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A Formin Homology Protein and a Profilin Are Required for Cytokinesis and Arp2/3-Independent Assembly of Cortical Microfilaments in *C. elegans*

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Summary

Background: F-actin is enriched at the cortex of embryonic cells in the nematode *Caenorhabditis elegans* and is required for multiple processes that include the establishment of an anterior-posterior (A-P) axis and cytokinesis. However, the mechanisms that regulate cortical microfilament (MF) assembly remain poorly understood.
Results: We show here that a profilin called PFN-1 accumulates at the cortex independent of the actin cytoskeleton and is required for the assembly or maintenance of cortical MFs and myosin. Reducing PFN-1 levels by RNAi results in cytokinesis and A-P polarity defects. PFN-1 binds to the Formin Homology (FH) protein CYK-1, which also is required for cortical MFs. In contrast to PFN-1 and CYK-1, the Arp2/3 complex appears to be dispensable for the assembly of cortical MFs, for A-P polarity, and for cytokinesis. Instead, the Arp2/3 complex is required for cell migrations that occur during gastrulation and may also be involved in cellular rearrangements required for epidermal enclosure prior to elongation of ovoid embryos into vermiform larvae.
Conclusions: We conclude that the FH protein CYK-1 and the profilin PFN-1 mediate the Arp2/3-independent assembly of MFs and are required for cytokinesis in the early embryo. These data suggest that CYK-1 and PFN-1 may nucleate MFs, as has recently been shown for an FH protein and a profilin in yeast.

Introduction

Spontaneous microfilament assembly occurs in vitro when the concentration of monomeric actin (G-actin) increases beyond a threshold level. However, cells can maintain G-actin concentrations that are 1000-fold higher than this critical concentration [1, 2]. This is accomplished by F-actin capping proteins, which inhibit extension at the barbed end of preexisting MFs, and by G-actin-sequestering proteins, which inhibit the nucleation of new MFs as well as elongation at exposed barbed ends [3]. These two classes of actin binding proteins hold the MF cytoskeleton in a metastable state in which nucleation of actin filaments is the rate limiting step in MF assembly. Actin polymerization occurs when free barbed ends are presented to the cytoplasm by uncapping or severing or when microfilaments are nucleated de novo [3]. The Arp2/3 complex, an assemblage of two actin-related proteins (Arp2 and Arp3) and five additional proteins (ArpC1–ArpC5), is one important nucleator of MFs. However, it remains unknown whether the Arp2/3 complex is generally required for MF assembly during development, or if multiple proteins or protein complexes each nucleate specific subsets of MFs.

Profilin was initially identified as an actin-sequestering protein that binds to G-actin with 1:1 stoichiometry [4, 5]. However, profilin can also stimulate MF polymerization by promoting the dissociation of ADP from G-actin, allowing the formation of assembly-competent ATP-actin [1, 2]. Studies in *Drosophila* and yeast have suggested that Formin Homology (FH) proteins transduce Rho signaling to the actin cytoskeleton through profilin, which binds to the proline-rich FH1 domain [6, 7]. Intriguingly, Bni1p and Bnr1p, two yeast Formin Homology proteins, mediate the Arp2/3-independent assembly of F-actin cables [8]. Moreover, two recent in vitro studies have shown that Bni1p can nucleate MF assembly in a process that is enhanced by profilin [9, 10]. Thus, the Arp2/3 complex and Bni1p may nucleate distinct populations of MFs in yeast cells.

To understand the mechanisms that regulate the cortical actomyosin cytoskeleton in animal cells, we have examined the requirements for the FH protein CYK-1 [11], a profilin that we have named PFN-1, and the Arp2/3 complex in the *C. elegans* embryo. Here, we show that both CYK-1 and PFN-1 are required for cytokinesis and for the assembly of cortical MFs. In contrast, the Arp2/3 complex is not required for MF assembly or for most known MF-dependent processes in the early embryo; instead, it is required for cell migrations during gastrulation. We propose a model in which CYK-1 and PFN-1 nucleate MFs required for cytokinesis, while the Arp2/3 complex nucleates MFs required for cell migrations.

Results

Profilin Is Required for Cytokinesis and Cell Polarity

Through BLAST sequence comparisons, we identified in the *C. elegans* genome three predicted genes, called F35C8.6, K03E6.6, and Y18D10A.20, which encode putative profilin proteins. (For sequence alignments, see the Supplementary Material available with this article online). We used RNA interference (RNAi) to test all three profilins for embryonic requirements (see the Experimental Procedures). RNAi of Y18D10A.20 resulted in 100% embryonic lethality beginning 20–26 hr postinjection. Injected worms became sterile approximately 26–30 hr postinjection, suggesting that Y18D10A.20 may be required for oogenesis or fertilization, in addition to embryogenesis. Microinjection of F35C8.6 and K03E6.6 dsRNA, either separately or together, did not result in embryonic lethality, and simultaneous RNAi of all three profilins did not enhance the defects resulting from

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depletion of Y18D10A.20 alone. Hereafter, we will refer to the product of the Y18D10A.20 open reading frame as PFN-1, and we will refer to embryos produced by worms injected with *pfn-1* dsRNA as *pfn-1(RNAi)* embryos.

Following fertilization, the wild-type *C. elegans* embryo is polarized along the anterior-posterior (A-P) axis by an MF-dependent process, resulting in the establishment of multiple asymmetries that include the position of pronuclear meeting and the position and shape of the first mitotic spindle (Figures 1A–1C) [12, 13]. These asymmetries are reduced or abolished in *pfn-1(RNAi)* embryos, and this finding suggests that polarization of the A-P axis is disrupted (Figures 1A–1C). Some embryonic asymmetries may be established by MF-dependent cytoplasmic flows. These flows move posteriorly in the central cytoplasm and anteriorly at the cortex and may carry ribonucleoprotein particles called P granules to the posterior of wild-type embryos (Figure 1D, n = 6) [13, 14]. We found that the rate of cortical flows was reduced from approximately 5.5 μm/min in wild-type to approximately 0.3 μm/min in *pfn-1(RNAi)* embryos (Figure 1E). Furthermore, P granules do not accumulate posteriorly in *pfn-1(RNAi)* embryos but instead aggregate in the central cytoplasm (Figure 1D, n = 15).

In addition to the polarity defects, cytokinesis fails with little or no furrow ingression in *pfn-1(RNAi)* embryos (Figure 1A). However, many mitotic events occur normally: robust astral, kinetochore, and midzone microtubules are present, and chromosome segregation appears normal (Figure 1C). In summary, PFN-1 is required for multiple MF-dependent processes but is not required for the formation or function of the mitotic spindle.

