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Involvement of fatty acid pathways and cortical interaction of the pronuclear complex in *Caenorhabditis elegans* embryonic polarity

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Abstract

Background: Cell polarity is essential for many decisions made during development. While investigation of polarity-specific factors has yielded great insights into the polarization process, little is known on how these polarity-specific factors link to the basic cellular mechanisms that function in non-polarity aspects of the cell. To better understand the mechanisms that establish embryonic polarity, we investigated genes required for polarity in the one-cell *C. elegans* embryo that are also required for other non-polarity functions. This has led to the identification of the Pod-class of mutants that are characterized by osmosensitive embryos and defects in anterior-posterior polarity.

Results: Mutation in either of two loci of this class, *emb-8* and *pod-2*, disrupts embryonic polarization and results in osmotically-sensitive embryos. Loss of *emb-8*, a previously uncharacterized polarity gene, causes mislocalization of PAR-3 and PAR-2 that molecularly mark the anterior and posterior cortices. *emb-8* encodes NADPH-cytochrome P450 reductase, a protein supplying electrons to cytochrome P450-family enzymes, some of which catalyze fatty acid modifications. Cloning of the previously characterized polarity gene *pod-2* reveals it encodes acetyl-CoA carboxylase, an enzyme that catalyzes the first step in de novo fatty acid synthesis. Depletion of fatty acid synthase, the next enzyme in the biosynthetic pathway, by RNA-interference (RNAi) also causes similar loss of one-cell polarity. Furthermore, *pod-2* polarity defects can be rescued by addition of exogenous fatty acids. By following the behavior of the pronucleus in *emb-8* and *pod-2* mutant embryos, we demonstrate that loss of polarity correlates with impaired interaction between the pronucleus-centrosome complex and the posterior cortex.

Conclusions: The characterization of *emb-8* and *pod-2* mutant embryos suggests that the pronucleus-centrosome complex interaction with the cortex plays a direct role in establishing polarity and that fatty acid pathways are important for this polarizing event.

Background

The formation and function of diverse cell types during development requires the establishment of cell polarity. Cell polarity can provide for the asymmetric localization of cell fate determinants, thereby ensuring their inheritance by only one of the two daughter cells [1]. Prominent examples of this process include ASH1 mRNA localization to the daughter cell in yeast, *Numb* and *Prospero* localization during *Drosophila* neuroblast determination, segregation of germline granules in *C. elegans*, and *Vg1* localization in *Xenopus* [2–6]. Once differentiated cell types and tissues have been formed, cell polarity continues to play an essential role in their function. For example, directional transport and sorting of molecules to either apical surfaces or basolateral regions are key features of epithelial cells [7,8].

Given the widespread requirement for cell polarity, it is no surprise its establishment involves a conserved set of proteins. Several studies have shown that a conserved protein complex, comprised of PAR-3 (ASIP/bazooka), PAR-6, and atypical PKC, functions in nematodes, fruit flies, and humans and in diverse cells ranging from embryos to epithelia (reviewed in [9,10]). One hallmark of this complex, hereafter referred to as the PAR-3 complex, is its asymmetric localization. In the *C. elegans* one-cell embryo, the PAR-3 complex becomes restricted to the anterior cortex and another polarity protein, PAR-2, localizes reciprocally to the posterior cortex [11,12]. This spatial distinction between anterior and posterior cortical domains defines the first body axis of the embryo. These molecular landmarks are thought to be translated into the physically asymmetric first division and differential segregation of developmental determinants such as germline granules (reviewed in [13]).

While the asymmetric localization of the PAR-3 complex appears to be essential for downstream polarity processes, the mechanisms underlying the polarization of the complex itself are poorly understood. Part of the difficulty lies in the fact such mechanisms probably involve the more generalized machinery of the cell. In contrast to polarity gene mutants that only affect polarity establishment, impairment of core cellular processes will likely produce more complex phenotypes with both polarity and non-polarity defects. Yet, in order to better understand the polarization process, the role of these factors will need to be defined.

Research in the *C. elegans* one-cell embryo is beginning to reveal some of these more general cellular functions required for polarity establishment. In *C. elegans*, the sperm, probably via its associated centrosome, provides the initial polarizing cue that defines the posterior end [14]. As might be expected, translation of this cue into the final polarization of the zygote requires basic elements of the cytoskeleton. Recent studies have implicated the microtubule cytoskeleton in this process. Mutations in the *spd-2* gene attenuate microtubule nucleation from the sperm-derived centrosomes and cause mislocalization of PAR-3 and PAR-2 [15]. Other mutants that create an ectopic microtubule array at the anterior end are sufficient to reverse the localizations of PAR-3 and PAR-2 [16]. These findings suggest that some aspect of the centrosome or microtubule array directs the localization of PAR-3 and PAR-2 to discrete parts of the cell, with PAR-3 adopting a localization opposite that of the microtubule organizing center. Studies with other polarity mutants further suggest that a close association of the sperm-derived centrosome with the cortex provides the polarity-generating cue required for normal polarity [17]. Disruption of actin or the actin-associated proteins, non-muscle myosin (*nmy-2*) and myosin light-chain (*mlc-4*), also cause loss of asymmetry at the first division and mislocalization of PAR-3 and PAR-2 although the underlying mechanism is still not understood [18–20]. Given the essential nature of the cytoskeleton in general, uncovering its polarity functions required transient disruption or partial protein depletion in order to circumvent other non-polarity phenotypes (*e.g.*, defects in cytokinesis or cell cycle arrest).

Our approach to understanding how the basic cellular machinery is involved in establishing polarity has led to the discovery and characterization of the Pod class of mutants (Polarity and Osmotic Defective; [17]). The inaugural member of this class is an actin-associated protein, POD-1. The loss of POD-1 causes polarity and non-polarity defects including osmotically sensitive (or osmosensitive) embryos, cytokinesis defects, abnormal endocytic structures, and transient appearance of abnormal cell peripheries [21]. The *pod* genes were defined as a class with the discovery of the *pod-2* gene that is also required for polarity and osmotic protection and functions in the same pathway as *pod-1* [22]. The osmotic sensitivity of *pod-1* and *pod-2* mutants likely results from defects in the secreted eggshell that normally protects the embryo from the external environments and make the embryo impermeable to most dyes. These phenotypes and the similarity of POD-1 to *coronin*, a gene involved in endocytosis and phagocytosis [23,24], suggest polarization of the zygote may involve membrane trafficking processes. Additional Pod class members encode subunits of the anaphase-promoting complex (APC) [17]. These are required to polarize PAR-3 and PAR-2, possibly through separin-dependent proteolysis of a centrosome/microtubule associated factor normally required to repulse PAR-3 from the posterior cortex.

Here we detail the characterization of two additional Pod-class polarity genes: *emb-8* and *pod-2*. We identified *emb-8*

as a member of the Pod class of polarity genes and show *emb-8* is required for establishing anterior-posterior polarity. The *emb-8* gene encodes the *C. elegans* NADPH-cytochrome P450 reductase (NCPR), which transfers electrons to cytochrome P450 monooxygenase enzymes. Our findings implicate NCPR, and thus P450-dependent reactions, in the establishment of cell polarity. The *pod-2* locus encodes acetyl-CoA carboxylase, a key enzyme in fatty acid biosynthesis. Furthermore, depletion of fatty-acid synthase, the next enzyme in the pathway, results in similar polarity phenotypes implicating fatty acids in the establishment of polarity. Both *emb-8* and *pod-2* act upstream of PAR-3 and PAR-2 polarization by enabling association between the paternal pronucleus/centrosome complex with the embryonic cortex. Our data provide important evidence that this association is a key event in polarizing the embryo along the anterior-posterior axis and suggest that the interaction depends on fatty acid pathways.

Results

emb-8, *a new gene required for embryonic polarity*

We identified *emb-8* as a Pod locus by screening through published osmosensitive mutants for those that also divide symmetrically at the first cell division. The *emb-8* allele, *hc69*, was initially isolated as a conditional embryonic lethal mutation required maternally for embryo viability [25]. Initial characterization of *hc69* showed mutant embryos were osmotically sensitive [26], and we verified that *hc69* mutant embryos placed in hypotonic medium became swollen and arrested similar to other Pod mutants. No polarity defect was reported in these earlier works.

