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pod-2, along with *pod-1*, Defines a New Class of Genes Required for Polarity in the Early *Caenorhabditis elegans* Embryo

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The asymmetric division of the one-cell *Caenorhabditis elegans* zygote gives rise to two cells of different size and fate, thereby establishing the animal's anterior–posterior (a-p) axis. Through genetics, a number of genes required for this polarity have been characterized, but many components remain unidentified. Recently, our laboratory discovered a mutation in the *pod-1* gene (for *p*olarity and *o*smotic *d*efective) that uniquely perturbed polarity and osmotic protection. Here, we describe a new *C. elegans* polarity gene identified during screens for conditional embryonic lethals. Embryos in which this gene has been mutated show a loss of physical and developmental asymmetries in the one-cell embryo, including the mislocalization of PAR and POD-1 proteins required for early polarity. Furthermore, mutant embryos are osmotically sensitive, allowing us to designate this gene *pod-2*. Thus, *pod-2*, along with *pod-1*, defines a new class of *C. elegans* polarity genes. Genetic analyses indicate that *pod-2* functions in the same pathway as *pod-1*. Temperature-shift studies indicate that *pod-2* is required during oogenesis, indicating that aspects of embryonic polarization may precede fertilization. *pod-2* mutant embryos also exhibit a unique germline inheritance defect in which germline identity localizes to the wrong spot in the one-cell embryo and is therefore inherited by the wrong cell at the four-cell stage. Our data suggest that *pod-2* may be required to properly position an a-p polarity cue. **© 2001 Academic Press**

***Key Words: C. elegans*; polarity; *pod*; embryogenesis.**

INTRODUCTION

The establishment of cell polarity is a biological process fundamental to unicellular and multicellular life. During development, cell polarization and the asymmetric segregation of cell determinants within a cell are important processes that allow daughter cells to take on different cell fates. Examples include segregation of determinants that establish mother–daughter fates in budding yeast, establishment of the body axes in plants, *Drosophila*, and *Xenopus*, and the segregation of embryonic versus extra-embryonic cell fates in the mouse embryo (Brownlee and Bouget, 1998; Chant, 1999; Johnson, 1996; Ubbels, 1997; van Eeden and St. Johnston, 1999).

Cell polarity plays a central role in the early development of the *Caenorhabditis elegans* embryo. The entry of the sperm into one end of the oocyte appears to be the initial cue for polarization of the anterior–posterior (a-p) axis, probably via a function of the sperm aster (Goldstein and Hird, 1996; O'Connell *et al.*, 2000; Sadler and Shakes, 2000; Wallenfang and Seydoux, 2000). A cascade of events then segregates various proteins required for normal development to either the anterior or posterior pole of the embryo and also causes the mitotic spindle to set up asymmetrically (reviewed in Bowerman, 1998; Rose and Kemphues, 1998). Because of the asymmetry in spindle location, the first cleavage of the zygote is unequal. The larger, anterior daughter cell is called AB and gives rise to most of the neuroectoderm of the animal; the smaller posterior daughter cell is called P_1 and gives rise to most of the mesoderm and all of the endoderm and germline. P_1 itself subsequently undergoes an asymmetric division to give rise to a larger anterior cell EMS, the mesoderm/endoderm precursor, and a smaller posterior cell P_2, the germline precursor.

The actual physical processes that generate developmental and physical asymmetry are poorly understood. Six genes required for these asymmetries in the one-cell em-

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bryo have been identified during screens for maternal effect embryonic lethal mutants (see above reviews). Mutations in these genes, called *par* genes for *par*titioning defects, lead to loss of asymmetric segregation in one-, two-, and four-cell embryos of developmental determinants, such as germline granules, transcription factors, and signal transduction pathway components. Furthermore, mutations in the *par* genes often lead to loss of physical asymmetry at the one-cell stage, producing two cells of equal size. Given the number of alleles in most of these *par* genes, genetic screens for strict maternal effect lethal have probably reached saturation for this class of polarity mutant (Cheng *et al.*, 1995; Etemad-Moghadam *et al.*, 1995; Kemphues *et al.*, 1988; Morton *et al.*, 1992; Watts *et al.*, 1996). Elimination of atypical protein kinase C and cytoplasmic myosin II heavy and light chains by RNA-mediated interference (RNAi) has a similar effect as mutation of the *par* genes, hinting at the existence of other genes important for polarity (Guo and Kemphues, 1996; Shelton *et al.*, 1999; Tabuse *et al.*, 1998).