PFN-1 Is Required for Cortical Actomyosin

The defects in *pfn-1(RNAi)* embryos resemble those observed in wild-type embryos exposed to MF-depolymerizing drugs [12, 14]. We therefore examined the localization of actin and the nonmuscle myosin II heavy chain NMY-2, which localize throughout the cortex of wild-type embryonic cells (Figure 2A) [15–17]. While some patches of cortical actin and myosin are still present in *pfn-1(RNAi)* embryos, they are discontinuous and well separated (Figure 2A; actin, 50/53 embryos; NMY-2, 17/20 embryos; 3 embryos had relatively normal levels of both cortical actin and NMY-2). Because some PFN-1 function may persist in *pfn-1(RNAi)* embryos, we have been unable to determine whether PFN-1 is required for the assembly of all cortical actin or if some cortical MFs assemble independent of PFN-1. However, PFN-1 is clearly essential for the formation of the majority of cortical microfilaments.

We raised polyclonal antisera against PFN-1 to examine its subcellular distribution. PFN-1 is present in the cytoplasm of wild-type embryos during interphase and mitosis and is slightly enriched at the cortex (Figure 2B). This pattern of localization is specific for PFN-1, as two independent sera give identical staining in >90% of stained embryos, and both cortical and cytoplasmic localization are undetectable in embryos stained with preimmune sera. Moreover, reduction of PFN-1 levels by RNAi (Figure 2B, 14/15 embryos) and preincubation of either antiserum with the immunogenic peptide (n = 15) both substantially reduced or eliminated cortical and cytoplasmic staining.

We next examined PFN-1 localization after using RNAi to deplete other components of the actomyosin cytoskeleton (Figure 3A). We found that PFN-1 and actin distributions were unchanged in embryos with reduced function of NMY-2 (14/15 embryos) or of the myosin II regulatory light chain MLC-4 (6/6 embryos). Surprisingly, PFN-1 also localizes normally after disruption of cortical actin by RNAi inhibition of the nonmuscle actin *act-5* (6/7 embryos) or by a strong combination of *cyk-1* alleles (7/7 embryos, see below). Because the *cyk-1* allelic combination retains some function, and RNAi depletion of components of the actomyosin cytoskeleton results in eventual sterility, we also examined PFN-1 localization in Latrunculin A-treated embryos. Cortical PFN-1 was detectable in embryos exposed to Latrunculin A both early (12/12 embryos) and late (5/5 embryos) in development (Figure 3A). Thus, the distribution of PFN-1 appears to be independent of the actomyosin cytoskeleton.

CYK-1 Is Required for Cortical Actomyosin

Because profilin binds to the proline-rich FH1 domain of Formin Homology proteins in other systems [6, 7], we used a yeast two-hybrid system [18] to show that PFN-1 can associate with the FH1 domain of CYK-1 (Figure 3B), a *C. elegans* FH protein that is required for embryonic cytokinesis [11]. We then compared the phenotype resulting from loss-of-function mutations in *cyk-1* to the *pfn-1(RNAi)* phenotype. It has been reported that CYK-1 is required late in cytokinesis, after substantial ingression of the cleavage furrow [11]. We have now identified a more severe loss-of-function *cyk-1* allele, called *s2833*, which results in hermaphrodite sterility (Figures 4A and 4B) [19]. Consistent with a requirement in oogenesis, we detect CYK-1 protein in wild-type gonads (Figure 4C), and oocyte cellularization is disrupted in *cyk-1(s2833)* hermaphrodites (Figure 4B).

Although *cyk-1(s2833)* homozygous mutant worms are sterile, many *cyk-1(s2833)/cyk-1(t1568)* worms are fertile and produce embryos that fail early in cytokinesis (Figure 4D) and have severely reduced F-actin localization (Figure 3A, n = 7). Remarkably, embryonic polarity appears normal, although cortical actin levels resemble those in *pfn-1(RNAi)* embryos (Figures 4D and 4E). Thus, CYK-1 may be required only for a subset of PFN-1 functions (see the Discussion).

The Arp2/3 Complex Is Not Required for Most MF-Dependent Processes in the Early *C. elegans* Embryo

Because FH proteins mediate Arp2/3-independent MF assembly in yeast (see the Introduction), we next examined the role of the Arp2/3 complex during early embryonic cell divisions in *C. elegans*. Of the seven proteins comprising the Arp2/3 complex, six are represented by single open reading frames in the *C. elegans* genome, while two paralogous genes encode ArpC5 homologs (see the Supplementary Material). Depleting any of the Arp2/3 components by RNAi resulted in >95% embryonic lethality, with the exception of one ArpC5 gene (see