Analysis of *emb-8(hc69)* mutant embryos in isotonic medium reveals *emb-8* mutant embryos have lost polarity in the one-cell embryo. In the wild-type embryo, the first cleavage produces a characteristic asymmetric two-cell embryo with a larger anterior and a smaller posterior blastomere (Figure 1A). These two cells subsequently divide asynchronously and in orthogonal orientations indicative of their dissimilar identities (only the posterior cell will give rise to germline, for instance). In contrast, half of *emb-8(hc69)* mutant embryos divide symmetrically, producing two blastomeres of roughly equal size (Figure 1E, Table 1). In embryos where the first division is symmetric, the second cell cycles are synchronous and the two blastomeres divide with roughly parallel orientations, transverse to the longitudinal axis (data not shown). A second *emb-8* mutant, *t1462*, was isolated in a chromosome III-wide genetic screen for embryonic lethal mutations [27]. Although *emb-8(t1462)* mutant embryos have penetrant osmotic defects, they divide asymmetrically (Table 1). Nonetheless, the symmetric first division in *emb-8(hc69)* mutants coupled with their osmosensitivity (both are also seen in *emb-8(RNAi)* embryos, see below), places *emb-8* in the Pod group of polarity genes. That *emb-8(t1462)* is fully penetrant for osmosensitivity but has no polarity defect indicates that the two functions are separable, as has been demonstrated for other *pod* genes to date. Mitotic timing of the first cell cycle in *emb-8* mutant embryos is not significantly different from wild-type (data not shown).

To determine if the symmetric division in *emb-8(hc69)* results from loss of one-cell polarity or from inability to position the first spindle, we examined the distribution of molecular markers of polarity in *emb-8(hc69)* mutant embryos. In wild-type one-cell embryos, PAR-3 occupies the anterior cortex and PAR-2 adopts a reciprocal location at the posterior cortex (Figure 1B and 1C, [11,12]). In response to this polarized state, germline granules are segregated to the posterior cytoplasm of the one-cell embryo (Figure 1D, [6]). The asymmetric localizations of these markers are disrupted in *emb-8(hc69)* mutant embryos. Reduction of *emb-8* function leads to either more uniform cortical localization of PAR-3 (Figure 1F, 5/13 embryos) or enrichment of PAR-3 along a cortical lateral edge that is sometimes reduced (Figure 1I, 5/13 embryos). The remaining three embryos had wild-type PAR-3 localization. Although PAR-2 is sometimes present at the posterior in *emb-8* mutant embryos, it is usually reduced to a small patch at the cortex (Figure 1G, 5/15) or mislocalized to a lateral edge (Figure 1J, 6/15). The remaining four embryos had wild-type PAR-2 localization. In half of *emb-8(hc69)* mutant embryos, germline granules are mislocalized, remaining more dispersed about the center of the embryo (Figure 1H, 6/22) or moving to a posterior-lateral position (Figure 1K, 5/22). Thus, *emb-8* functions in establishing the overall polarity of the one-cell embryo as judged by physical and molecular characteristics.

emb-8 *encodes the NADPH-cytochrome P450 reductase*

To gain insight into the role of *emb-8* in establishing one-cell polarity, we determined the molecular identity of the *emb-8* locus. Previous genetic mapping placed *emb-8* in a central region of chromosome III [25]. We narrowed the region through three-factor mapping crosses which placed the *emb-8* locus between *dpy-17* and *unc-32* (Figure 2A); 25 of 28 Uncs and none of 20 Dpy F2 hermaphrodites from *dpy-17+unc-32/+emb-8(hc69)+* segregated F3 progeny that produced osmosensitive embryos. Furthermore, the rarity with which we found Unc animals that did not carry the *emb-8(hc69)* allele suggested that *emb-8* was located very close to *dpy-17*.

We used the osmosensitive phenotype of *emb-8* mutants to identify a candidate gene near *dpy-17*. Gönczy, et al. recently documented the phenotypic analysis of individually depleting 2,232 of the 2,315 predicted open reading frames on chromosome III by RNA-interference (RNAi,

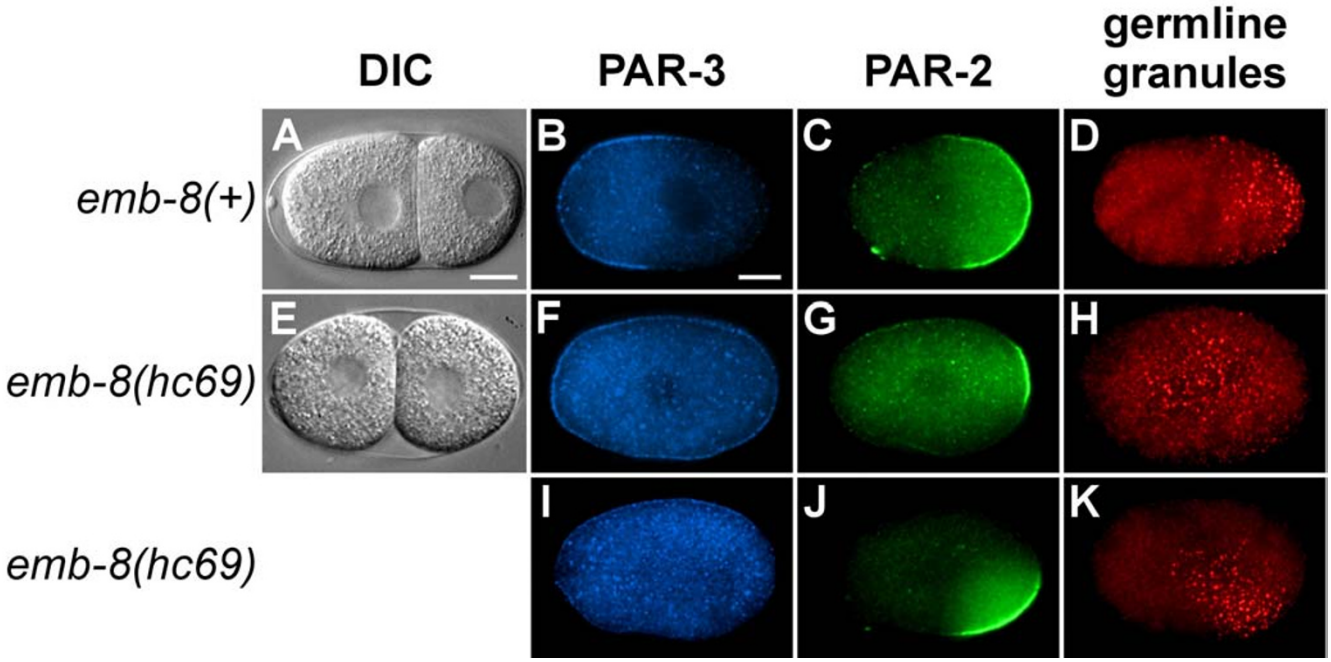

Figure 1
Physical and molecular anterior-posterior polarity requires *emb-8* function. Images of *emb-8(+)* (A-D) and *emb-8(hc69)* mutant embryos (E-K). Representative DIC images of two cell embryos (A, E) and immunolocalization of PAR-3 (B, F, and I), PAR-2 (C, G, and J), and germline granules (D, H, and K) in one-cell embryos. All embryos are oriented with anterior to the left and each image represents a different embryo. All fluorescent images and data represent analysis of one-cell embryos between pronuclear meeting through anaphase of the first cell cycle when PAR-3, PAR-2, and germline granules are normally asymmetrically distributed. All Wild-type embryos (A) divide asymmetrically and restrict (B) PAR-3 and (C) PAR-2 to the anterior and posterior cortices, respectively. (D) Germline granules are segregated to the posterior cytoplasm. (E) Half of *emb-8(hc69)* mutant embryos divide symmetrically. Some *emb-8* mutant embryos show (F) uniform cortical PAR-3 localization; 5/13; (G) reduced PAR-2 domains at the posterior; 5/15; and (H) centrally dispersed germline granules; 6/22. Other *emb-8* mutant embryos show (I) reduced, lateral localization of cortical PAR-3; 5/13 ; (J) mislocalization of PAR-2 to the posterior-lateral cortex; 6/15; (K) and mistargeted segregation of germline granules to posterior-lateral corners; 5/22.