Recently, we characterized a new polarity gene, *pod-1*, which stands for *p*olarity and *o*smotic *d*efective. POD-1 was identified biochemically by using an F-actin affinity column (Aroian *et al.*, 1997). Loss of maternal *pod-1* results in loss of a-p polarity in half of *C. elegans* one-cell embryos (Rappleye *et al.*, 1999). Unlike other polarity mutants, *pod-1* mutant embryos also display alterations in the internal and external structure of embryonic cells, including an abnormal eggshell that results in osmotic sensitivity, the formation of abnormal endocytic vesicles, the presence of hyaline zones, and the deposition of an extracellular layer on the embryo. These defects indicate that *pod-1* plays a role in a physical process, e.g., membrane trafficking and/or actin organization, that is also important for establishing a-p polarity. It was not known whether *pod-1* was a unique polarity gene or whether other genes with similar phenotypes existed.

Here, we describe a new polarity gene identified during large-scale screens for conditional embryonic lethal mutants. Mutation of this gene causes defects in a-p polarity in one-cell *C. elegans* embryos. Like loss of *pod-1*, loss of this gene function also results in embryos sensitive to their osmotic environment. Hence, this gene defines a second *pod* locus, *pod-2*. Our data indicate that the *pod-1* and *pod-2* genes function in a common pathway to polarize the *C. elegans* embryo. *pod-2* mutant embryos also display a unique defect in the segregation of germ cell potential.

MATERIALS AND METHODS

Strains and Growth Conditions

Maintenance of *C. elegans* strains, mutagenesis, and genetics were carried out by using standard techniques (Brenner, 1974). The strains used are listed by linkage group (LG) as follows: LGI, *emb-20(g27)*; LGII, *ccDf11/dpy-25(e817)*, *unc-85(e1414) dpy-10(e128)*, *dpy-10(e128) unc-4(e120)*; LGIII, *unc-32(e189) pod-1(ye11)/qC1* (Rappleye *et al.*, 1999), *dpy-18(e364)*; LGX, *lin-2(e1309)*.

For all experiments involving *pod-2(ye60)* at the nonpermissive temperature, *ye60* hermaphrodites were grown at 24°C and shifted as L4 hermaphrodites to 15°C at least 24 h prior to the experiments, unless otherwise noted. For *ye162* and *ye180* mutations, animals were grown at 15°C and shifted as L4 hermaphrodites to 25°C overnight.

Screen for Embryonic Lethal Cold-Sensitive Mutations

A screen for cold-sensitive (*cs*) embryonic lethal mutants was carried out as follows. A synchronous population of L4 *lin-2* hermaphrodites (*lin-2* animals develop internally hatched larvae and form a "bag of worms" due to lack of a vulva) were mutagenized with 30 mM ethylmethanesulfonate, grown overnight at 24°C, and then bleached *en mass* the next day to isolate F_1 embryos. These embryos were hatched off without food overnight at 24°C and plated at 24°C as synchronous L1 larvae on high-growth plates. When gravid, these hermaphrodites were bleached *en mass* for embryos, which were hatched off overnight at 24°C. To maintain independence of mutants, only 5% of these F_2 larvae were plated on high-growth plates, and allowed to grow to the L4 stage at 24°C. The plates were then shifted to 15°C. After 82–84 h, the animals were washed off plates and older *lin-2* hermaphrodites that contained only dead eggs and had not formed a bag of worms (and therefore carry potential maternal effect embryonic lethal mutations) were enriched for by allowing the worms to sink in 15-ml conical tubes for 5 min (smaller F_3 larvae tend to float and thus are selected against). These nonbagged, older hermaphrodites were then replated at 24°C on 100-mm NG agar plates at a density of 4000 animals per plate. After 20 h, animals that have formed a bag of worms are then picked. These are candidates for reversible, *cs* embryonic lethal mutations. Out of 250,000 F_2 mutagenized hermaphrodites screened over a 6-month period, 191 *cs* embryonic mutants were verified. The *ye60* mutant was selected out of these mutants by staining mutant embryos with DAPI and tubulin antibody and looking for polarity defects. Of the 160 mutants analyzed, *ye60* was the only polarity mutant found. *ye162* and *ye180*, which fail to complement each other, were isolated in two separate screens for temperature-sensitive osmotic mutants (C.A.R. and R.V.A., manuscript in preparation).