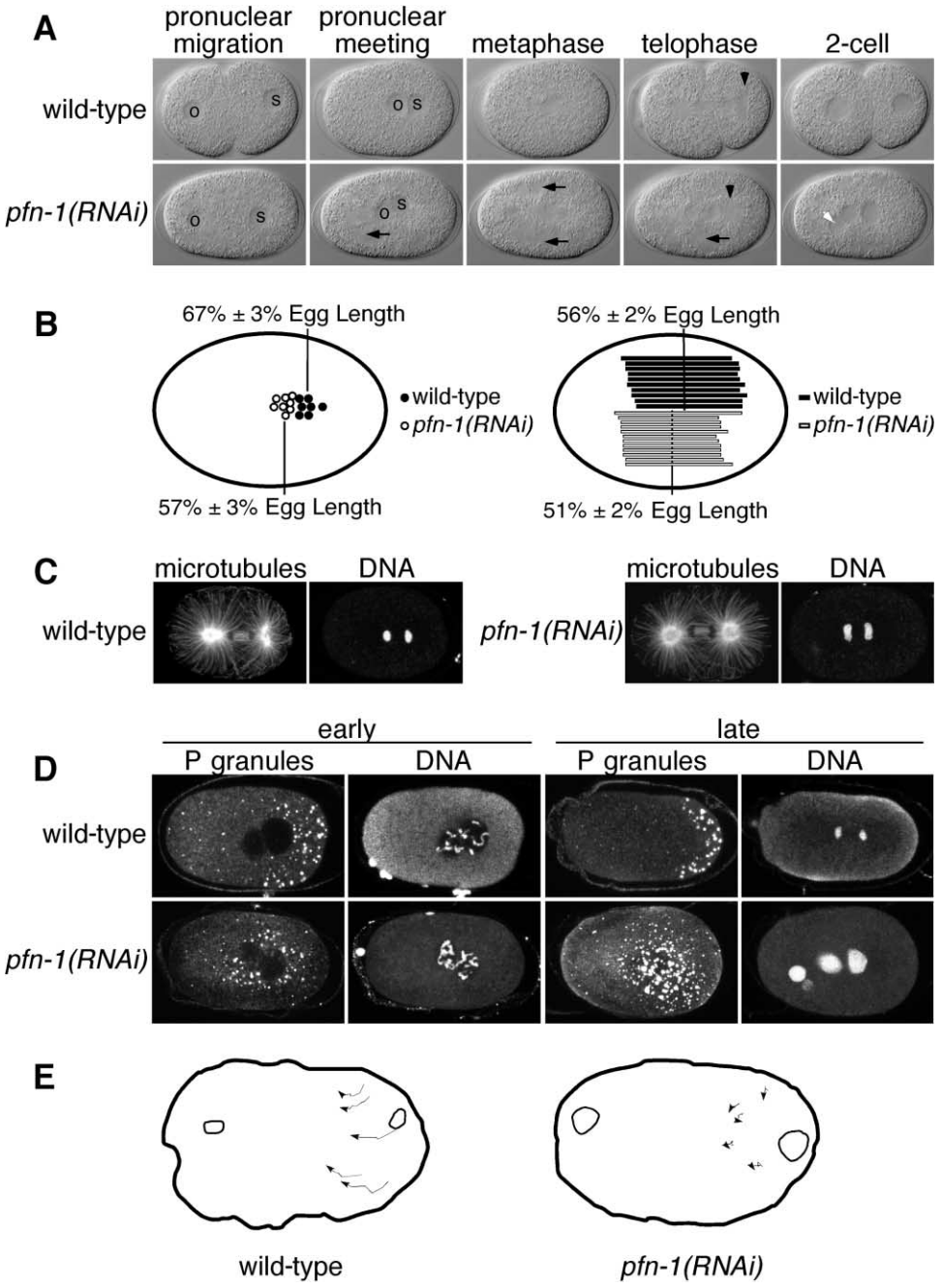

Figure 1. PFN-1 Is Required for Multiple MF-Dependent Processes

(A) Nomarski micrographs of wild-type and *pfn-1(RNAi)* embryos. Following fertilization in wild-type, the oocyte pronucleus (o) meets the sperm pronucleus (s) near the posterior pole, the first mitotic spindle becomes posteriorly displaced, and the posterior centrosome becomes flattened (arrowhead). These asymmetries are reduced or abolished in *pfn-1(RNAi)* embryos, cytokinesis fails, and abnormal cytoplasmic clearings are present (arrows). Extra nuclei form in *pfn-1* embryos as a result of defects in meiotic cytokinesis (white arrowhead). In this and all subsequent images, anterior is oriented toward the left.

(B) Schematic representations of the position of pronuclear meeting (left) and of the first mitotic spindle (right). Spindle position was measured in telophase, when the reforming daughter nuclei first became visible.

(C) Spindle structure in wild-type and *pfn-1(RNAi)* embryos. Robust astral, kinetochore, and midzone microtubules are visible in both wild-type and *pfn-1(RNAi)* embryos. The posterior centrosome becomes flattened in wild-type but remains rounded in *pfn-1(RNAi)* embryos.

(D) During pronuclear migration, P granules accumulate in the posterior of wild-type embryos (early), where they remain throughout the first cell cycle (late). P granules fail to localize posteriorly in *pfn-1(RNAi)* embryos.

(E) Cortical flows are dramatically reduced in *pfn-1(RNAi)* embryos. Each arrow indicates the path of a single yolk granule during 1 min.

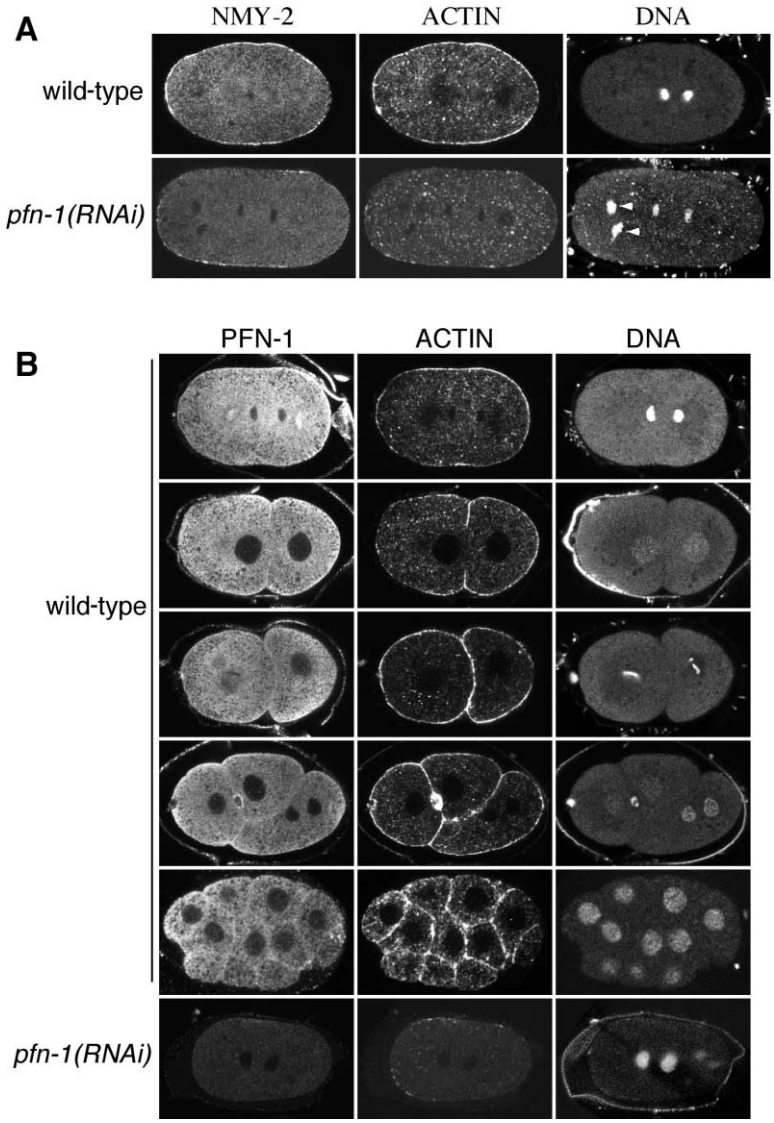

Figure 2. PFN-1 Is Required for the Cortical Localization of Actin and Myosin

(A) Actin and NMY-2 localize around the cortex of wild-type embryos. The levels of both proteins are reduced at the cortex of *pfn-1(RNAi)* embryos.