Table 1: Polarity and osmotic defects of *emb-8* and fatty acid biosynthesis mutants

genotype	symmetric first cleavage (n)[a]	osmotically sensitive embryos[b]
emb-8(+)	0% (21)	0%
emb-8(hc69)	50% (22)	100%
emb-8(t1462)	0% (16)	100%
emb-8(RNAi)	72% (18)	100%
pod-2(ye60)[c]	43% (28)	100%
pod-2(RNAi)	70% (30)	100%
FAS(RNAi)[d]	89% (19)	100%
HMG-CoA reductase(RNAi)[e]	0% (14)	100%

[a] determined by comparison of cross sectional areas as in Rappleye, et al., 1999. [b] osmotic sensitivity determined by absorption of Nile Blue dye by embryos (Rappleye, et al., 2002) or by swelling in hypotonic media. n>50 embryos in all cases. [c] data from Tagawa, et al., 2001 for comparison. [d] open reading frame F32H2.5. [e] open reading frame F08F8.2

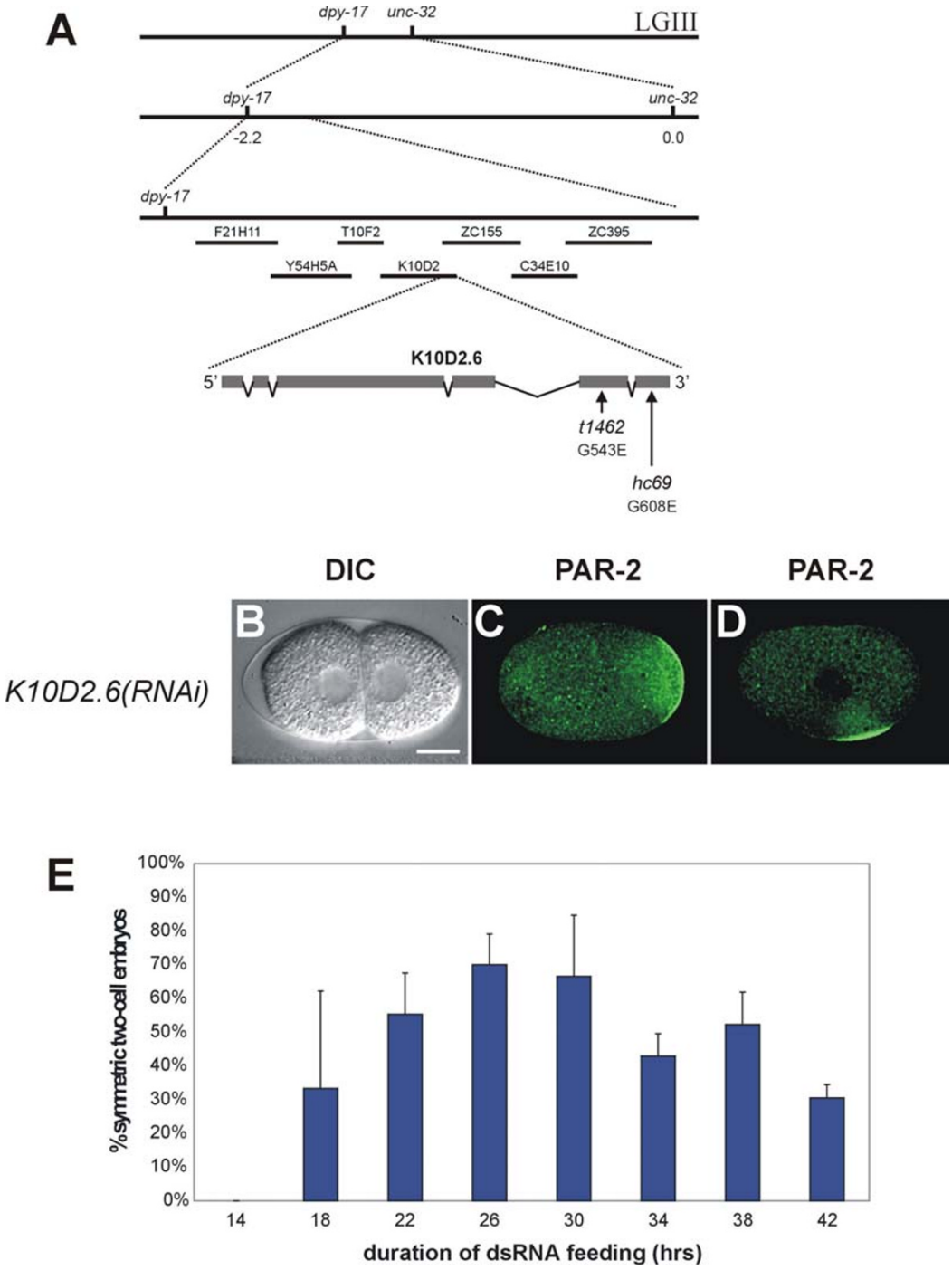

Figure 2
The *emb-8* locus is encoded by K10D2.6, the *C. elegans* NADPH-cytochrome P450 reductase homolog. (A) Schematic representation of mapping results which place *emb-8* in the *dpy-17* – *unc-32* interval on chromosome III. Sequencing of *emb-8* alleles *hc69* and *t1462* revealed two missense mutations in the predicted gene K10D2.6 which encodes the *C. elegans* NADPH-cytochrome P450 reductase. Sequencing of two independent clones from wild-type cDNA libraries confirmed the predicted gene structure. RNA-interference of K10D2.6 phenocopies the *emb-8(hc69)* mutant (B-D). RNAi of the K10D2.6 sequence causes embryos to (B) divide symmetrically at the first division, 41/59; and mislocalization of PAR-2 to (C) reduced posterior domains, 10/31 or (D) posterior-lateral regions of the cortex 14/31. (E) Results of time course experiments for K10D2.6 RNAi using synchronized mid-L4 staged hermaphrodites. Bars represent average proportion of embryos dividing symmetrically from 2–4 experiments with 8 hermaphrodites per experiment per time point (on average 10–15 embryos per time point).

[28]). Of 45 open reading frames in the putative *emb-8* region, only four showed embryonic lethal phenotypes. RNAi depletion of one of these, K10D2.6, produced embryos that "lose structural integrity upon dissection" and were placed in a phenotypic category that included the *pod-1* locus, an osmosensitive mutant described previously [21,28]. To determine if the K10D2.6 locus contained molecular variations associated with the *emb-8* mutants, we sequenced the *hc69* and *t1462* alleles and identified missense mutations that result in glycine to glutamate changes in residues 608 and 543, respectively, of the predicted protein (Figure 2A).

To confirm the K10D2.6 open reading frame encoded *emb-8*, we phenocopied the *emb-8* mutant phenotypes by RNA-mediated interference (RNAi) of K10D2.6. Reduction of maternal K10D2.6 function by RNAi made embryos osmosensitive and permeable to their external environment – embryonic cells shriveled in hypertonic medium and absorbed the lipophilic dye, Nile Blue, when depleted of K10D2.6 (Table 1). RNAi of K10D2.6 also reproduced the polarity defects of *emb-8(hc69)* mutants, causing up to 72% of one-cell embryos to divide symmetrically (Table 1, Figure 2B). Furthermore, RNAi of K10D2.6 reproduced the mislocalization of PAR-3 and PAR-2 seen in *emb-8(hc69)* mutants (Figure 2C and 2D). Together with the sequencing of alleles, these data indicate that the open reading frame K10D2.6 represents the *emb-8* gene and that osmotic sensitivity and symmetric cleavage phenotypes are caused by reduction of EMB-8 function.

