos, mom-5 embryos exhibited markedly lower levels of p-rMLC staining at the apical surface of Ea and Ep throughout all stages of gastrulation movements (Figure 8). The apical surfaces of Ea and Ep in mom-5 embryos stained in few

cases, and staining was weak in these cases (2/11 embryos), whereas apical staining in wild-type embryos is more robust (10/20 embryos showing strong staining, as shown in the Figure 8, and 6/20 embryos showing weak staining equivalent to the mom-5 embryos). We conclude that in the absence of Wnt signaling, there is a significant reduction in the level of phosphorylated regulatory myosin light chain on the apical sides of the Ea and Ep cells.

Discussion

The study of morphogenesis is one of the key areas where cell and developmental biology meet [1] and [40]. Although embryonic patterning mechanisms can play crucial roles in determining cytoskeletal behaviors, there are few examples where the coupling between embryonic patterning and cytoskeletal behaviors is well understood [41]. We have found that the well-studied pathways that specify endoderm in C. elegans act upstream of mechanisms that internalize the endodermal precursors. We have demonstrated that Wnt-Fz signaling regulates C. elegans gastrulation in addition to specifying endodermal cell fate and cell-cycle timing. The mechanism by which Wnt-Fz signaling acts is not through the generation of the polarized distribution of PAR proteins or apical-myosin accumulation, suggesting that Wnt-Fz signaling might directly affect apical constriction downstream of myosin accumulation. In support of this, we found an enrichment of an activated form of myosin at the apical cortex of wild-type Ea and Ep during gastrulation, and we found that this activation of myosin is dependent on signaling through Fz.

Our results rule out the simple possibility that Wnt signaling affects C. elegans gastrulation solely through its well-documented role in endoderm specification because we found that Ea and Ep often produce endoderm by multiple measures, but fail to gastrulate, in the absence of Wnt signaling. However, it remains possible that rMLC phosphorylation could be a higher threshold response to Wnt-Fz signaling at the four-cell stage. Alternatively, rMLC phosphorylation could depend on an independent, later Wnt-Fz interaction. Signaling to rMLC downstream of Dsh in either way could occur by a transcriptional mechanism or by signaling that is more direct to rMLC. It will be of interest to identify more members of this signaling pathway and to determine when they function so that these questions can be answered.

Our results, together with previous results from us and others [7], [9] and [10] outline a molecular and mechanical model for C. elegans gastrulation (Figure 9). The apicobasal polarity of all or most cells is determined by the positions of cell-cell contacts and is reflected in the localization of PAR proteins. The pathways that specify endodermal fate in C. elegans, involving many of the genes listed in Table 1, determine which cells will enrich myosin heavy chain apically in response to PAR-protein localization. Having myosin enriched at the apical, contact-free surfaces likely primes these cells for internalization. Wnt-Fz signaling leads to phosphorylation of a conserved residue on rMLC, and this phosphorylation can result in the formation of active myosin filaments. The contraction of the apical actomyosin machinery then shrinks the contact-free apical areas, pulls neighboring cells under the Ea and Ep cells, and results in the ingression of Ea and Ep into the center of the embryo. In this way, ingression appears to depend on the combinatorial information from cell-fate specification, apico-basal polarity, and reception of a cell signal that results in contraction of the actomyosin network on a specific side of specific cells.

The role of cell-fate-specification mechanisms and Wnt signaling in activating ingression of specific cells in C. elegans gastrulation has both striking parallels and critical differences with Drosophila gastrulation. In Drosophila, the mesoderm-specification protein Twist activates apical secretion of a different intercellular signaling protein, Folded-gastrulation (Fog) [41] and [42]. Fog acts through a presumed but unidentified cell-surface receptor and a G α protein, Concertina, to cause myosin localization to the apical surface of cells. Loss of the Fog

downstream targets, DRhoGEF or the Rho kinase Drok, causes much more severe defects in ventral furrow formation than does loss of Concertina, suggesting that Fog must act redundantly with another, unidentified pathway to drive apical constriction [41]. Myosin motor activity is required for its apical localization in Drosophila gastrulation [41]. Whether Fog signaling regulates myosin activity by rMLC phosphorylation in Drosophila gastrulation like the Wnt pathway does in C. elegans has not been examined, but Rho kinase is known to cause phosphorylation of rMLC or myosin activation in other settings in Drosophila [43], [44] and [45] and other organisms [36].

In vertebrates, the actin-binding protein Shroom functions to localize both actin filaments and myosin and can cause apical constriction when expressed in MDCK cells and during normal neural-tube closure [46], [47] and [48]. Shroom functions in apical constriction by restricting myosin localization, and it is not known to affect myosin activity. Shroom acts through a small GTPase like Fog, but does so through a different GTPase, Rap1. Fog, Shroom, and Wnt-dependent control of apical constriction appear to work independently rather than as parts of a single pathway because Fog and Shroom do not have homologs in the C. elegans genome, and removal of the function of the Rap1 homolog in C. elegans has no effect on gastrulation (T. Grana and J.H., unpublished data). Also, Shroom is required for apical constriction of only some cells in the organisms where it functions; for example, Xenopus bottle cells do not require Shroom during gastrulation [48]. These data suggest that apical constriction is regulated during animal development by multiple, independent mechanisms that can affect either myosin distribution or activity, or both.