The *ye60* and *ye162* mutants were outcrossed four times to wild type; *ye180* was outcrossed twice. All *ye60* experiments were carried out with this version, except for PAR-3 stainings and some germline granule staining, which were collected with a double outcrossed version. We have confirmed that both versions behave identically by lineage analysis and by various antibody stainings.

Mapping, Strain Constructions, and Genetic Tests

The *ye60* and *ye180* mutations were mapped to LGII and excluded from all other chromosomes by standard linkage tests. *ye60* and *ye180* were mapped to the left arm of LGII by three-factor mapping relative to *dpy-10* and *unc-4*. *ye60* and *ye180* were then crossed into the high transposon copy strain RW7000, homozygous Pod mutant F_2 animals were selected, and then assayed for the presence of polymorphic markers at the left end of LGII [from left to right: stP100, stP196, stP101 (Williams *et al.*, 1992)]. For *ye60*, out of 56 animals analyzed, 1 displayed stP100 only, 1 displayed stP196 only (presumably a double recombinant), 6 displayed stP101

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only, and 11 displayed both stP196 and stP101. For *ye180*, out of 17 animals, 1 displayed stP100 only, 1 displayed stP196 and stP101, and 3 displayed stP101 only. These data indicate that *ye60* and *ye180* lie between stP196 and stP100. To narrow the region further, *ye60* and *ye162* were mapped relative to the single-nucleotide polymorphism, pkP2015 (present in the strain CB4856), which is located on cosmid ZC204 between stP100 and stP196. To perform this mapping, *ye60 unc-4(e120)* and *ye162 unc-4(e120)* strains were constructed (by placing in *trans* to *dpy-10 unc-4* and picking Unc non-Dpy recombinants) and CB4856 males were crossed into each. For *ye60*, out of 99 Unc non-Pod F_2 recombinants, 6 showed the wild-type pattern for pkP2015; for *ye162*, out of 93 Unc non-Pod F_2 recombinants, 4 showed the wild-type pattern for pkP2015. Therefore, both *ye60* and *ye162-ye180* are to the left of pkP2015 and to the right of stP100.

The *ye60 dpy-10(128)* double was constructed by placing *ye60* in *trans* to *unc-85 dpy-10* and picking Dpy non-Unc recombinants. Embryos from this strain were verified to be Pod. The *pod-2(ye60); pod-1(ye11)* double mutant was constructed as follows. *pod-2(ye60)* males at 24°C were crossed with *unc-32(e189) pod-1(ye11)/qC1*, wild-type F_1's were picked, and *pod-2(ye60)/pod-2(ye60); qC1/+* was identified in the F_2. This strain was crossed with *unc-32(e189) pod-1(ye11)/qC1* males, and *pod-2(ye60)* homozygotes were identified in the F_2 that also segregate *unc-32* and *qC1*. The presence of *pod-2* was verified by placing non-Unc non-qC1 animals at 15°C. The presence of *pod-1(ye11)* was verified by PCR (Rappleye *et al.*, 1999). The strain is maintained at 24°C. To study the double mutant, Unc Pod-1 animals were shifted to 15°C as L4 larvae.