K10D2.6 encodes the *C. elegans* ortholog of the NADPH-cytochrome P450 reductase (NCPR). The K10D2.6 encoded protein shows high sequence similarity to NADPH-cytochrome P450 reductase enzymes from a wide range of species; K10D2.6 is 46% identical and 63–64% similar to NCPR proteins from human, mouse, and *Drosophila*. This similarity extends along the length of the protein and includes the recognizable FMN-, FAD-, and NADPH-binding domains that are involved in transferring electrons from NADPH to cytochrome P450. The only region lacking significant similarity is the first 60 residues which comprises a hydrophobic membrane anchor in NCPR [29,30]. Metazoan species typically possess only one form of NCPR and this appears true in nematodes as well (data not shown). Given the high degree of sequence similarity to NCPR enzymes and the recognizable functional domains of the protein, we conclude that *emb-8*, encodes the *C. elegans* NCPR ortholog. Studies in other systems have shown that NCPR catalyzes the transfer of electrons from NADPH to cytochrome P450 enzymes. One of the physiological roles of NCPR-dependent enzymes is to catalyze the modification of fatty acids. This role has interesting implications given the *pod-2* gene, identified below.

In the course of our RNAi experiments, we found that only feeding mid-L4 staged larvae reliably produced these polarity defects (Figure 2E). dsRNA-feeding of late-L3 to early L4-stages produced less penetrant polarity defects although osmosensitive embryos were obtained (data not shown). To date, we have not been able to demonstrate any essential larval functions for *emb-8* since dsRNA feeding from the L1 stage does not prevent growth to adulthood.

The pod-2 *polarity gene encodes acetyl-CoA carboxylase*

The previously characterized Pod-class locus, *pod-2*, directly implicates fatty acids in the generation of polarity. Using single-nucleotide polymorphisms, we mapped the *pod-2* gene to the region around cosmid W09B6 (data not shown). *pod-2* was further shown to reside on cosmid W09B6 using transformation rescue. To determine which gene on W09B6 encodes POD-2, we performed RNAi of all open reading frames (ORFs) present on the cosmid. RNAi of a single ORF, W09B6.1, phenocopied the *pod-2* mutant loss of asymmetric first cleavage and osmotic sensitivity (Table 1). To confirm that W09B6.1 encodes *pod-2*, cDNA from the W09B6.1 open reading frame was amplified from three *pod-2* alleles and sequenced. For each, a single missense mutation was identified providing important evidence that *pod-2* corresponds to W09B6.1 (Figure 3A). W09B6.1 encodes the sole *C. elegans* homolog of acetyl-CoA carboxylase (ACC) and contains all the domains conserved in ACC from other organisms (Figure 3A).

To further confirm that the previously observed polarity phenotypes in the *pod-2* mutants resulted from loss of ACC-function, we analyzed characteristics of anterior-posterior polarity in embryos depleted of W09B6.1/ACC by RNAi. Wild-type adult hermaphrodites fed control bacteria produce embryos with normal polarity that divide asymmetrically at the first cell division (not shown). In contrast, adult nematodes fed bacteria expressing double-stranded RNA (dsRNA) encoding the ACC gene produce embryos that divide symmetrically at the first cell division (Figure 3B, Table 1). Feeding of W09B6.1 dsRNA to *pod-2(ye60)* animals at 15° can increase the number of symmetric embryos to greater than 90% symmetric cleavage (data not shown). As expected, embryos depleted of POD-2/ACC by RNAi display 100% penetrant osmotic sensitivity (Table 1). RNAi of the other gene with similarity to *pod-2* in the *C. elegans* genome (T28F3.5 that encodes carbamoyl-phosphate synthase) resulted in neither symmetric 2-cell nor osmotically sensitive embryos (data not shown).

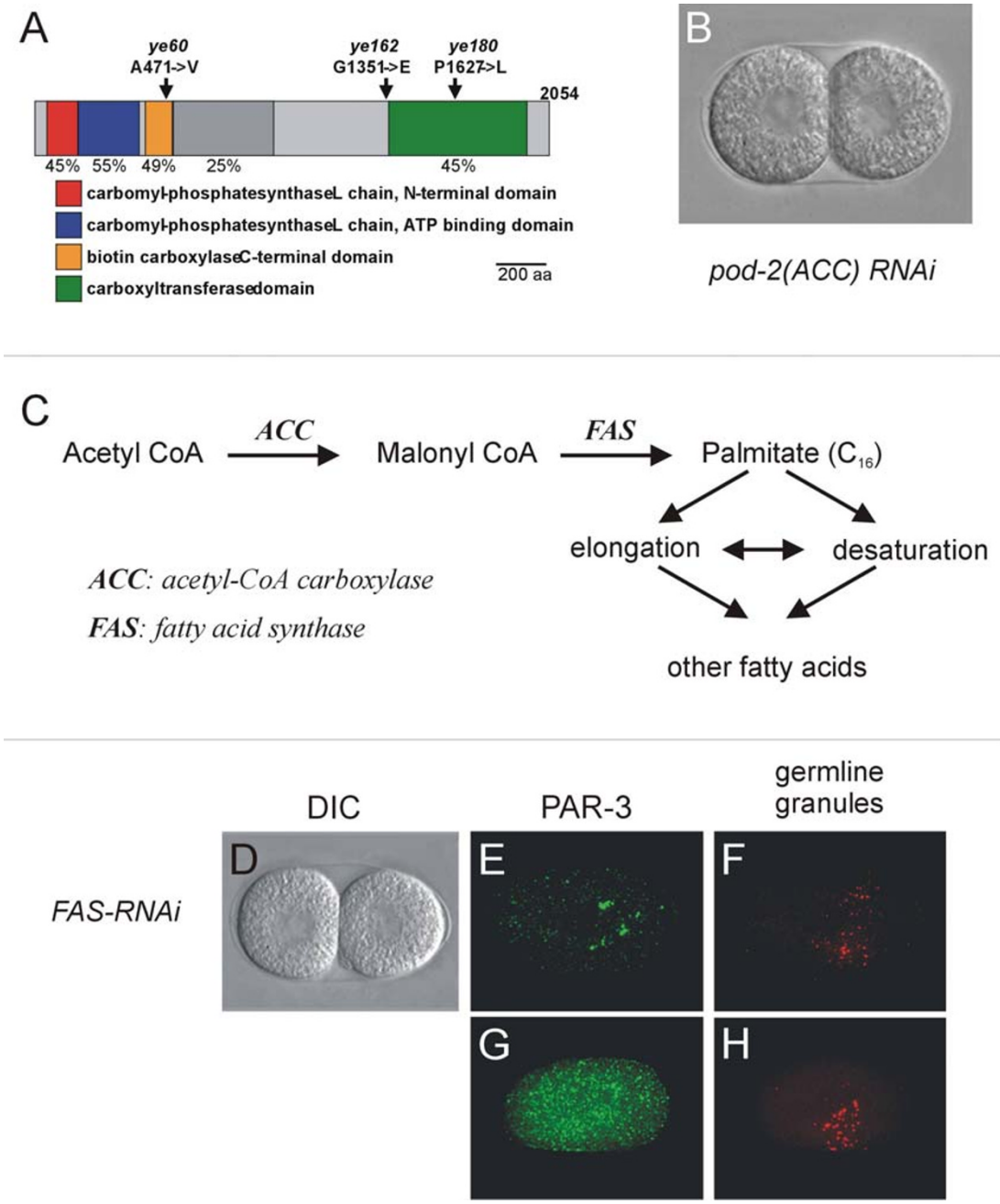

Figure 3

pod-2 encodes acetyl-CoA carboxylase (ACC) which, along with fatty acid synthase (FAS), is required for anterior-posterior polarity. (A) Schematic representation of the POD-2/ACC protein indicating regions homologous with ACC of other species. The number below each box represents the percent amino acid identity between POD-2 and human ACC1 in that domain. (B) RNA-interference of W09B6.1/ACC phenocopies the symmetric division of *pod-2(ye60)* mutant embryos. (C) Overview of the fatty acid biosynthetic pathway. ACC and FAS catalyze the first two committed steps in fatty acid biosynthesis. Supplementary fatty acids can feed into this pathway where they become interconverted through the action of elongases and desaturases. Ultimately fatty acids can become incorporated into lipids or can be catabolized for metabolic energy. (D-H) Images of embryos depleted of fatty acid synthase (FAS) by RNA-interference. Loss of FAS function causes embryos to (D) divide symmetrically at the first division (17/19 embryos) and results in mislocalization of PAR-3 (E and G) and germline granules (F and H). RNAi of FAS causes PAR-3 to either lose cortical localization (E; 15/33 embryos) or to become more symmetric about the cortex (G; 10/33 embryos). Germline granules are missegregated to the center of the embryo (F; 14/33 embryos) or to a lateral location (H; 2/33).