Although ours is the first report of intercellular Wnt signaling regulating morphogenesis through rMLC phosphorylation, there is precedent for FrizzlDrosophila, suggesting that this might be an ancient mechanism of morphogenetic regulation. Frizzled can affect ommatidial polarity and actin bundle number in Drosophila planar-cell polarity (PCP) signaling pathways via Dsh, RhoA, Drok, and rMLC phosphorylation [45]. This pathway suggests some molecular players that might act between Dsh and rMLC phosphorylation in C. elegans gastrulation. The Drosophila PCP pathways differ from what we have outlined in this report in that Frizzled is probably not responding to intercellular Wnt signals in PCP signaling pathways [49] and [50] and in that PCP signaling is not known to drive apical constriction. The mechanism by which myosin activation regulates ommatidial polarity and actin bundle number in PCP pathways is unknown.

In zebrafish and frog embryos, PCP signaling is required during gastrulation for convergence and extension movements [17] and [18]. Vertebrate PCP genes are known to regulate the activity of the cytoskeletal modifiers Rho and Rac [51], [52] and [53], but the direct effect of PCP signaling on the cytoskeleton has not yet been analyzed in detail. Both the vertebrate PCP pathway and C. elegans Wnt/Fz signaling result in modification of cell shape and cell behavior during gastrulation. These pathways differ in that vertebrate PCP signaling affects cell polarity [17] and [18], whereas cell polarity is undisturbed in mom-2/Wnt and mom-5/Fz mutant embryos (this report) and RNAi experiments targeting C. elegans homologs of the PCP genes fat and flamingo (D.M., unpublished) and van gogh/strabismus (T.W. and J.H., unpublished data) have not resulted in any gastrulation defects, suggesting that C. elegans Wnt/Fz signaling in gastrulation through conventional PCP signaling is unlikely.

Why would myosin activity in C. elegans gastrulation be regulated by a cell-cell signal? It seems plausible that constitutively activating myosin could achieve the same goal because myosin is enriched in the apical cortex of the Ea and Ep cells, and hence, activation of myosin throughout these cells might cause an imbalance of forces that could drive apical constriction reliably. One possible explanation is that signaling ensures that apical constriction occurs at the right time. For example, signaling could ensure that myosin activation occurs at a time

when the actomyosin network is apically enriched and is not being used for cell division or at a time when adhesion to neighboring cells is sufficiently strong for constriction to pull neighboring cells under the Ea and Ep cells. Signaling might also contribute spatial specificity. For example, it is possible that there are stages in development when several cells have the potential to ingress but only the appropriate ones do so because they contact a Wnt signaling cell.

Others have speculated that morphogenesis may more often depend on redundant pathways than embryonic patterning does [2]. Very few backgrounds that we examined prevented gastrulation in all embryos, suggesting that C. elegans gastrulation is regulated by multiple, partially redundant mechanisms. Morphogenesis depends on diverse mechanisms that are of interest in the field of cell and developmental biology, including spatial and temporal gene regulation, cell signaling, cell polarization, cell adhesion, and cytoskeletal dynamics. It will be of interest to explore how such mechanisms work together in morphogenesis in C. elegans gastrulation.

Conclusions

We have used a combination of genetics, cell manipulations, and in vivo imaging to investigate the regulation of the cytoskeleton by cell-fate-specification genes and by intercellular signaling pathways during the morphogenetic movements of C. elegans gastrulation. We have shown that the pathways required for endoderm specification, including a Wnt/Frizzled pathway, are required for gastrulation to occur. Furthermore, we have shown that this Wnt/Frizzled pathway functions in gastrulation in addition to specifying endodermal cell fate. In the absence of Frizzled, apical constriction of the endoderm precursors fails to occur. Additionally, embryos lacking Frizzled show reduced levels of phosphorylated myosin in the apical domains of the endodermal precursors compared to wild-type embryos. Because this phosphorylation of myosin is likely to drive actomyosin contraction, and thus the ingression in these cells at this site, we hypothesize that this failure to phosphorylate myosin underpins the gastrulation defect in Wnt/Frizzled signaling defective embryos. Thus, we have demonstrated a novel role for Wnt signaling during morphogenesis through understanding its modulation of the cytoskeleton and how this impacts upon cell movements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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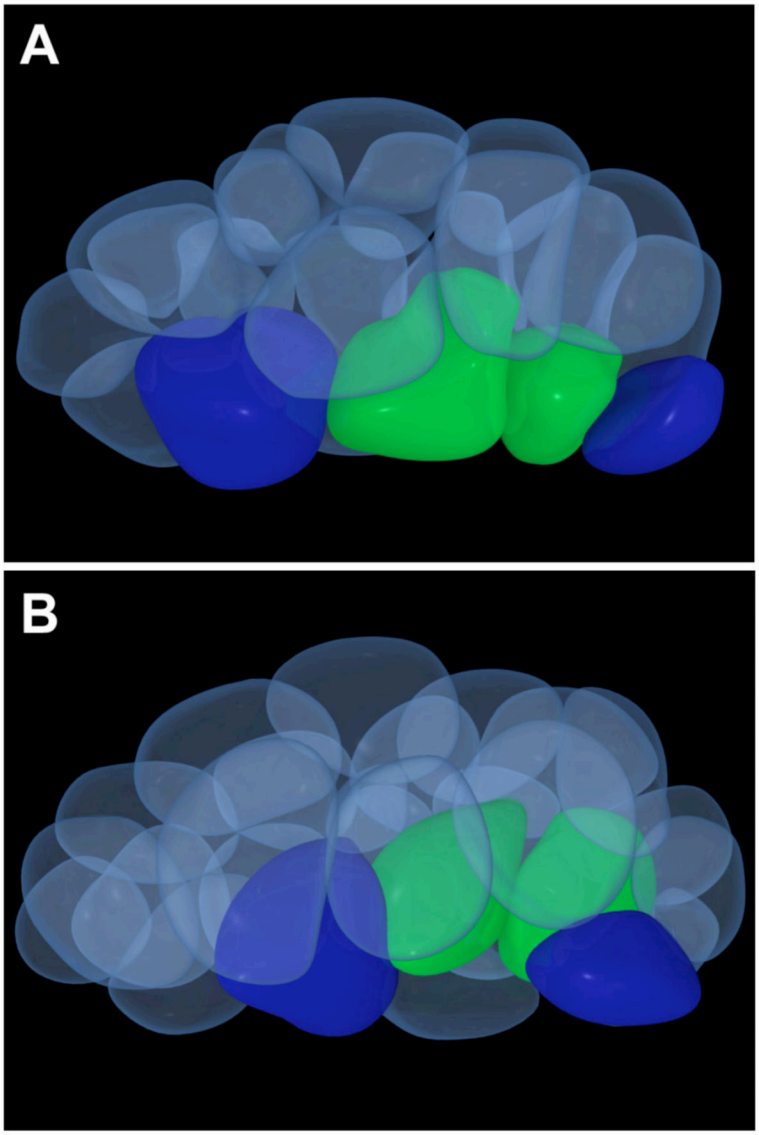