For testing of *ye60* in *trans* to deficiency, *ye60* males were crossed to *ccDf11/dpy-25(e817)* hermaphrodites and non-Dpy F_1 progeny were placed as L4's at 15°C (since *dpy-25* is semidominant, these will be *ccDf11/ye60*). All of the animals segregated dead embryos, verifying the genotype *ye60/ccDf11* and that *ccDf11* deletes *pod-2*. For testing *ye60* zygotic requirement, wild-type males were mated into *ye60 dpy-10* hermaphrodites. Non-Dpy F_1's were shifted as L4 hermaphrodites to 15°C, allowed to lay eggs, and then removed. As expected, about one quarter of the next generation were Dpy [these were sampled to verify that *ye60* was still linked to *dpy-10(e128)* in most cases]. To test for maternal requirement, *ye60* animals were grown at 24°C, mated as L4 hermaphrodites to N2 males at 15°C, and allowed to lay eggs. All adults were removed. Of the embryos that hatched, 6/16 were male, verifying that efficient mating had taken place. To test for paternal requirement, *dpy-18(e364)* mothers were mated with *ye60* males at 15°C, allowed to lay eggs, and then removed. About 20% non-Dpy progeny appeared in the next generation, verifying that mating had occurred.

Microscopy of Live Embryos

Embryos were dissected from gravid hermaphrodites in 0.8× egg salts (94 mM NaCl, 32 mM KCl, 2.7 mM $CaCl_2$, 2.7 mM $MgCl_2$, 4 mM Hepes, pH 7.5) for *ye60* experiments and 0.75× egg salts for *pod-2;pod-1* double mutant studies. These conditions were empirically derived. Embryos were mounted pressure-free by including Vaseline between the coverslip and slide. Embryos were observed from pronuclear migration through the four-cell stage on a Zeiss axiovert (100×, 1.30 NA lens) or Olympus BX60 (100×, 1.30 NA lens) with DIC optics and recorded with a time-lapse VCR. For 15°C lineaging, the microscope room was kept at 15 ± 1°C. Osmotic sensitivity of embryos was determined by mounting embryos in water or 300 mM KCl. As a control, we lineaged 10 *emb-20(g27ts)* embryos at 25°C in 0.8× egg salts from before pronuclear meeting to the two-cell stage and verified that our salt conditions do not cause osmotically sensitive embryos to display polarity defects in general (similar to the test in Rappleye *et al.*, 1999). To determine whether a one-cell embryo divided symmetrically, the cross-sectional areas of the daughter cells were calculated using NIH Image and analyzed as described (Rappleye *et al.*, 1999). Cytoplasmic flows were qualitatively ascertained as described (Rappleye *et al.*, 1999).

Determination of Cold-Sensitive Period

For shift-down analyses, *ye60* hermaphrodites embryos were grown at 24°C and then shifted for 1, 4, 7, 10, 13, 16, and 19 h to 15°C. Two-cell embryos were then dissected out of mothers in a 15°C room, transferred to plates, and placed in a 15°C incubator. The embryos were allowed to develop and scored for viability. For shift-up experiments, *ye60* L4 hermaphrodites were shifted to 15°C for 1.5 days and then shifted for 1, 4, 7, 10, 13, 16, and 19 h to 24°C. Two-cell embryos were dissected out at 24°C, transferred to plates, and placed in a 24°C incubator. Each data point represents a minimum of 40 embryos. The time points of shift-down experiments are normalized to the speed of growth at 24°C, which is approximately twice as fast as at 15°C.

Immunofluorescent Stainings

PAR-3, PAR-1, P granule (K76), and POD-1 (11-1) freeze-fracture staining were performed as published (Etemad-Moghadam *et al.*, 1995; Guo and Kemphues, 1995; Rappleye *et al.*, 1999), except that embryos were cut from mothers in 0.8× egg salts. For PIE-1 (P4G5) and P granule co-staining, embryos were fixed in −20°C MeOH for 10 min and 4°C acetone for 10 min. The embryos were then processed according to the published protocol beginning with the 30-min formaldehyde fix (Tenenhaus *et al.*, 1998). DAPI was included with all stainings. Fluorescent stainings were observed with a deconvolution system (Delta Vision 2.10; Applied Precision) on an Olympus IX-70 inverted microscope (60×, 1.40 NA objective) and a Micromax camera (Princeton Instruments). Image stacks were collected with 0.6-μm sections and deconvolution performed according to the Agard and Sedat inverse matrix algorithm. All data for one-cell embryos were collected between pronuclear meeting and anaphase.

pod-2;pod-1 Double Mutant Analysis

Polarity was scored in these mutants by freeze-fracture permeabilization, fixation in room temperature methanol for 4 min, and staining with DAPI. Fluorescence was visualized as above. Asymmetry and symmetry of early embryos were scored by using the location and cell cycle state of the DNA between anaphase of one-cell through telophase of two-cell embryo, except during prophase and prometaphase of the two-cell embryo when both AB and P_1 can display similar DAPI staining in wild type. Lineage analysis was performed to confirm our findings in live embryos.