Table 2: Rescue of *pod-2* polarity defects by fatty acids and lipids

Fatty acid or lipid	[ ] in plate mg/ml	%symmetric *pod-2* 2-cell embryos (n)	Rescue of osmotic defect?[1]
none[2]		54 (24)	No
none[3]		43 (35)	No
none[4]	1.0	39 (23)	No
palmitic (16:0)	0.5	**0 (80)**	No
stearic (18:0)	1.0	**0 (10)**	No
oleic (18:1)	1.0	**9 (11)**	No
linoleic (18:2)	1.0	**9 (11)**	No
arachidonic (20:4)	1.0	**0 (14)**	No
docosahexaenoic (22:6)	1.0	**10 (52)**	No
diacylglycerol[5]	0.1	36 (14)	No
ceramide[6]	1.0	41 (22)	No
ceramide[7]	1.0	39 (23)	No
cardiolipin[8]	1.0	32 (19)	No
sphingomyelin[9]	1.0	34 (38)	No
sphingomyelin[10]	1.0	41 (22)	No
phosphatidylcholine	1.0	31 (16)	No
phosphotidylinositol[11]	1.0	**3 (35)**	No
phosphotidylinositol[12]	1.0	**0 (12)**	No
palmitic[13]	1.0	**0 (5)**	No
palmitic[13]	0.5	**0 (6)**	No
palmitic[13]	0.1	**8 (12)**	No
palmitic[13]	0.01	29 (7)	No

Compounds that rescued polarity defects are shown in bold. [1] Scored by looking at whether embryos shrunk in hypertonic medium. In all cases, >80% osmosensitive embryos were detected. [2] Solvent control. Ethanol alone, the solvent for sphingomyelin and all the fatty acids except stearic acid was spread on the plate. [3] Solvent control. Chloroform alone, the solvent for stearic acid and all lipids but sphingomyelin, was spread on the plate. [4] Glycerol 3-phosphate in water at 1 mg/ml in plate. [5] Stearic and arachidonic acids. [6] Bovine; stearic and nervonic acids. [7] Chicken; palmitic acid. [8] 80% lineoleic acid content. [9] Non-hydroxy fatty acid; stearic and nervonic acids. [10] Hydroxy fatty acid. [11] Crude (50%). [12] 99% pure; linoleic and palmitic acids. [13] Separate set of experiments studying dose-dependence of fatty acid rescue

POD-2/ACC's role in establishing the anterior-posterior (a-p) axis in the embryo appears to be specific. This conclusion is supported by the following observations. First, embryos depleted for POD-2/ACC are healthy and show no overt phenotypes (*e.g.*, cytokinesis defects, loss of cell integrity) apart from loss of polarity and osmotic sensitivity. When placed pressure-free in osmotically balanced medium, these embryos are essentially indistinguishable from other polarity mutants (*e.g.*, *par*'s) and exhibit first cell cycle mitotic timings statistically equivalent to wild type (data not shown). Second, the cold-sensitive *pod-2(ye60)* allele is a strict maternal affect lethal, and temperature shift studies with this allele suggest that the embryonic defects can be traced to a POD-2 function ~3 hours prior to fertilization [22]. These data suggest the *pod-2*/ACC gene can be mutated to uncover a specific role in a-p axis polarization. However POD-2/ACC must also have functions in addition to a-p axis formation since first stage larvae fed *pod-2*/ACC dsRNA grow slowly and often die at the L3 stage (6/9).

Polarity establishment is linked to fatty acids

The above data suggest that fatty acids, which are synthesized by ACC and potentially modified downstream by EMB-8/NCPR-dependent reactions, play an important role in a-p axis formation. If so, then elimination of the next enzyme in the biosynthetic pathway, fatty acid synthase (FAS; Figure 3C), would also be predicted to lead to loss of a-p polarity. In *C. elegans*, a single open reading frame, F32H2.5, encodes FAS. Elimination of maternal FAS in embryos by dsRNA feeding results in a loss of a-p polarity in the progeny as judged by symmetric 2-cell embryos (Figure 3D, Table 1). The polarity phenotype became apparent at 28 hours after the initiation of feeding at 15° C (33%; n = 24) and reached a maximum (89%; n = 19) at 64 hours. FAS(RNAi) embryos also displayed completely penetrant osmotic sensitivity.

We also looked at molecular markers of a-p polarity. Out of 33 embryos depleted for FAS, 15 showed PAR-3 protein localization clustered in the cytoplasm (Figure 3E), ten showed lateral, cortical staining (Figure 3G), two had no cortical staining, one had staining all around the cortex, and five had wild-type staining. As for germline granules,

14/33 embryos had staining near the middle of the embryos (Figure 3F), two showed lateral, posterior staining (Figure 3H), one had staining all over the embryo, and 14 were wild-type in localization. Similar PAR-3 and P granule defects are seen in *pod-2(ye60)*/ACC mutant embryos [22]. The loss of PAR-3 staining at the cortex observed in *pod-2* mutants is intriguing and may be indicative of significant changes in membrane composition. Nonetheless, as indicated below, the primary polarity defect in *pod-2* mutant embryos is caused by events that precede PAR-3 localization.

As loss of ACC or FAS function would be expected to impair fatty acid synthesis, dietary supplementation with exogenous fatty acids might be able complement the *pod-2*/ACC polarity defects. We found that adding the fatty acids palmitic acid, stearic acid, oleic acid, linoleic acid, arachidonic acid, or docosahexaenoic acid to the diet of *pod-2(ye60)* mothers rescues the a-p polarity defects in their progeny (Table 2). Rescue by palmitic acid was shown to be dose-dependent. Conversely, neither solvents nor glycerol 3-phosphate (required for making phosphatidic acid) rescue. The polarity defect appears to be specific for fatty acids or the fatty acid moieties of lipids as addition of diacylglycerol, the other major component of lipids, to the diet also did not rescue. Although all fatty acids tested rescued the *pod-2(ye60)* polarity defects, none rescued osmotic sensitivity, confirming that the function of *pod-2*/ACC required for osmotic protection (*i.e.*, normal eggshell) is separable from its function in a-p polarity. That the addition of fatty acids rescues only a subset of ACC phenotypes has also been seen in yeast [31].

These data demonstrate that fatty acids play an important role in establishing *C. elegans* polarity. However, since acetyl-CoA is also used in cholesterol biosynthesis, it was possible that the *pod-2*/ACC polarity defect might be caused by cholesterol deficiencies. To test this, we used RNAi to deplete the function of hydroxylmethylglutanyl (HMG) CoA reductase, an enzyme that uses acetyl-CoA and catalyzes the first committed step in the cholesterol biosynthesis. HMG CoA reductase in *C. elegans* is encoded by the open reading frame F08F8.2. Injection of F08F8.2 dsRNA into wild-type hermaphrodites resulted in embryos that were osmotically sensitive (100% penetrance 32 hours after injection) but had normal two-cell asymmetry (Table 1). This result confirms previous demonstrations that osmotic sensitivity itself does not lead to polarity defects [17,21,22]. After this time, the mothers became sterile as previously described [32]. Thus the *pod-2* polarity defect appears to be specific for defects in fatty acid biosynthesis.