Figure 1. Three-Dimensional Illustrations of Embryos prior to and during Gastrulation

Ea and Ep (green) become completely enveloped by neighboring cells as gastrulation proceeds. These 3D renderings are based on tracings of optical sections of 24-cell (A) and 28-cell (B) embryos that were stained with labeled phalloidin to mark the cell cortex in each cell. Six cells extend into the gap vacated by the ingressing Ea and Ep cells. Two of these—one granddaughter of MS, left, and the P4 cell, right—are shown here in opaque blue. Illustrations by Janet Iwasa (janet@onemicron.com).

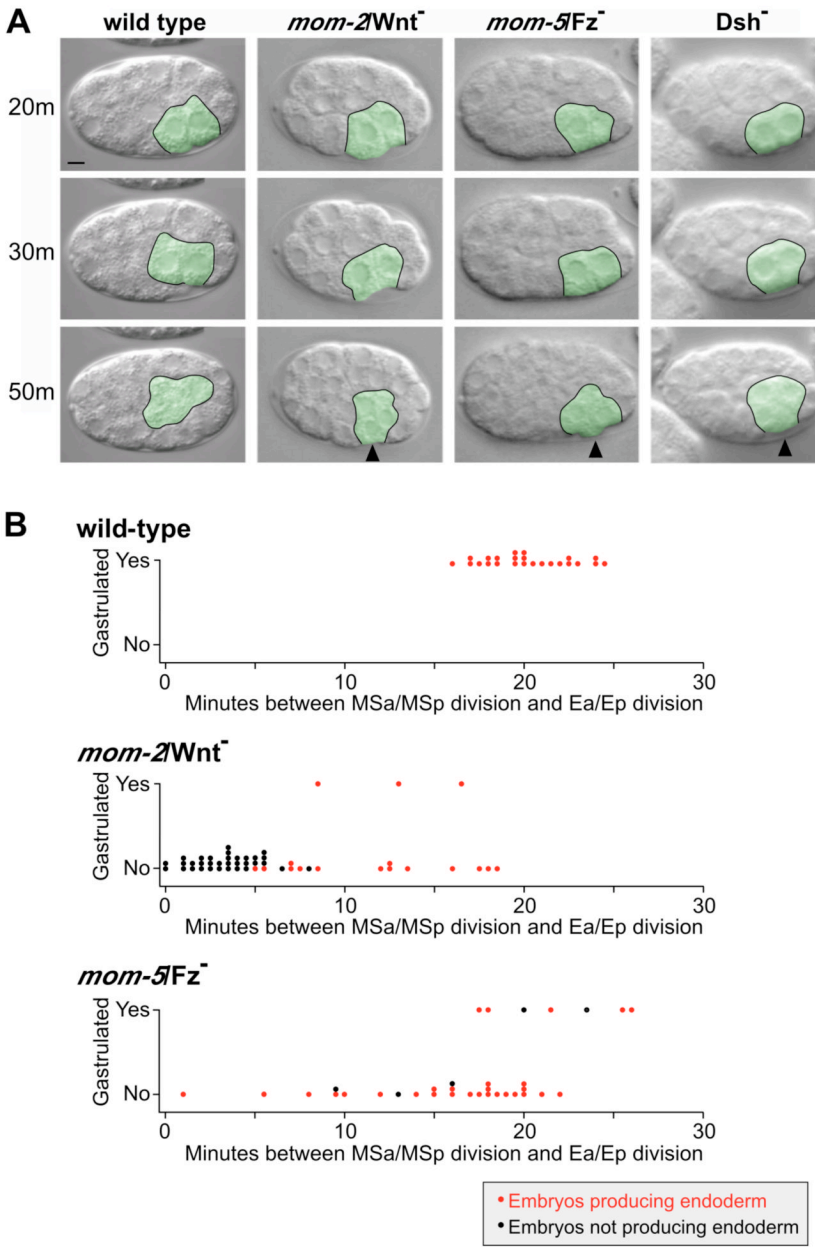

Figure 2. mom-2/Wnt, mom-5/Fz, and Dsh Are Required for Gastrulation

(A) Gastrulation in wild-type embryos occurs with the ingression of Ea and Ep, and the embryos divide once they are completely surrounded by neighboring cells. Each panel is a single midsagittal optical section from a Nomarski time-lapse movie, with the minutes elapsed since Ea-Ep birth noted on the left side. All sections were examined to ensure that cells internalized in one section were internalized in three dimensions. E cells are pseudocolored green in all frames. mom-2 and mom-5 embryos are mutants, and the Dsh embryo shown is triple dsh-1;dsh-2;mig-5(RNAi). Arrowheads point to E lineage cells that failed to ingress. In this and all figures, embryos are oriented with anterior to the left and dorsal side up. Scale bar represents 5 μm.