PIE-1:GFP Study

pod-2(ye60) strain was mated into a line carrying *PIE-1:GFP* on an extrachromosomal array (Reese *et al.*, 2000). *pod-2(ye60);PIE-1:GFP* was selected in the second generation and maintained at 24°C

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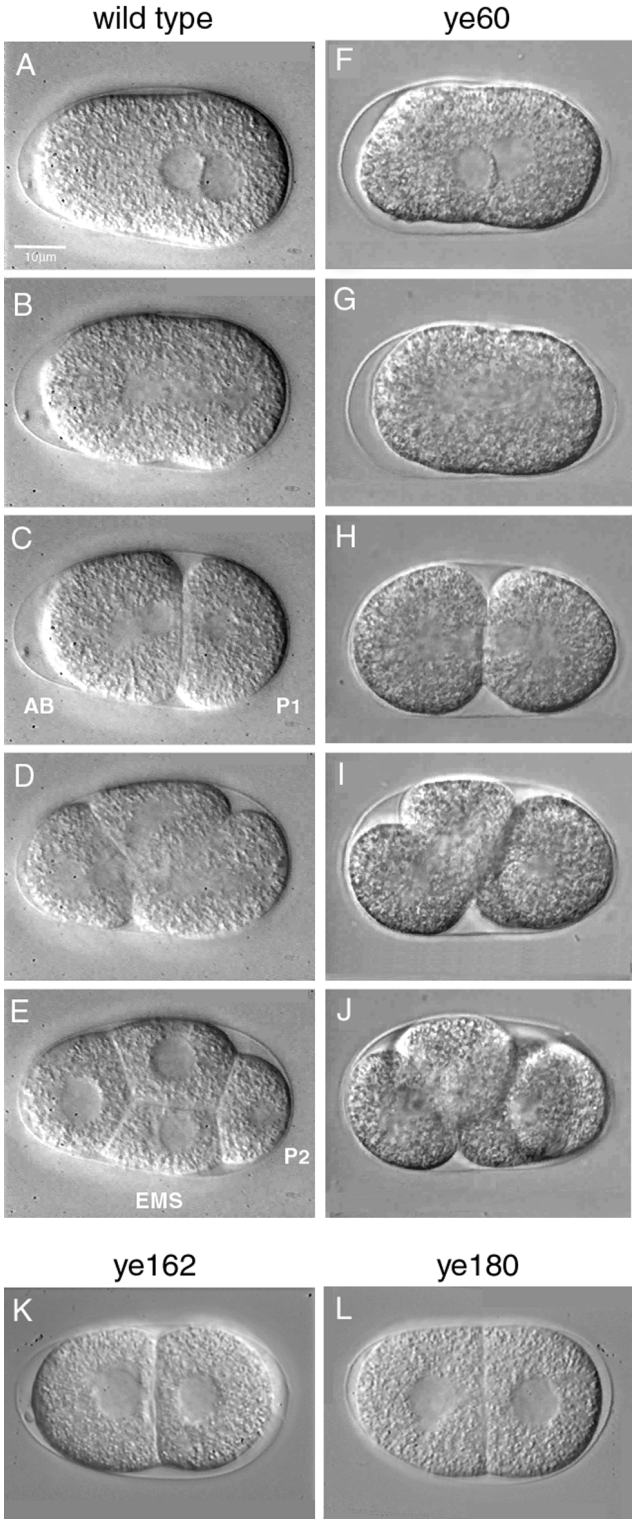