Loss of either emb-8 *or* pod-2 *impairs the interaction between the paternal pronucleus and the posterior cortex*

The fact that *emb-8* and *pod-2* have similar phenotypes and are either implicated in (*emb-8*) or directly involved in (*pod-2*) fatty acid biosynthesis, respectively, raises the issue as to whether they function in the same pathway. Although for temperature reasons, an *emb-8*; *pod-2* double mutant is difficult to construct and score (one is temperature sensitive and the other is cold sensitive, including by feeding of dsRNA), we have previously shown that the *pod-2* mutant functions genetically in the same pathway as a deletion allele of *pod-1*. We similarly tested whether *emb-8* functions in the same pathway as *pod-1* by constructing a *pod-1(ye11) emb-8(hc69)* double mutant and examining the penetrance of the polarity defect. We found no significant enhancement of the loss of a-p polarity in the double mutant as compared to the single mutants when two cell symmetry was scored (29% symmetric, n = 41 for pod-1(*ye11)*; 42% symmetric, n = 24 for *emb-8(hc69)*; and 29% symmetric, n = 31 for the double mutant). This result, together with the similar polarity phenotypes, suggests that *pod-1*, *pod-2*, and *emb-8* all function in a similar pathway with regards to polarity.

This finding predicts that both *emb-8* and *pod-2* mutants should manifest similar defects in the events leading to the polarization of the one-cell embryo. We have previously reported that the APC Pod-class of mutants are defective in the interaction of the pronucleus centrosome complex (PNCC) with the posterior cortex and that our observations, as well as important observations from other laboratories, suggest interaction of the PNCC with the cortex was responsible for repelling PAR-3 from the posterior, thereby establishing anterior-posterior asymmetry. This interaction is characterized as movement of the paternal pronucleus (with associated centrosome) towards the posterior (Figure 4A) and subsequent tight juxtaposition with the posterior cortex (Figure 4B,4C). This association lasts for approximately five minutes and coincides temporally with the exclusion of PAR-3 from this region of the cortex [12,33], after which the paternal pronucleus dissociates from the cortex to meet the maternal pronucleus in the posterior cytoplasm (Figure 4D).

To determine if *emb-8* and *pod-2* were required for this process, we examined this polarizing interaction in embryos that had been depleted of *emb-8* or *pod-2* functions. In embryos depleted of *emb-8* function by RNAi, the paternal pronucleus initiated posterior movements (Figure 4E) but close association with the cortex was often abbreviated or prevented entirely (Figure 4F,4G). Lack of interaction was followed by meeting of the pronuclei near the middle of the embryo (Figure 4H) and symmetric division at first cleavage. Loss of *pod-2* function similarly impaired this cortical interaction (Figure 4I,4J,4K,4L).

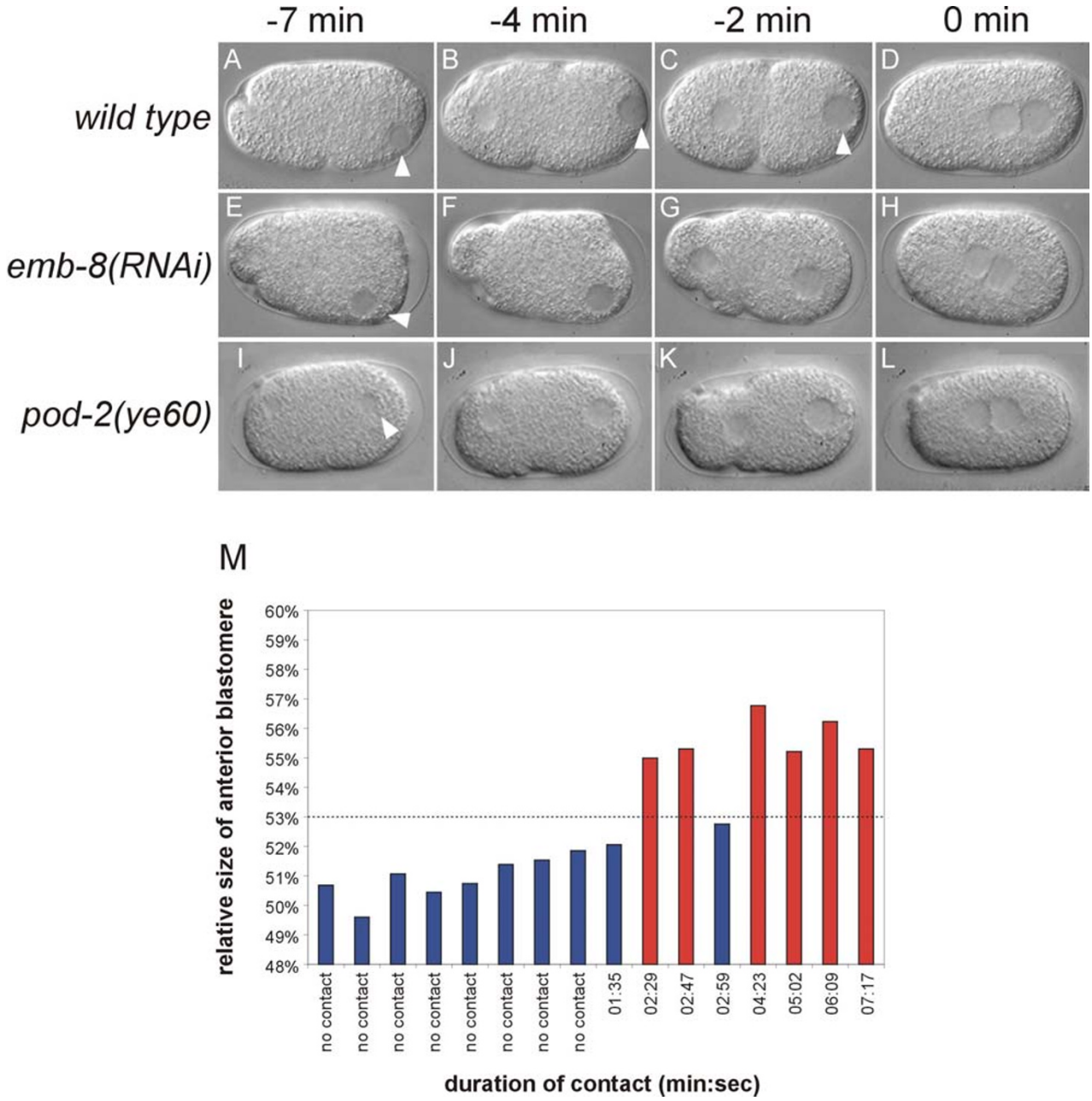

Figure 4

Loss of *emb-8* or *pod-2* impairs cortical interaction by the paternal pronucleus which correlates with loss of polarity. Representative wild-type (A-D), *emb-8(RNAi)* (E-H), and *pod-2(ye60)* embryos (I-L). In wild type, (A) the paternal pronucleus initially appears in the posterior cytoplasm and (B) moves to the posterior where it closely associates with the cortex. This cortical interaction is maintained for approximately 5 minutes before (C) the pronucleus dissociates and (D) migrates to meet the maternal pronucleus in the posterior cytoplasm. In 8/16 *emb-8(RNAi)* embryos, (E) the paternal pronucleus appears in the posterior cytoplasm and some posterior movement occurs; however (F-G) contact with the cortex is not made and (H) the paternal and maternal pronuclei meet near the center of the embryo. In *pod-2(ye60)* mutants, (I-K) the paternal pronucleus does not become juxtaposed with the cortex. Subsequently, (L) the pronuclei meet in the center of the embryo. (M) Correlation between impaired cortical interaction and symmetric division in *emb-8(RNAi)* embryos. Relative size of the anterior blastomere (cross-sectional area) of individual embryos is indicated on the y-axis. Duration of pronucleus-cortex interaction is indicated along the x-axis. For 8 embryos, no interaction occurred ("no contact"). Symmetric division was defined as two-cell embryos in which the anterior blastomere comprised less than 53% of the total area. Blue bars = symmetric division. Red bars = asymmetric division.