(B) There is no strict correlation between endoderm production and gastrulation or between Ea-Ep cell-cycle timing and gastrulation in mom-2 or mom-5. Each dot represents one embryo (24 wild-type, 50 mom-2, and 34 mom-5 embryos). The length of Ea-Ep delay, whether it gastrulated, and whether it produced endoderm are indicated.

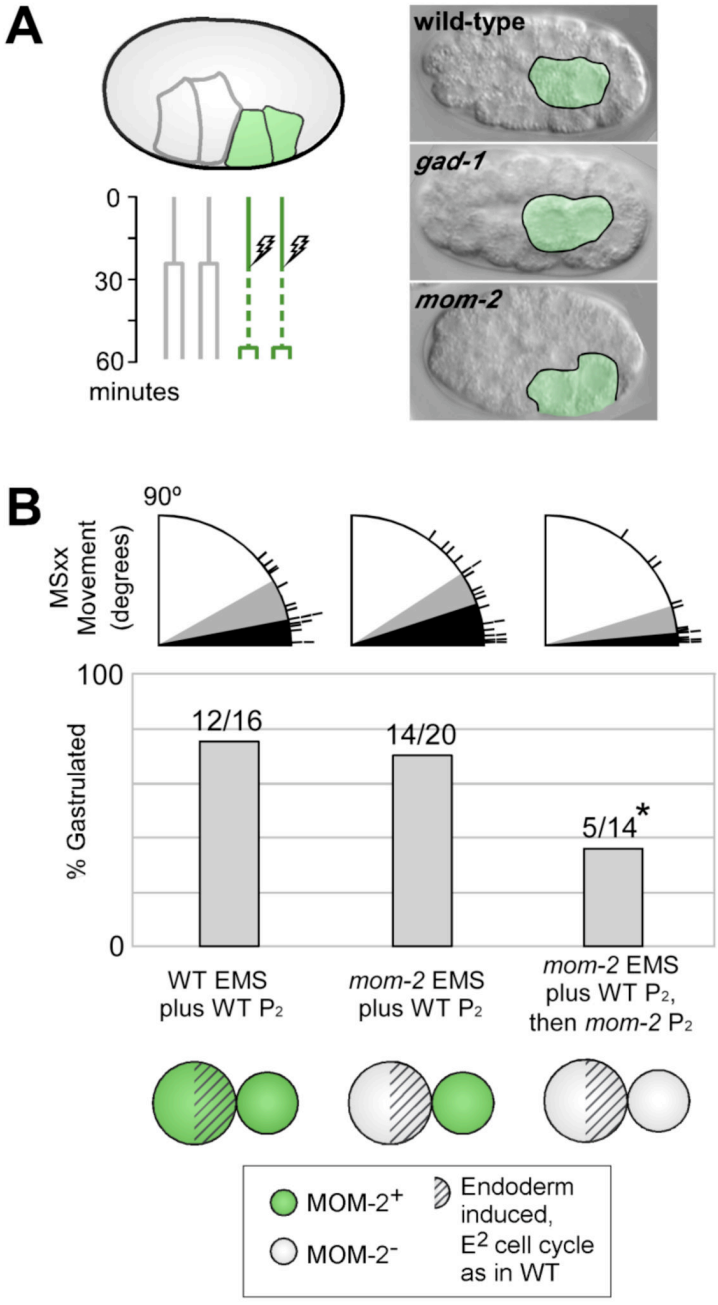

Figure 3. mom-2 Is Required for Gastrulation in Addition to its Effect on Ea-Ep Cell-Cycle Timing

(A) Laser-mediated delay of Ea-Ep cell division does not prevent gastrulation in wild-type embryos (9/9 cell pairs ingressed), and it can sometimes rescue gastrulation in gad-1 embryos (9/19 cell pairs), but it cannot rescue gastrulation in mom-2 embryos (0/14 cell pairs, significantly lower than the proportion of untreated mom-2 embryos that produced endoderm in Figure 2, chi-square test, $p < 0.05$). A schematic of MSa-MSp (gray outlines) and Ea-Ep (green) cell positions is shown above cell-division patterns. Lightning bolts represent approximate time of irradiation.

(B) In vitro cell-recombination experiments suggest that MOM-2 functions in gastrulation after endoderm specification has occurred. Three experiments are diagrammed at bottom. In these

experiments, wild-type P2 cells recombined with mom-2 EMS cells always rescued endoderm and rescued normal cell-cycle division timing in Ea-Ep cells. The top of the panel shows quarter-pie graphs depicting the extent of movement by MSxx relative to Ea-Ep during the 30 min after MSxx birth in each experiment. Each short line represents the degree of movement in one recombination experiment. The black area encompasses 50% of the data, and the gray area encompasses a further 25% of the data. Results are quantified in the bar graph below, in which the proportions that gastrulated are shown (more than 8° of MSxx movement was required to score movement as gastrulation, based on previous results [7]). Wild-type P2 cells more successfully rescued movement. The asterisk indicates that the results from this manipulation was statistically different from each of the other two manipulations with chi-square tests, at $p < 0.05$.