FIG. 1. *pod-2* mutants perturb physical polarity in the early embryo. Lineage of a single wild-type embryo (A–E) and a single *ye60* embryo (F–J). Anterior is to the left in all panels. (A) In wild-type, the two pronuclei meet in the posterior of the zygote, and (B) an asymmetric spindle sets up that is biased toward the posterior. (C) When division occurs, the posterior cell P_1 is smaller than the anterior cell AB. (D) The AB cell divides first along a transverse axis, and then P_1 along the longitudinal axis. (E) The location of P_1's anterior daughter, EMS, and posterior daughter, P_2, in the early four-cell embryo. (F) In *ye60* embryos, the two pronuclei sometimes meet in the middle (5/22 embryos). (G) In this embryo, the spindle set up symmetrically such that (H) both daughter cells are the same size. (I) Both cells enter division at approximately the same time, and divide along transverse axes, one dividing from top to bottom of the embryo the other dividing in and out of the plane of the paper. (J) The arrangement of cells at the four-cell stage. The cell cycle is slightly delayed in *ye60* embryos. At 15°C, the average time from pronuclear meeting to completion of first cleavage is 15.6 ± 2.7 min in the mutant (n = 11) versus 13.7 ± 1.5 min in wild type (n = 7). (K, L) Images of two-cell *ye162* and *ye180* embryos, respectively, showing symmetric cleavage. In both cases, the two daughters subsequently divided synchronously and in parallel orientations. Scale bar in this and all other figures is 10 μm.

(*PIE-1:GFP* animals can be identified by a rolling phenotype). Prior to the observation, rolling animals were shifted to 15°C overnight and one-cell embryos dissected out. Images were collected in 5-μm sections every 1 min by using the DeltaVision system (see above). Out of 74 GFP-staining *ye60* embryos lineaged, only 7 showed clear segregation of GFP to the anterior daughter of P_1. We do not know why the percent live embryos showing this phenotype (10%) is lower than that found in fixed embryos (30%) but may reflect fluorescent damage of live embryos, different temperature conditions caused by live fluorescent imaging, or superior signal:noise of antibody staining.

RESULTS

The ye60, ye162, and ye180 Alleles Perturb a-p Polarity in the Early Embryo

To identify new components required for antero-posterior (a-p) asymmetry in the early *C. elegans* embryo, we screened 250,000 mutagenized F_2 hermaphrodites for cold-sensitive (*cs*) embryonic lethal mutants. Although a number of screens for temperature-sensitive (*ts*) embryonic lethal mutants have been performed in *C. elegans*, none to our knowledge have been performed for *cs* mutants. We hypothesized that screening for cold sensitivity might allow us to identify mutants not found in *ts* screens, as, for example, was found in yeast screens for *cs* cell-division-cycle mutants (Moir *et al.*, 1982).

Among the 160 *cs* embryonic lethals characterized from our screen, we found 1 mutant allele *ye60* with defects in establishing a-p polarity. Normally, the first cell division of the *C. elegans* zygote results in a large anterior cell (AB) and a small posterior cell (P_1) (Figs. 1A–1C). This physical asymmetry correlates with differences in the behavior of the two cells in the next cell cycle. The anterior cell AB divides first and along the transverse axis, while the poste-

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TABLE 1

ye60, *ye162*, and *ye180* Mutants Cause a Loss of Physical Polarity

Genotype of mother	% Symmetric two-cell embryos[a]	*n*[b]
+/+	0	9
ye60/ye60	43 (46)[c]	28
ccDf11/+	0	10
ye60/ccDf11	53 (71)[c]	17
ye162/ye162	17	23
ye180/ye180	11	36

[a] As measured by the cross-sectional area of two cells after first cleavage. All lineages were performed at 15°C, except for *ye162* and *ye180* data which were collected at 25°C.

[b] Number of embryos lineaged from the one-cell stage to two-cell stage.