Table 3: Correlation between cortical interaction and embryo polarization in *pod-2(ye60)*

	number of embryos	cortical interaction[a]	asymmetric first spindle
pod-2(ye60)	5/10	yes	yes
	5/10[b]	no	no
pod-2(ye60) + palmitate	17/18[c]	yes	yes

[a] defined as juxtapositioning of paternal pronucleus with the cell cortex. [b] in one embryo the spindle initially set up symmetrically but subsequent division was asymmetric. [c] one embryo divided asymmetrically but with reversed polarity

The variability in the interaction of the pronucleus with the cortex in the *emb-8* and *pod-2* mutants allowed us to investigate whether the quality and duration of the interaction directly correlated with polarity defects. Indeed, in *emb-8(RNAi)* embryos, strong correlation was observed between impaired pronucleus-cortex interaction and loss of first division asymmetry (Figure 4M). Embryos that did not contact the cortex always divided symmetrically (relative area of the anterior blastomere less than 53%) and those that exhibited significant cortical interactions (longer than 2–3 minutes), with one exception, produced asymmetric divisions (Figure 4M). In *emb-8*-depleted embryos that subsequently divided symmetrically, posterior-directed cytoplasmic flows were also qualitatively absent (data not shown). Similar correlation between loss of polarity and lack of pronucleus-cortical interaction was also observed in *pod-2* mutant embryos (Table 3). Importantly, dietary supplementation of *pod-2(ye60)* mutants with palmitic acid rescued both the cortical interaction and a-p axis polarization (Table 3). Thus, *emb-8* and *pod-2* are each required for the cortical interaction by the pronucleus/centrosome complex, and this interaction correlates strongly with the establishment of a-p polarity.

Discussion

Although numerous polarity factors required for anterior-posterior axis formation in *C. elegans* have been identified, the next challenge facing developmental biologists is connecting these components to the basic cellular machinery. This study reveals two of these central components that are essential for generating asymmetries in the early *C. elegans* embryo: (1) *emb-8* which encodes NADPH-cytochrome P450 reductase (NCPR) and (2) the fatty acid biosynthetic pathway represented by *pod-2*/ACC and FAS. Depletion of any of these functions causes loss of the asymmetry of the first division and missegregation of germline granules. These defects are not simply a consequence of an inability to translate cell polarity downstream of PAR-3 and PAR-2 but instead result from ineffective polarization of the one-cell embryo as evidenced by mislocalization of PAR-3 and PAR-2 in *emb-8* or PAR-3 in FAS mutant embryos. Although PAR-2 still localizes to the cortex in *emb-8*/NCPR mutant embryos, it is often restricted to a small patch. We speculate that this narrowed PAR-2 domain might reduce the number or quality of the stronger PAR-2-dependent spindle pulling forces exerted on the posterior pole of the one-cell mitotic spindle, thereby preventing its posterior displacement [34]. In *emb-8*/NCPR mutants and FAS-depleted embryos, germline granule segregation is impaired or mistargeted to lateral regions. This lateral localization of germline granules was also observed in *pod-2* mutant embryos [22]. These results indicate *emb-8*/NCPR and the fatty acid biosynthetic pathway function in establishing polarity in the one-cell embryo.

As additional evidence supporting previous findings with Pod mutants defective in the APC, we demonstrate that the source of the loss of polarity correlates with defects in the interaction between the paternal pronucleus/centrosome complex and the posterior cortex. We found a near perfect correlation between the duration of this interaction and the resultant physical polarity in both *emb-8* and *pod-2* mutant embryos. These data, together with other research in the one-cell *C. elegans* embryo field [14–16], suggests that contact between short microtubules from the paternal pronucleus-associated centrosome and the cortex provides the cue which polarizes the embryo, most likely via repulsion of PAR-3 from the posterior cortex. Lack of PAR-3 at the posterior cortex permits PAR-2 localization thereby establishing spatial differences in the anterior versus posterior domains.

The similarity in phenotypes shared by the known Pod genes – *emb-8*/NCPR, *pod-1*, *pod-2*/*ACC*, and APC class (*emb-30*, *pod-3*, *pod-4*, *pod-5*, and *pod-6*) suggests each represents a different aspect of a pathway that polarizes the one-cell embryo and that mutates to the Pod phenotype. Indeed, all show defects in the interaction of the paternal pronucleus/centrosome complex and the posterior cortex. We hypothesize that *emb-30* and the other APC *pod* genes represent one aspect of the polarization pathway [17]. Given the APC pathway ultimately regulates separin, which has been shown to affect microtubule stability [35], we propose that the APC *pod* mutants represent the microtubule aspect of the polarizing interaction between the paternal pronucleus/centrosome complex with the posterior cortex. Although recent data reports that APC-class *pod* mutants also show failures in second polar body extrusion [36], it is unclear how this would impair the key cortical interaction by the paternal pronucleus at the opposite end of the embryo. Furthermore, polarity defects in an APC mutant do not correlate with defects in meiotic timings, and RNAi of separin, which has disrupted polar-

ity, has normal meiotic timings [17]. Although we cannot exclude the possibility that meiosis defects indirectly contribute to the lack of association of the pronucleus with the cortex in Pod mutants, our data suggest otherwise. A second aspect of the POD polarization pathway, represented by *pod-1*, we hypothesize is indicative of a requirement for the actin cytoskeleton at the cortex or possibly spatial control of membrane trafficking events required for the interaction [21]. A third aspect is represented by EMB-8/NCPR and POD-2/ACC (see below).

Understanding how *emb-8*/NCPR contributes to the interaction of the pronucleus with the cortex and the subsequent asymmetric localization of PAR-3 and PAR-2 requires dissection of the myriad of functions downstream of NADPH-cytochrome P450 reductase. The best characterized function of NCPR is the transfer of electrons to cytochrome P450 (CYP) family enzymes, although cytochrome b5 can also act as an acceptor [37,38]. General types of CYP-mediated reactions have been identified and include hydroxylation, desaturation, and epoxidation [39]. Most of these P450-dependent reactions pertain to the oxidation of xenobiotics such as drugs and pesticides. Of the few endogenous substrates identified, cytochrome P450 reactions are largely directed towards hydrocarbons such as fatty acids and sterols. For example, mammalian CYP4 and CYP2 subfamily members can promote omega- and omega-1 hydroxylation of laurate and arachidonic acid [40,41] as well as desaturation of laurate [42]. Consistent with this, elimination of yeast NCPR causes changes in the fatty acid profile including chain lengths and levels of unsaturated fatty acids [43].

This function of NCPR-dependent reactions in maintaining proper chain lengths and levels of desaturation of cellular fatty acids is intriguing given the molecular identification of the *pod-2* gene as acetyl-CoA carboxylase, the first and rate-limiting step in de novo fatty acid biosynthesis. The fact depletion of another fatty acid biosynthetic enzyme, FAS, shows similar loss of polarity and that dietary supplementation with fatty acids can rescue the polarity defects suggests that polarization of the embryo is linked to a third class of POD components in addition to those mentioned above: fatty acid pathways.

We therefore hypothesize that *emb-8*/NCPR and *pod-2*/ACC influence embryonic polarity via effects on the fatty acid composition of membrane lipids. It is well established that the biochemical properties of membranes are altered by changing the fatty acid composition of the constituent lipids. The fatty acid balance achieved through the combined functions of *emb-8*/NCPR and *pod-2*/ACC might be required to (1) establish a particular membrane composition or membrane subdomain (e.g., membrane microdomains [44]) necessary to promote interaction between the pronucleus/centrosome complex and the cortex, or (2) maintain the proper membrane tension or curvature required for this interaction as has been suggested [31]. The importance of lipids and cellular asymmetry is best exemplified by polarized epithelial cells where apical and basolateral domains are characterized by distinct membrane compositions (reviewed in [45]). The fact that both epithelial cells and the *C. elegans* one-cell embryo require the PAR-3/ASIP complex for polarity suggests additional parallels, including membrane subdomains, may exist between these cell types. Alternatively, fatty acid composition could influence membrane fluidity, which could in turn affect endo- and exocytosis abilities of the cell [46].