(B) PFN-1 is detectable in the cytoplasm and at the cortex of wild-type embryonic cells. Profilin is present at similar levels in the cleavage furrows of dividing cells and elsewhere at the cortex. Cortical and cytoplasmic staining are both reduced in *pfn-1(RNAi)* embryos. Nonspecific centrosomal staining is sometimes observed in both wild-type and *pfn-1(RNAi)* embryos.

the Experimental Procedures). Nevertheless, the A-P axis was polarized, and both meiotic and mitotic cytokinesis were completed normally (Figures 5A and 5B, and data not shown; $n \geq 6$ for each component). Both actin and NMY-2 accumulated throughout the cortex of *arp-2; arp-3(RNAi)* embryos (Figure 5C, $n = 10$) and in each single mutant ($n \geq 5$ for each, data not shown). However, prominent membrane blebs formed in all mutant embryos, suggesting that the Arp2/3 complex is required for cortical stability (Figure 5A; see the Supplementary Material). While it remains possible that residual Arp2/3 function is sufficient for cytokinesis and polarization in these embryos, depletion of any one component of the Arp2/3 complex results in the same phenotype, and simultaneous depletion of Arp2 and Arp3 or other combinations of Arp2/3 complex components did not enhance any defects (Figure 5A, data not shown).

We next examined the terminal phenotype of depleted embryos. Mutant embryos differentiated but failed to elongate into long, thin larvae (Figure 6A). Furthermore, gut and pharyngeal cells were nearly always present on the surface of mutant embryos (Figure 6A). To determine whether the Arp2/3 complex is required for gastrulation, we observed cell migrations in embryos expressing a histone::GFP fusion that labels chromosomes (Figure 6B). In 9/12 *arp-2; arp-3* embryos, the endodermal precursor cells Ea and Ep failed to ingress and divided on the surface of the mutant embryo (Figure 6B; see the Supplementary Material). In 3/12 embryos, Ea and Ep began to ingress into the blastocoel, but neighboring cells did not spread over their apical surfaces and gastrulation failed (see the Supplementary Material). We also observed defects in the ingression of the germline progenitor P_4 (Figure 6B) and the descendents of the mesodermal founder cell MS (data not shown).

In addition to the defects in gastrulation, *arp-2; arp-3*-depleted embryos failed to elongate. Elongation of wild-type embryos occurs in two MF-dependent steps [20]. First, the hypodermal (or skin) cells intercalate dorsally and elongate while migrating ventrally to enclose the embryo. Subsequently, the hypodermal cells constrict circumferentially to elongate the embryo. We

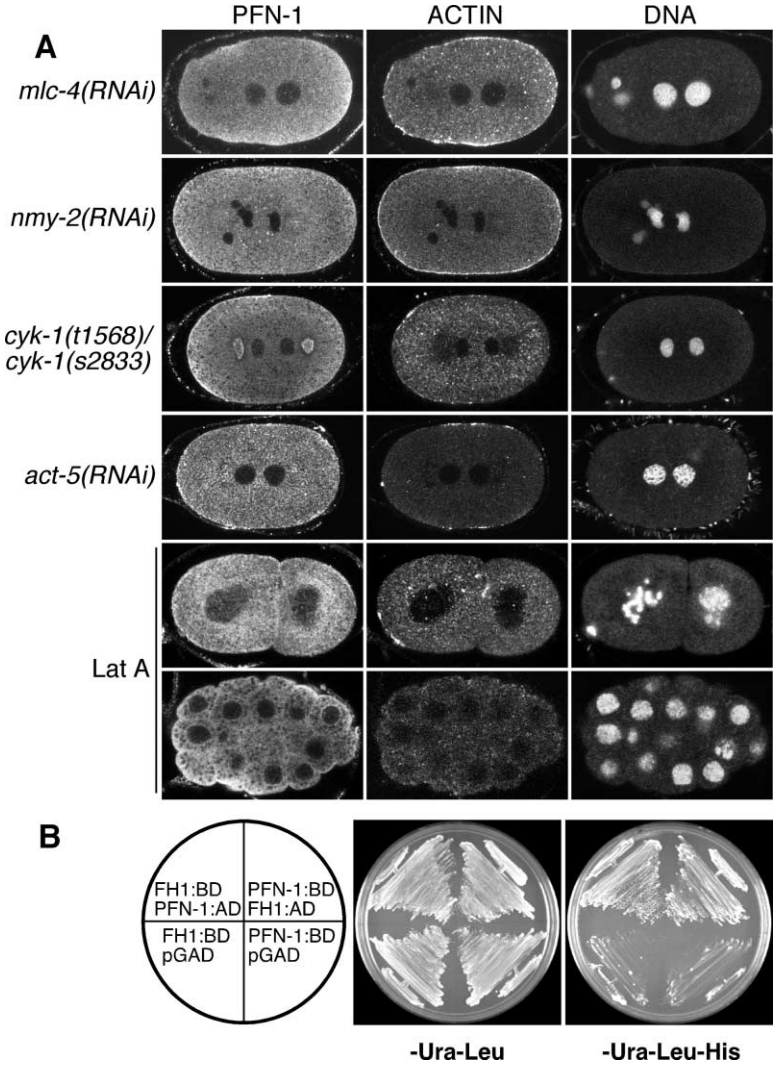

Figure 3. PFN-1 Distribution in Cytokinesis Mutants

(A) PFN-1 localizes at the cortex of embryos with reduced levels of NMY-2, its regulatory light chain MLC-4, the FH protein CYK-1, or the nonmuscle actin ACT-5. PFN-1 also appears normal in embryos treated with Latrunculin A.

(B) PFN-1 can bind to the FH protein CYK-1 in a yeast two-hybrid assay.

found that hypodermal cells were present in a clump on one surface of *arp-2; arp-3(RNAi)* embryos after 5 hr of development at 25°C, while similarly staged wild-type embryos were completely enclosed (Figure 6C, $n \geq 10$). Thus, the Arp2/3 complex may be required for MF-dependent cell migrations and/or shape changes during morphogenesis. Alternatively, enclosure may fail due to defects in the substrate over which hypodermal cells migrate, which could be disrupted by the earlier failure in gastrulation.

Discussion

We have shown that the *C. elegans* profilin PFN-1 and the FH protein CYK-1 are required for assembly or stability of the cortical actomyosin cytoskeleton and for cytokinesis. In contrast, the Arp2/3 complex is required for cell migrations during gastrulation, but not for cortical accumulation of actomyosin or for cytokinesis. These findings suggest that PFN-1 and CYK-1 either nucleate MFs or stabilize MFs nucleated by an Arp2/3-independent mechanism.