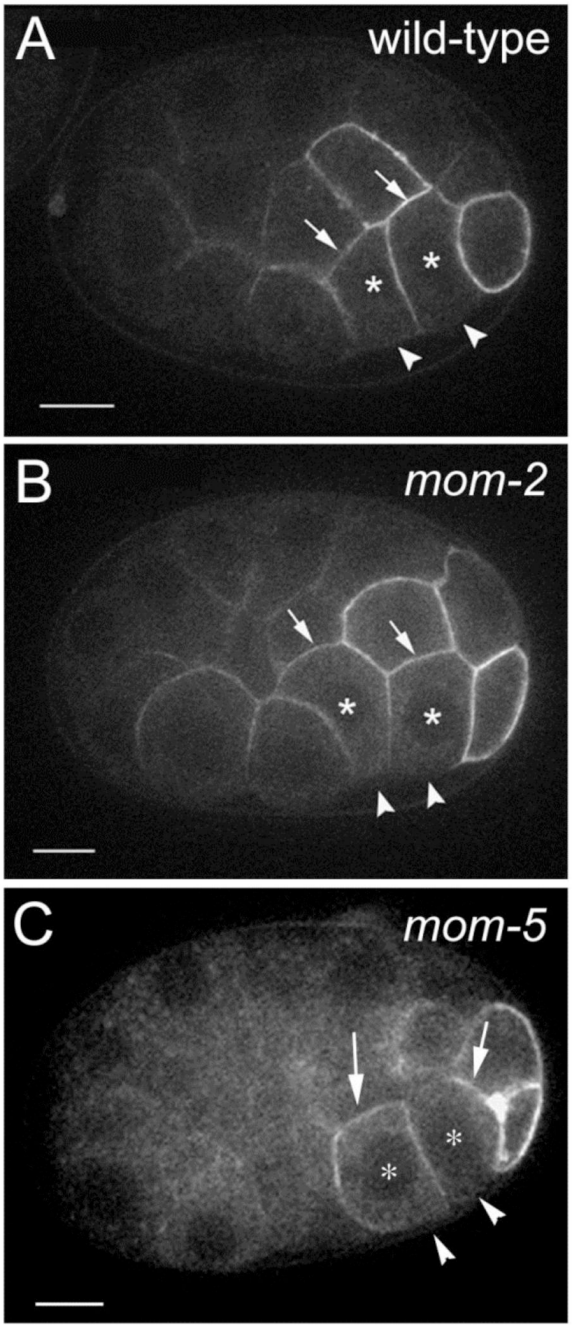

Figure 4. GFP::PAR-2 Distribution Shows that Apicobasal Polarity Is Established Normally in the Absence of mom-2/Wnt or mom-5/Fz

GFP::PAR-2 is enriched basolaterally in Ea and Ep in wild-type (A), mom-2 (B), and mom-5 (C) embryos. Ea-Ep are labeled with asterisks. Arrows mark the basal sides of Ea-Ep cells, where GFP::PAR-2 is enriched, and arrowheads mark the apical sides. P4, on the posterior side of the embryo, exhibits nonlocalized GFP::PAR-2 because the GFP construct is driven by a PIE-1 promoter that is most active in the P lineage. Confocal images are shown; scale bars represent 5 μm.