The identification of *emb-8*/NCPR (and presumably cytochrome P450) and fatty acid biosynthesis pathways as necessary players in polarity establishment in the *C. elegans* embryo highlight the need to better understand the role of basic cellular processes and how they function in developmental contexts. Because of the powerful genetics in *C. elegans*, nearly all of the identified developmental components represent proteins and protein complexes. The potential link between membrane composition and polarity provided by the *emb-8* and *pod-2* mutants serves as a reminder that efforts must also be made to understand the contributions of non-protein elements of the cell (i.e. lipids and fatty acids) in the establishment of polarity. Our *emb-8*/NCPR results also provide evidence that NCPR has important functions beyond the metabolism of exogenous chemicals. The further merging of development with cell biology studies should continue to reveal additional roles for such cellular processes as well as better define the underlying mechanisms used by development to generate complex multicellular organisms and cell polarity.

Conclusions

The characterization of emb-8 and pod-2 mutants suggests that a key event in anterior-posterior axis polarization in the C. elegans embryo is the interaction between the pronucleus/centrosome complex with the posterior cortex. This finding uncovers an important link between the polarization cue provided by sperm entry and the resultant polarization of the anterior and posterior landmarks, PAR-3 and PAR-2, upon which all known downstream asymmetries depend. Furthermore, the molecular identities of these Pod-class polarity genes suggest establishment of polarity requires fatty acid pathways – an unexplored element of the fundamental cellular machinery.

Methods

Strains and Genetics

Culture of *C. elegans* strains and complementation tests were performed using standard techniques [47]. Nematodes harboring the temperature sensitive *emb-8(hc69)* allele were maintained at 15°C and placed at non-permissive conditions (25°C) as L4-staged hermaphrodites overnight to produce mutant phenotypes. Nematodes harboring the cold-sensitive *pod-2(ye60)* allele were maintained at 25°C and were shifted to non-permissive conditions (15°C) for at least 24 hours to examine mutant embryos. Osmosensitive phenotypes were assayed using Nile Blue uptake and verified by placement of mutant embryos in hypertonic media [17]. Alleles used in this study were obtained from the Caenorhabditis Genetic Center (CGC) and from H. Schnabel and included the chromosome III loci: *emb-8(hc69)*, *emb-8(t1462)*, *dpy-17(e164)*, *unc-32(e189)*, *him-5(e1490)*. Genetic mapping of the *emb-8* locus was performed by crossing *emb-8(hc69)* males into *dpy-17(e164) unc-32(e189)* hermaphrodites, isolation of Unc nonDpy and Dpy nonUnc F2-hermaphrodites, and monitoring the production of osmosensitive embryos by F3-hermaphrodites using NGM plates with Nile Blue [17]. Complementation of the *pod-2* mutation was done through cosmid rescue carried out by injecting cosmid W09B6 (20 μg/μl) with the dominant marker *rol-6* DNA (50 μg/μl) into *pod-2(ye60)* and *pod-2(ye162)* mutant animals. Double mutant embryos for the *emb-8(hc69)* and *pod-1(ye11)* alleles were generated by first marking *emb-8(hc69)* chromosomes with *unc-32(e189)* and then crossing the *pod-1(ye11)* allele into homozygous *emb-8 unc-32* animals at the permissive temperature. Progeny were immediately mated to *unc-32(e189) / qC1* males to maintain *emb-8(hc69)* and *pod-1(ye11)* in the heterozygous state over the balancer chromosome *qC1*. Verification of this *emb-8(hc69) pod-1(ye11)* chromosome was done by test crosses to *emb-8(hc69)* males and by PCR verification of the *pod-1(ye11)* deletion allele. Homozygous double mutants were isolated as Unc hermaphrodites and their progeny tested for osmosensitivity and loss of two-cell asymmetry.

Microscopy

Live analysis of embryos was performed by dissecting embryos from gravid hermaphrodites into isotonic medium (EBGM, [48]) and placing the embryos on slides with coverslips supported by petroleum jelly to maintain pressure-free conditions. Embryos were observed using 100X DIC optics. Asymmetry of the two-cell embryo was determined by comparison of the cross-sectional areas of the anterior and posterior blastomeres as described previously [21]. For determination of pronuclear behavior and timing, recently-fertilized embryos were monitored and scored for meiosis completion (reformation of the maternal pronuclear envelope) and juxtaposition of the paternal pronucleus with the posterior cortex. Immunolocalization of PAR-3, PAR-2, and germline granules (O1C1D4 epitope) were performed as described [11,12] except embryos were released into isotonic EBGM buffer. Embryos were co-stained with antibodies to tubulin (DM1a, Sigma) and DAPI to allow unambiguous determination of cell cycle state. Immunofluorescence images were collected with an Olympus IX-70 microscope and deconvolution system (softWoRx, v2.5, Applied Precision, Inc.).

Molecular characterization of the K10D2.6 and W09B6.1 loci

The 5' end of the K10D2.6 mRNA was deduced by amplification and sequencing of K10D2.6 clones from a cDNA library (RIKEN DNA Bank) using a K10D2.6-specific reverse primer and a primer anchored in the flanking vector sequences. It is possible that the 5' end extends beyond this clone, but we note that the predicted gene structure extends the entire length of other NCPR proteins. The W09B6.1 5' end was similarly deduced however two clones were isolated: one matched the predicted transcript end for W09B6.1 and the other contained an additional 333 coding nucleotides derived from genomic regions several kb upstream of the predicted start. Thus *pod-2* may have two forms alternatively spliced at the 5' end.

For examination of mutant alleles, mRNA was isolated from a population of homozygous *emb-8(hc69)* or *pod-2* mutant animals by TRIZOL (Gibco BRL)-extraction and reverse-transcription using gene-specific reverse primers and Superscript II reverse-transcriptase (Stratagene). Full length cDNA copies were generated using a 5' end-anchored primer and PCR amplified for sequencing. For sequencing of the *emb-8(t1462)* allele, genomic DNA was prepared from single Unc hermaphrodites homozygous for the *t1462* mutation as described [49] and the full length genomic region amplified by PCR and cloned into pBluescript(SK+) (Stratagene) for sequencing. Our sequencing efforts have confirmed the amino acids and intron/exon boundaries as predicted by the Genome Center for both K10D2.6 and W09B6.1

RNA interference (RNAi)

RNAi was performed by plating L4-staged hermaphrodites onto NG + Ampicillin (100 μg/ml) plates containing 1 mM IPTG and seeded with *E. coli* HT115 expressing specific open reading frame dsRNA from plasmid L4440 [50,51]. Open reading frames used included: K10D2.6 cDNA (*emb-8)*, W09B6.1 cDNA (*pod-2*), F32H2.5 cDNA (*FAS*), T28F3.5 cDNA (*CPSase*), and F08F8.2 cDNA (*HMG CoA Reductase*). Embryos were dissected from gravid hermaphrodites in EBGM and visualized by DIC microscopy or processed for immunofluorescence with

PAR-2 antibodies. For determination of the optimal feeding duration, synchronized wild-type hermaphrodites were plated as early L4-, mid L4-, late L4-, and young adult stages for varying durations of time at 20°C. Hermaphrodites were individually dissected in 100 μl EBGM and two-cell embryos scored for symmetry. A drop (approximately 25 μl) of 5 M NaCl was added to determine osmosensitivity.

Fatty acid and lipid supplementation

Fatty acids and lipids (except for diacylglycerol; BIOMOL, PA) were purchased from SIGMA and resuspended typically at 100 mg/ml (notable exception is DAG at 10 mg/ml) in the solvent indicated in Table 2. 100 μl of each was spread over a 10 ml NG agar plate. 40 μl of E. coli OP50 were added as food source and ~25 *pod-2(ye60)* L4 hermaphrodites were plated, grown at 15° for 72 hours, and then dissected to release embryos. In control experiments, 100 μl of either ethanol, chloroform, or water was spread.

Authors contributions

CR carried out the characterization, cloning, and phenotypic analysis of the emb-8 mutants and drafted the manuscript. AT performed the cloning of pod-2, phenotypic characterizations of fatty acid enzyme mutants, and fatty acid feeding analysis. Cloning of pod-2 was aided by unpublished information provided by NL and JA. RA was the primary investigator and coordinated the research efforts. All authors read and approved the final manuscript.

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