Profilin and FH Proteins Regulate Actomyosin Assembly

In *S. cerevisiae* and *S. pombe*, profilin and FH proteins are dispensable for the assembly of cortical actin patches but are required for patch polarization and contractile ring formation [21–24]. Likewise, mutations in FH proteins or in profilin disrupt contractile ring assembly in *Drosophila* [25] but only weakly affect MF enrichment at the embryonic cortex [26, 27]. In contrast, both CYK-1 and PFN-1 are essential for the assembly of the cortical actin network that encircles *C. elegans* embryonic cells. Moreover, membrane ingressions form around the entire cortex of wild-type embryos following fertilization, and deep invaginations that resemble cleavage furrows form at ectopic locations in some mutant backgrounds [28]. We propose that the entire cortical actomyosin cytoskeleton in *C. elegans* embryos resembles a contractile ring and is required not only for cytokinesis but also for embryonic polarity. Furthermore, the Arp2/3 complex is dispensable for contractile ring assembly in *S. cerevisiae* [29], and we suggest that this is also true for cortical microfilament assembly in worm embryos.

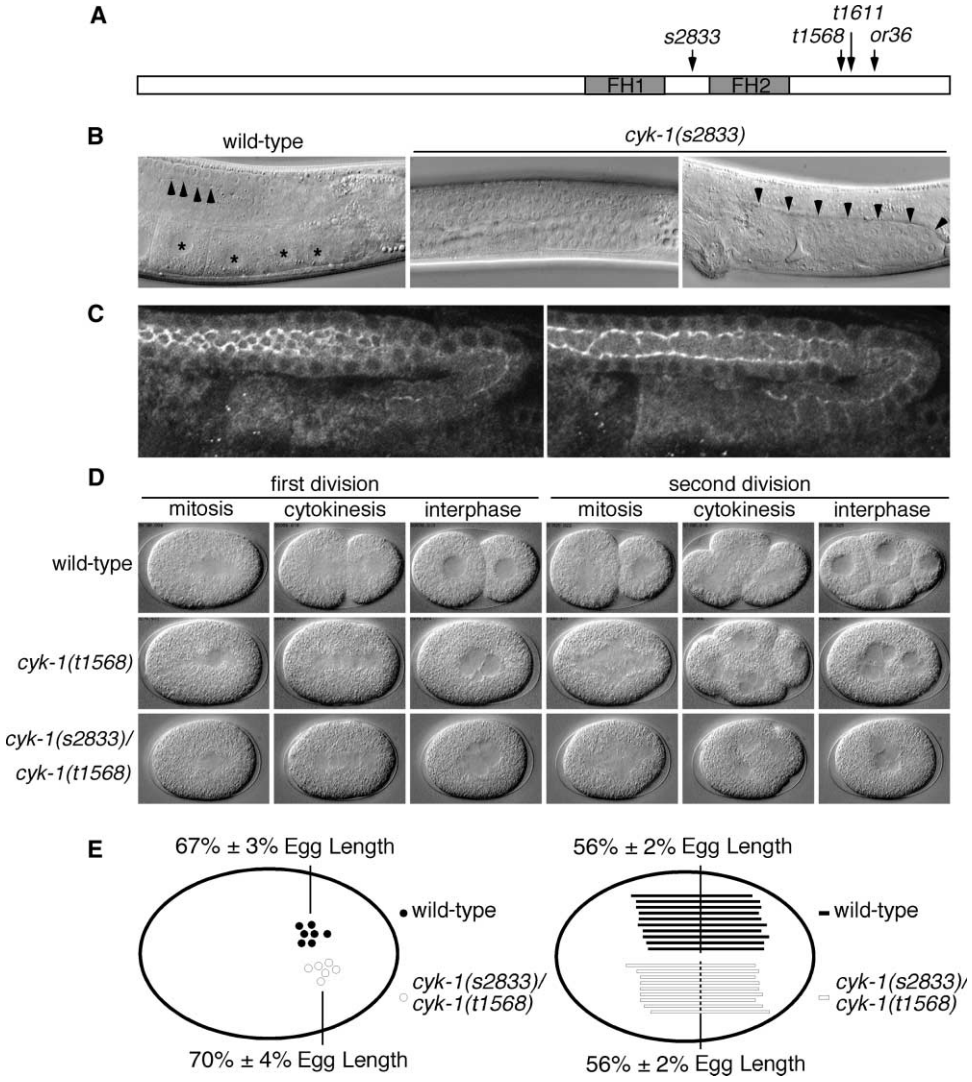

Figure 4. The FH Protein CYK-1 Is Required for Oogenesis and Embryonic Cytokinesis

(A) A schematic representation of the CYK-1 protein, showing the positions of the four known mutations, which introduce premature stop codons.

(B) Left panel: in wild-type hermaphrodites, the germline occupies two symmetrical gonad arms, each of which reflexes on itself to form a U-shaped organ. Germline nuclei occupy the periphery of the syncytial distal gonad and are separated by membrane invaginations (arrowheads). Oocytes are cellularized in the proximal gonad (asterisks). Center panel: while gonadogenesis appears normal in some *cyk-1(s2833)* animals, oocytes are not cellularized. Right panel: morphogenesis of the gonad fails in the most severely affected *cyk-1(s2833)* animals. Arrowheads delimit a shortened, nonreflexed gonad arm. The membrane invaginations around germline nuclei appear disorganized or absent in *cyk-1(s2833)* worms, and germline nuclei are present throughout the gonad.

(C) CYK-1 is detectable at the leading edge of membrane cubicles surrounding germline nuclei in a wild-type ovary. Left: optical section through the gonad at a level just above the peripheral germline nuclei. A meshwork of membrane invaginations containing CYK-1 protein is visible. Right: medial section. CYK-1 protein is detectable at the apex of membrane invaginations and around newly formed oocytes.

(D) The first cleavage furrow is severely reduced in *cyk-1(t1568)* mutant embryos, but deep furrows bisect each arm of the tetrapolar second spindle. Embryos of *cyk-1(s2833)/cyk-1(t1568)* mutant worms produce only shallow furrows during both divisions.

(E) Left panel: pronuclei meet posteriorly in *cyk-1(s2833)/cyk-1(t1568)* embryos, as in wild-type. Right panel: the first mitotic spindle is asymmetrically positioned in wild-type and *cyk-1* embryos.