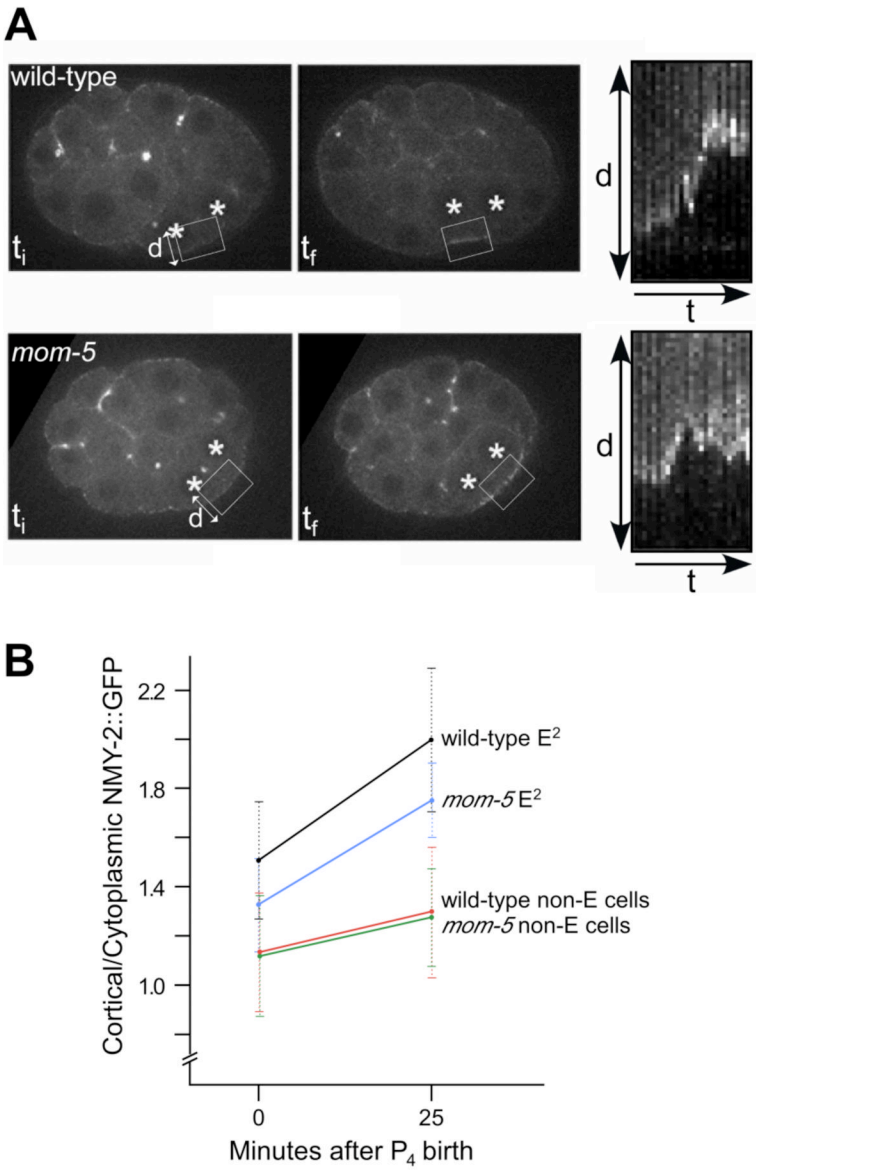

Figure 5. Apical NMY-2 Accumulation Occurs in Both Wild-Type and mom-5/Fz Ea and Ep Cells and at Similar Rates

(A) Confocal images from recordings of wild-type and mom-5 embryos expressing NMY-2::GFP. Asterisks mark Ea and Ep cells. d represents distance, t represents time, where ti is the initial time point analyzed and tf is the final time point analyzed 25 min later. Boxes around the Ea and Ep cortex represent the areas used to make each kymograph at right, in which the maximum pixel value along each d position in each box is shown over 25 min. In the kymographs, the cortex in wild-type can be seen accumulating NMY-2::GFP and moving toward the interior of the embryo, and the cortex in mom-5 can be seen accumulating NMY-2::GFP but moving only erratically. Similar areas were analyzed on three other quadrants of the embryo.

(B) Graph of cortical to cytoplasmic NMY-2::GFP level over time. Five wild-type embryos, and four mom-5 embryos that failed to gastrulate, were analyzed. NMY-2::GFP levels were quantified by calculation of the ratio of cortical to cytoplasmic pixel level above background. A ratio of one therefore indicates that the cortex is at the same intensity as the cytoplasm. By

25 min after P4 birth, E cell cortexes have enriched significantly more NMY-2::GFP than non-E cells, in both backgrounds ($p < 0.05$). The wild-type Ea-Ep GFP ratio is not statistically distinguishable from the mom-5 Ea-Ep GFP ratio at either time point. Error bars indicate ± 1 SD.

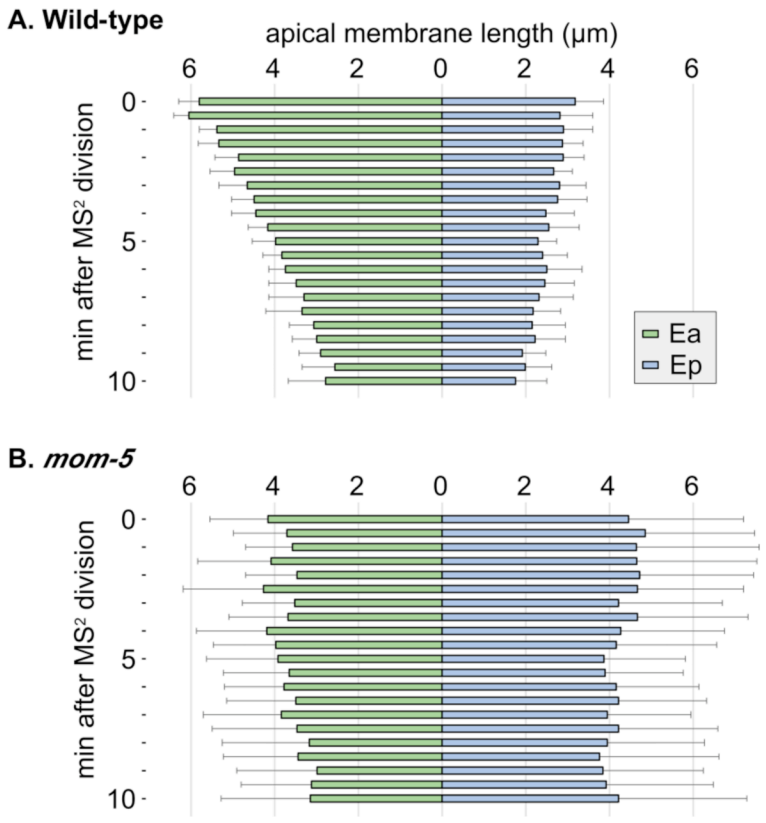

Figure 6. Apical Domains of Ea-Ep Fail to Constrict in mom-5 Mutant Embryos

Lengths of the apical domains of Ea and Ep over time are shown for (A) 13 wild-type and (B) six mom-5 mutant embryos that produced endoderm but failed to gastrulate. Error bars are 95% confidence bars.