The Arp2/3 Complex Acts in Gastrulation

The Arp2/3 complex is thought to play a major role in MF nucleation, based on in vitro assays of MF assembly and studies of crawling cells and of the rocket-like motility of some bacterial pathogens [3, 30, 31]. In contrast, this complex is not required for the assembly of certain MF structures in *Drosophila* and yeast [8, 29, 32] Likewise, the Arp2/3 complex is dispensable for the assembly or function of cortical MFs in the early *C. elegans* embryo; however, it is required for cell migrations during gastrulation and perhaps for epidermal cell migrations or shape changes during morphogenesis. Mutations in *gad-1*, *gex-2*, and *gex-3* also result in the presence of superficial gut cells and defects in hypodermal enclosure [33, 34]. However, *gex-2* and *gex-3* are not required for gastrulation but are required for hypodermal enclo-

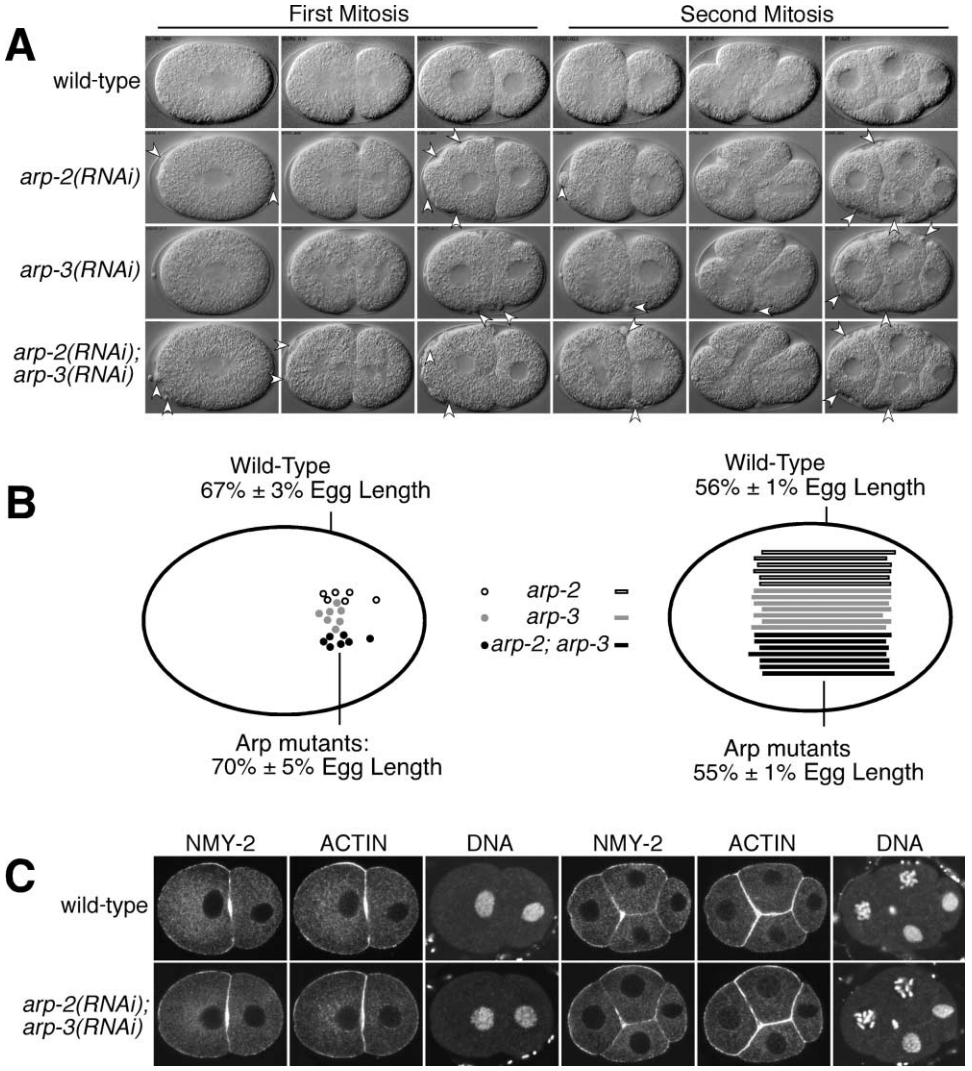

Figure 5. The Arp2/3 Complex Is Not Required for Cytokinesis or Polarity

(A) Membrane protrusions form in *arp-2*- and *arp-3*-depleted embryos (arrowheads), but cytokinesis appears normal. Simultaneous depletion of ARP-2 and ARP-3 does not result in a stronger phenotype. The blebbing phenotype is best viewed in time-lapse Movies 4–6, which can be found in the Supplementary Material available with this article online.

(B) Embryonic polarity is established normally in embryos mutant for components of the Arp2/3 complex. The positions of pronuclear meeting and of the first mitotic spindle are shown for *arp-2* and *arp-3* single mutants and for *arp-2; arp-3* double mutants. The positions for wild-type embryos are shown in Figure 1B.

(C) Cortical MFs and myosin accumulate at normal levels at the cortex of *arp-2; arp-3* embryos.

sure and possibly for organ morphogenesis. In *gad-1* mutants, Ea and Ep divide prematurely, while the cell cycle timing of the E lineage is unaffected in Arp2/3-deficient embryos (data not shown). Thus, reducing Arp2/3 function results in a novel gastrulation defect. To our knowledge, this is the first demonstration of a requirement for the Arp2/3 complex in gastrulation in any animal.

CYK-1 Is Required throughout Cytokinesis

Our analysis indicates that CYK-1 is required early in cytokinesis, like FH proteins in other organisms [6, 7]. Because CYK-1 is first detectable only after extensive furrow ingression [11], it presumably can function at levels not detected with currently available antisera, both during formation of the cortical actomyosin cytoskeleton and early in cytokinesis. CYK-1 accumulates dramatically in the contractile ring as furrow ingression progresses. We do not detect equatorial CYK-1 in *pfn-1*, *mlc-4*, or *nmy-2* mutant embryos (data not shown), suggesting that PFN-1 and myosin may be important for the accumulation of CYK-1 late in cytokinesis. Alternatively, perhaps interactions between the cleavage furrow and the central spindle are required for the late localization of CYK-1, as FH proteins affect both the actin and microtubule cytoskeleton in other organisms [25, 35–37]. Consistent with this possibility, CYK-1 is not detectable during ingression of the pseudocleavage furrow, a transient furrow that does not encircle a mitotic spindle (AFS, unpublished data).

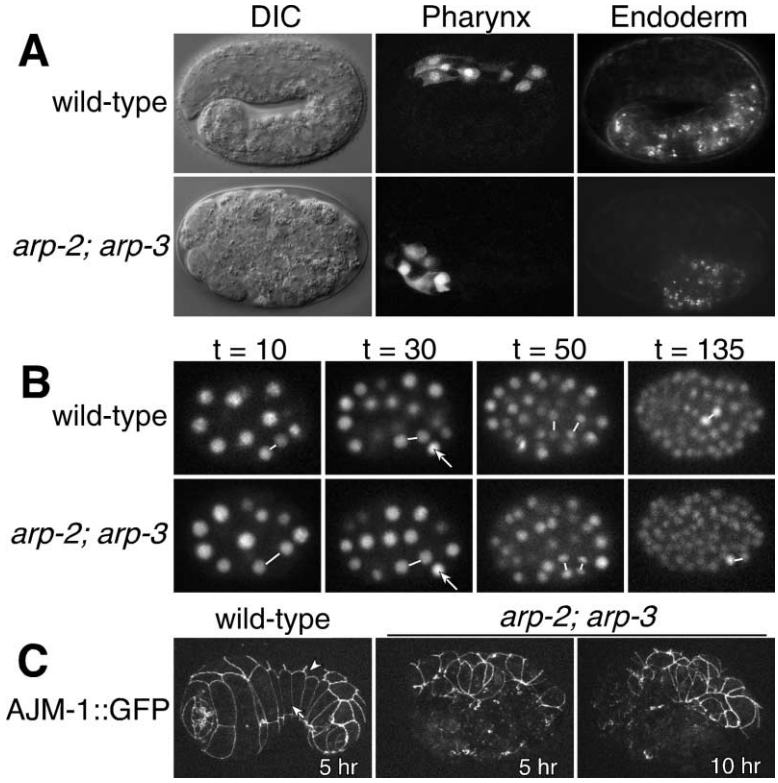

Figure 6. The Arp2/3 Complex Is Required for Gastrulation and Epidermal Morphogenesis

(A) Pharyngeal cells expressing a CEH-22::GFP transgene are present internally in wild-type embryos but are present on the surface of *arp-2; arp-3* embryos. Similar defects are observed in the distribution of endodermal cells, as shown by the birefringence of gut granules.

(B) Time-lapse analysis of gastrulation. Times are given in minutes after metaphase in the endodermal precursor cell E. The positions of embryonic cells were detected by a histone::GFP fusion that marks chromosomes, and sister cells are joined by white bars. In wild-type embryos, Ea and Ep ingress into the blastocoel (t = 10 min, 30 min), then continue to divide inside the embryo (t = 50 min). P_4 (arrow, t = 30 min) migrates over the apical surface of Ep, then its descendants later ingress into the embryo (t = 135 min). Ea and Ep divide on the surface of *arp-2; arp-3(RNAi)* embryos. The descendents of P_4 are also present externally.

(C) Enclosure of wild-type and *arp-2; arp-3(RNAi)* embryos expressing AJM-1::GFP, which labels the periphery of hypodermal cells [46, 47]. Times are hours of development at 25°C (see the Experimental Procedures). Hypodermal cells migrate and undergo epiboly to completely enclose the wild-type embryo. In this view, the ventral and lateral rows of hypodermal cells are visible (arrow and arrowhead, respectively). Hypodermal enclosure fails in *arp-2; arp-3(RNAi)* embryos.

Although the cortex of *cyk-1(s2833)/cyk-1(t1568)* mutant embryos contains very low levels of actin, embryonic polarity appears normal. However, this allelic combination does not completely eliminate *cyk-1* function and may therefore not reveal the null phenotype. CYK-1 is required for polarity in the absence of the Mitotic Kinesin-Like Protein (MKLP) ZEN-4, suggesting that CYK-1 is involved in polarizing the embryo, even if it is not itself essential for embryonic asymmetries [38].

A Hierarchical Mechanism of Cortical Assembly

CYK-1 and PFN-1 are required for the accumulation of cortical actin, suggesting that they function at a high level in a hierarchy of proteins that control the assembly of cortical actomyosin (Figure 7). Moreover, PFN-1 can localize at the cortex independent of the actin cytoskeleton. Interactions with phosphatidylinositol 4,5 bisphosphate (PIP2) are required for membrane association of yeast profilin [39]. While the functional significance of this interaction remains poorly understood, it is a conserved property of profilins from yeast to humans [2, 40]. Because RNAi depletion of inositol synthase or of phosphotidylinositol 4-phosphate 5-kinase, enzymes involved in PIP2 biosynthesis, does not result in lethality, we have been unable to demonstrate such a role in *C. elegans* (data not shown).

Like PFN-1, NMY-2 and MLC-4 are required for cytokinesis and embryonic polarity. However, neither myosin subunit is required for cortical MF assembly, suggesting that myosin occupies a lower rank in a hierarchy of actomyosin assembly (Figure 7). A more rigorous analysis of this model will require identification of conditional null alleles affecting myosin function, as RNAi may not deplete myosin completely. However, our data indicate that cortical actin can assemble in embryos with sub-

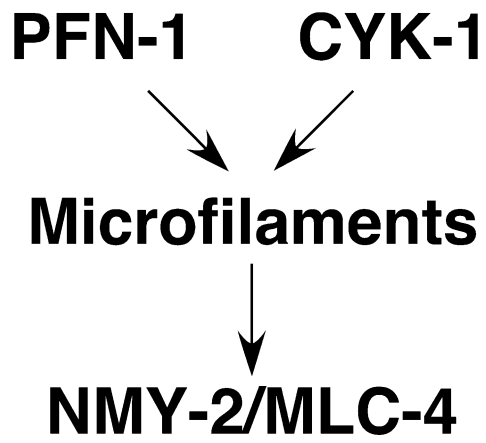

Figure 7. A Model for Assembly of the Actin Cytoskeleton

PFN-1 and CYK-1 are required for cortical localization of MFs and NMY-2. Although PFN-1 and CYK-1 interact in a yeast two-hybrid assay (Figure 3), they may share only a subset of functions in the regulation of MF assembly and are therefore shown as separate inputs to MF assembly in this model. F-actin can accumulate at the cortex of embryos lacking NMY-2 or MLC-4, suggesting that myosin functions downstream of actin localization.

stantially reduced levels of NMY-2 or MLC-4. We therefore propose that formation of the cortical MF cytoskeleton in *C. elegans* proceeds through a series of discrete, genetically separable steps (Figure 7). This pathway is consistent with recent data showing that a yeast FH protein and profilin nucleate MFs. We propose that formins including CYK-1 function similarly in metazoan cells.