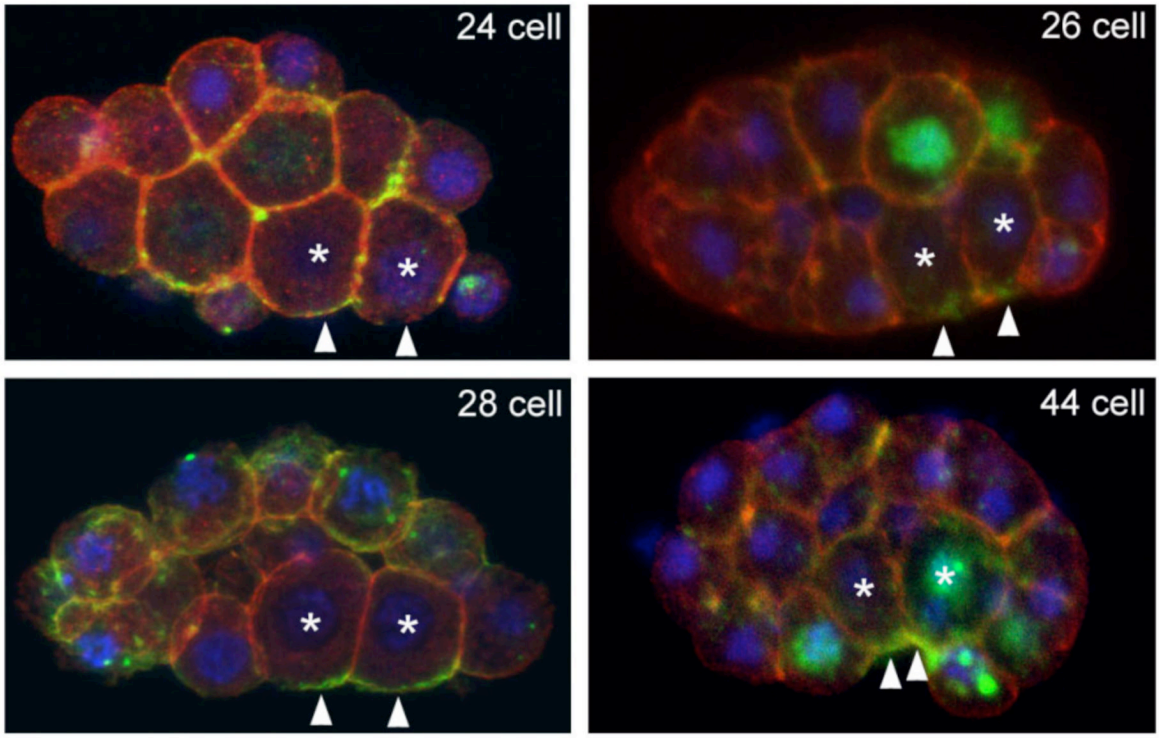

Figure 7. Phosphorylated Regulatory Myosin Light Chain Accumulates Apically in the Endoderm Precursors Near the Time that Gastrulation Occurs

Gastrulation movements begin near the 26-to 28-cell stage in most embryos. Ea-Ep cells are indicated by asterisks, and apical-domain membranes are labeled with arrowheads. p-rMLC is shown in green, F-actin is shown in red, and DAPI staining is shown in blue.

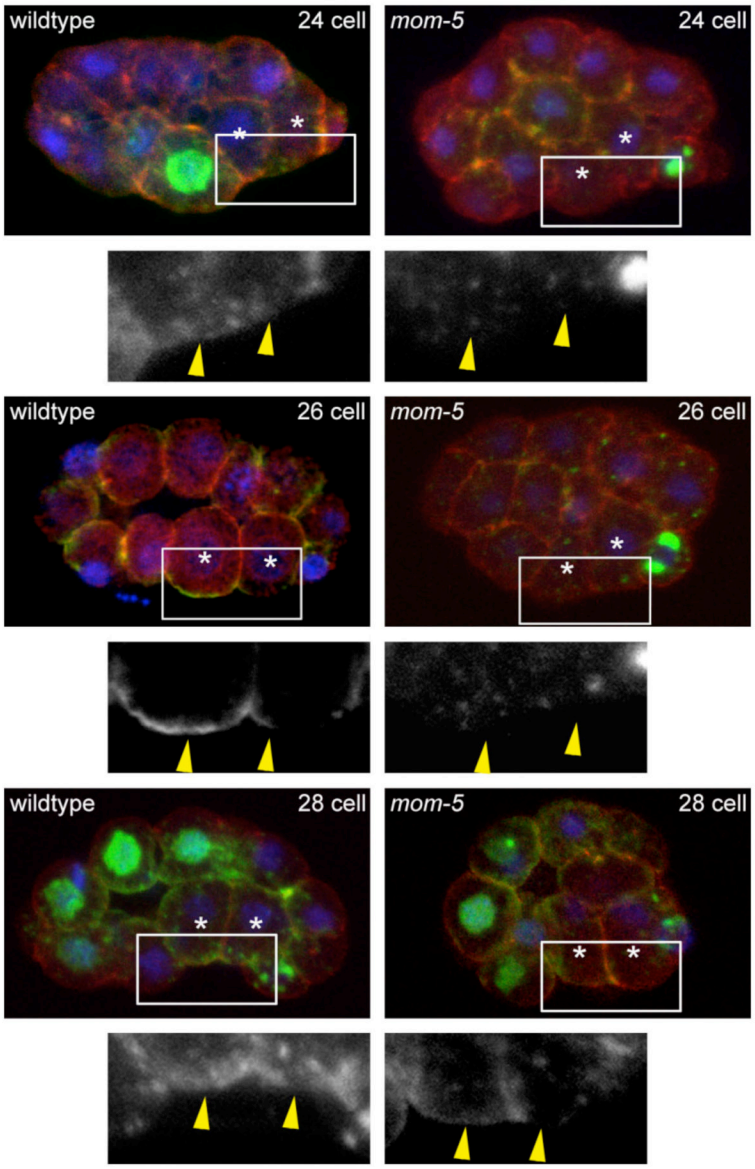

Figure 8. Regulatory Myosin Light-Chain Phosphorylation Depends on MOM-5/Fz

Wild-type embryos show apical accumulation of phosphorylated rMLC as gastrulation occurs, particularly in 26- and 28-cell stage embryos. In contrast, mom-5 embryos show reduced accumulation of phosphorylated rMLC compared to wild-type embryos of the same stages. Ea-Ep cells are indicated by asterisks. p-rMLC is shown in green, F-actin is shown in red, and DAPI is shown in blue. In the bottom panels, Ea-Ep apical membranes are expanded to show p-rMLC staining. Apical membranes are denoted by arrowheads.