Conclusions

We conclude that PFN-1 and CYK-1 function at a high level in a hierarchical pathway of MF assembly and may act to nucleate MFs that are required for cleavage furrow ingression during cytokinesis. In contrast, the Arp2/3 complex is dispensable for most MF-dependent processes in the early embryo and instead appears to be required in motile cells during gastrulation and morphogenesis. Thus, distinct mechanisms may nucleate MFs that function in cytokinesis and those required for cell migration.

Experimental Procedures

Strains and Alleles

C. elegans strains were cultured as described previously; N2 Bristol was used as the wild-type strain [41]. The following alleles and balancer chromosomes were used in this study: LGIII: *dpy-17(e164)*, *lon-1(e185)*, *cyk-1(t1568)*, *cyk-1(s2833)*, *unc-36(e251)*, *unc-32(e189)*, and *qC1(inversion balancer: dpy-19, glp-1)*; LGIV: *him-8(e1489)*. The identification of *cyk-1(s2833)*, previously known as *let-794(s2833)*, has been reported elsewhere [19].

RNA Interference

Double-stranded RNA was prepared and injected by standard methods [42]. To ensure that all embryos examined had the lowest protein levels possible, we obtained embryos from dsRNA-injected worms at time points at which many injected worms were already sterile, approximately 24 hr postinjection. The following clones were used: *act-5*, yk170f4; *mlc-4*, yk167f10; *nmy-2*, yk45d7; *pfn-1*, yk402e3; F35C8.6, yk124e8; K07C5.1 (Arp2), yk377e3; Y71F9AL.16 (Arp3), yk221f3; Y79H2A.6 (ARPC1), yk147h9; Y6D11A.2 (ARPC2), yk277h3; Y37D8A.1 (ARPC3), yk508a3; C35D10.16 (ARPC4), yk393h10; M01B12.3 (ARPC5), yk311a10. The cDNA encoding the profilin K03E6.6 was cloned by RT-PCR with the SuperScript II Reverse Transcriptase (Life Technologies). For C46H11.3 (ARPC5), the entire open reading frame was amplified by PCR, then cloned into the pGEM-T plasmid (Promega). In two independent experiments, RNAi of C46H11.3 did not result in embryonic lethality.

Production of Antisera that Recognize PFN-1

Two independent rabbit polyclonal antisera were generated against the C-terminal PFN-1 peptide CAQVRKAVESMQTYLNNA (Quality Controlled Biochemicals). A PCR product containing the full-length PFN-1 coding sequence from the cDNA clone yk402e3 was ligated into pGEX-3x (Pharmacia), and GST-PFN-1 was purified as described [43]. The PFN-1 antibody was affinity purified by binding to GST-PFN-1 protein immobilized on nitrocellulose [44]. For peptide competition assays, antisera were incubated with 100 μg/ml of the immunogenic peptide overnight at 4°C, then used for immunofluorescence as described below.

Immunofluorescence and Microscopy

Embryos were fixed and stained as described [38]. The following antibodies were used: anti-PFN-1 diluted 1:500, anti-NMY-2 [17], anti-CYK-1 [11], mouse anti-actin (ICN clone C4), and a monoclonal anti-α-tubulin antibody (clone DM1α; Sigma). DNA was labeled with 0.2 μM TOTO3 (Molecular Probes). Fluorescent images were obtained on a BioRad MRC 1024 laser scanning confocal microscope. Hypodermal morphogenesis was examined by the expression of AJM-1::GFP [20]. One- and two-cell-stage wild-type or *arp-2; arp-3* embryos were incubated at 25°C for 4–5 hr, then GFP localization was observed by confocal microscopy.

Time-lapse microscopy and analyses of cortical flows were performed as described elsewhere [15, 38]. Velocities were calculated for 20 yolk granules, representing 5 wild-type and 5 *pfn-1(RNAi)* embryos. For Latrunculin A treatment, embryos were adhered to a polylysine-coated slide in distilled water, then stained with Coomassie Brilliant Blue [44] for 30 s to increase light absorption of the eggshell. Embryos were rinsed in SGM + FCS [15], then immersed in SGM + FCS containing either 10 μM Latrunculin A (from a 10 mM stock in DMSO, Molecular Probes) or DMSO alone as a control. The eggshell was permeabilized with 3–4 pulses of laser irradiation from a sealed nitrogen-pulsed laser (VSL-337, Laser Science). Permeabilized embryos were incubated for >10 min, then processed for immunocytochemistry as described above. Staining of negative controls was identical to wild-type.

Lineaging of Gastrulating Embryos

Embryos expressing HIS-11::GFP [45] were observed with a spinning disc confocal microscope (Perkin Elmer). Briefly, 12 focal planes were acquired at 2-μm intervals. One complete Z-series was taken every minute. These settings were not lethal when embryos were viewed at approximately 10% of maximum laser power for 4–5 hr.

Yeast Two Hybrid

The full-length *pfn-1* cDNA was amplified from yk402e3 by PCR and was cloned into the yeast two-hybrid vectors PGAD-C1 and PGBDU-C1 [18]. The 692-bp SalI/NcoI fragment of the *cyk-1* cDNA yk471h10 was cloned into PGAD-C3, and the 764-bp SalI/PvuII fragment was cloned into PGBDU-C3. These fragments contain the entire FH1 domain. Each binding domain vector was transformed into the yeast strain PJ69-4A, either with the AD fusion from the other protein or with a plasmid encoding the AD alone. Yeast were plated on SD-Ura-Leu to select for both plasmids. Healthy colonies were restreaked on SD-Ura-Leu-His + 2.5 mM 3-AT to test for interactions.

Supplementary Material

Supplementary Material including sequence alignments for profilin and the components of the Arp2/3 complex, as well as movies of the early embryonic divisions of the wild-type and mutant embryos described in this manuscript is available at http://images.cellpress.com/supmat/supmatin.htm.

Acknowledgments

We thank Ken Kemphues and Susan Strome for providing antibodies, Yuji Kohara for sharing cDNA clones, and the *C. elegans* Genetics Center, funded by the National Institutes of Health (NIH) National Center for Research Resources, for providing strains. We thank Michael Glotzer and Chris Doe for comments on the manuscript. A.F.S. was funded in part by a training grant from the NIH (5T32 HD07348), D.L.B. was funded by a grant from the Natural Sciences and Engineering Research Council of Canada, and A.F.S. and B.B. were funded by the NIH (R01GM058017).

Received: August 21, 2002
Revised: October 1, 2002
Accepted: October 3, 2002
Published: December 23, 2002

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