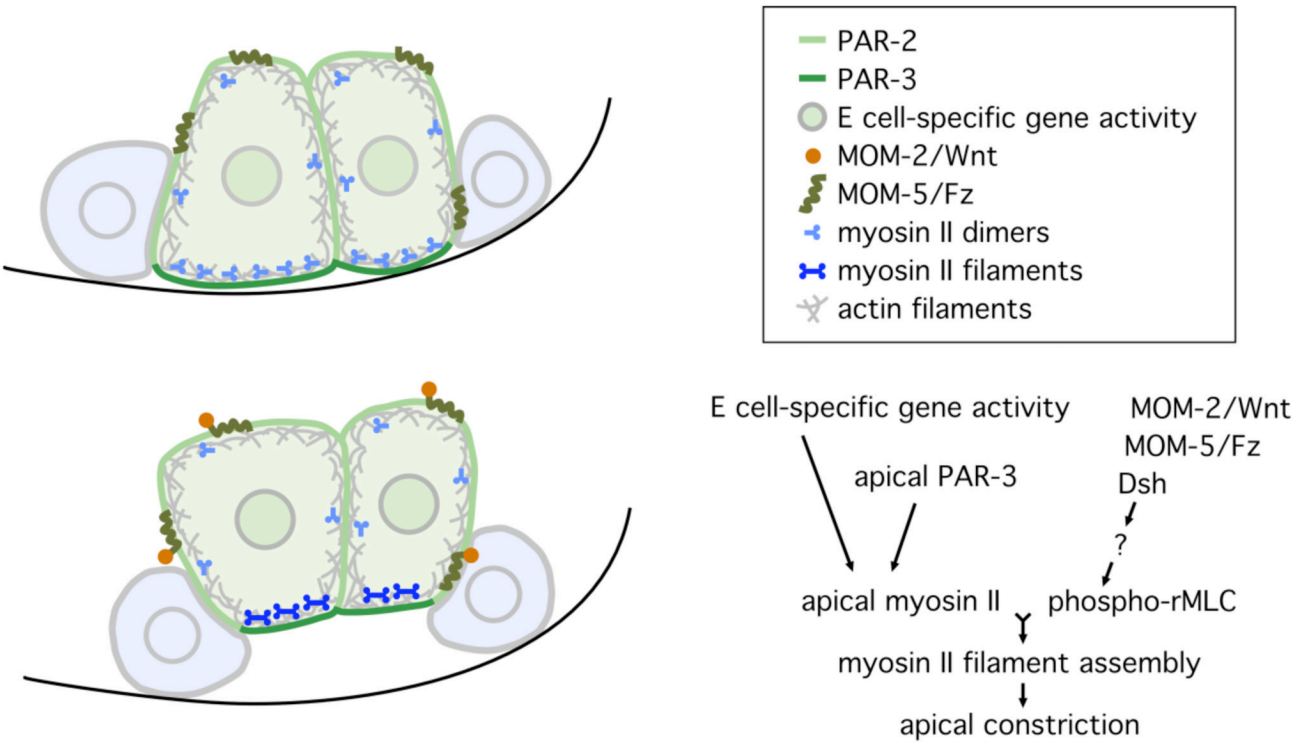

Figure 9. Model of the Mechanisms Controlling C. elegans Gastrulation

At left, genetic and cellular pathway regulating apical constriction.

At bottom right, diagrams of the events at left without (top) and with (bottom) a Wnt signal. See Discussion for explanation. MOM-5 distribution is not detected asymmetrically in cells during gastrulation [54], and MOM-2 distribution is not known. All other proteins are known to localize as diagrammed.

Table 1

Gastrulation Movements in Endoderm-Specification Mutants

Genotype	Homolog/Conserved Domains	Gastrulated/Total	Percentage Gastrulating	Allele Type[a]
Wild-type		17/17	100%	
mom-1	porcupine	2/18	11%	strong loss-of-function
mom-2	wnt/wingless	3/50	6%	deletion; genetic null
mom-3	wntless	3/7	43%	strong loss-of-function
mom-4	MEKK/TAK1	0/10	0%	strong loss-of-function
mom-5	frizzled	7/34	21%	TC1 transposon
lit-1	nemo-like kinase	0/10	0%	protein null
pie-1	CCCH zinc-finger protein	10/11 (Ea-Ep); 11/11 (P4-D)*	91%	strong loss-of-function
skn-1	bZIP transcription factor	2/27	7%	strong loss-of-function
end-1	GATA factor	1/7	14%	deficiency; genetic null
end-3	GATA factor	0/12	0%	genetic null
elt-2	GATA factor	12/12	100%	TC1 transposon; genetic null

*Ea-Ep ingression is followed within the next 30 min by P4-D ingression in pie-1 embryos only. In wild-type, 0/17 embryos showed P4-D ingression during this period.

[a]References are as follows: mom-1, mom-3, and mom-4 [23]; mom-5 [33]; lit-1 [55]; pie-1 [55]; skn-1 [24]; end-1 [25]; end-3 [25]; and elt-2 [27]. See the Supplemental Experimental Procedures for allele designations.