[c] Number in parentheses refers to percent of embryos in which the spindle set up symmetrically. In a few embryos, the symmetric spindle shifted posterior as the cell was dividing, resulting in asymmetric cleavage.

rior cell P_1 divides second and along the longitudinal axis (Figs. 1D and 1E). In the *ye60* mutant, these physical asymmetries are lacking in nearly half of all mutant embryos at the nonpermissive temperature (Table 1). The first cell division of *ye60* embryos at the nonpermissive temperature often proceeds symmetrically (Figs. 1F–1H). Phenotypes associated with loss of physical polarity continued during the next cell cycle. In 9 of the 12 embryos where the first division proceeded symmetrically, the second divisions proceeded synchronously and transversely (Figs. 1I and 1J). Such polarity phenotypes have been described before in the *par* or *par*titioning mutants and indicate a loss of a-p polarity (Kemphues *et al.*, 1988).

The loss of polarity associated with *ye60* does not appear to be a unique aspect of the *ye60* allele. This conclusion is based on the fact that, in an unrelated screen for temperature-sensitive osmotic embryonic mutants (C.A.R. and R.V.A., manuscript in preparation), we isolated two independent mutants, *ye162* and *ye180*, that failed to complement each other and therefore defined a single gene. Furthermore, like *ye60*, they display a-p polarity defects (Table 1, and Figs. 1K and 1L) and are osmotically sensitive (see below). Although we cannot perform complementation tests between *ye60* and *ye162-ye180* since the former is cold-sensitive and the latter are temperature-sensitive, all three very likely mutate the same gene since they share the same phenotypes and map to the same interval on chromo-

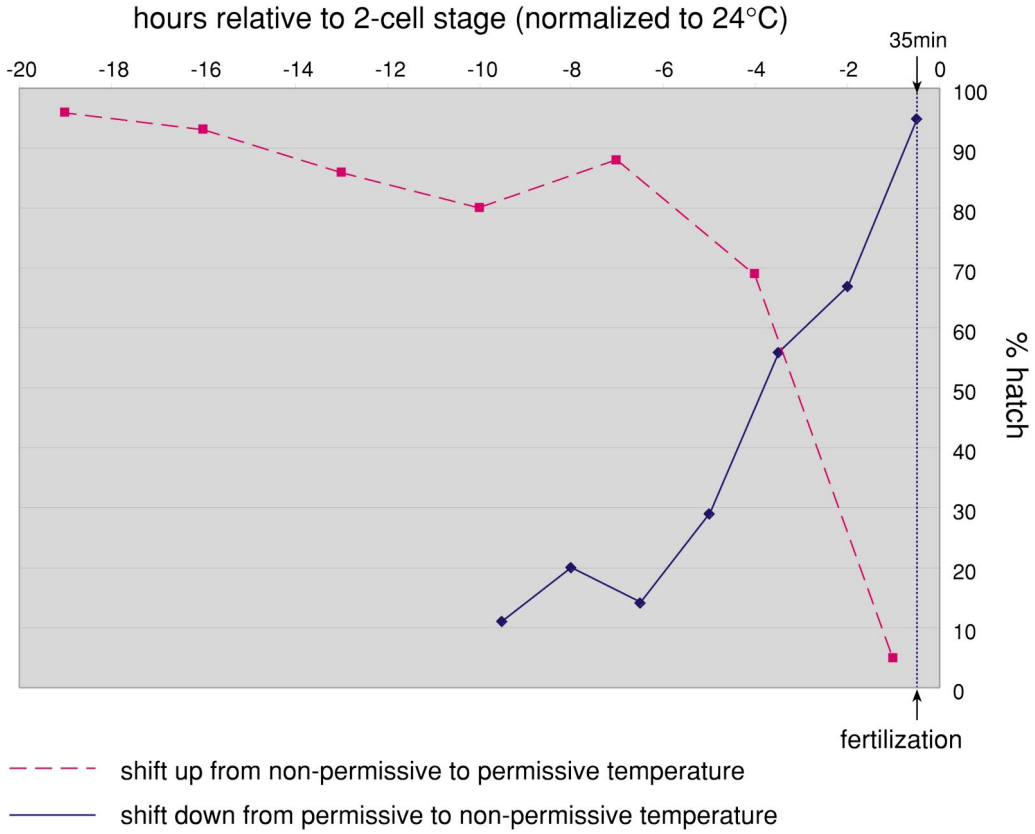

FIG. 2. *ye60* cold-sensitive period. Results of shift-up experiments to permissive temperature (red) and shift-down experiments to nonpermissive temperature (blue).

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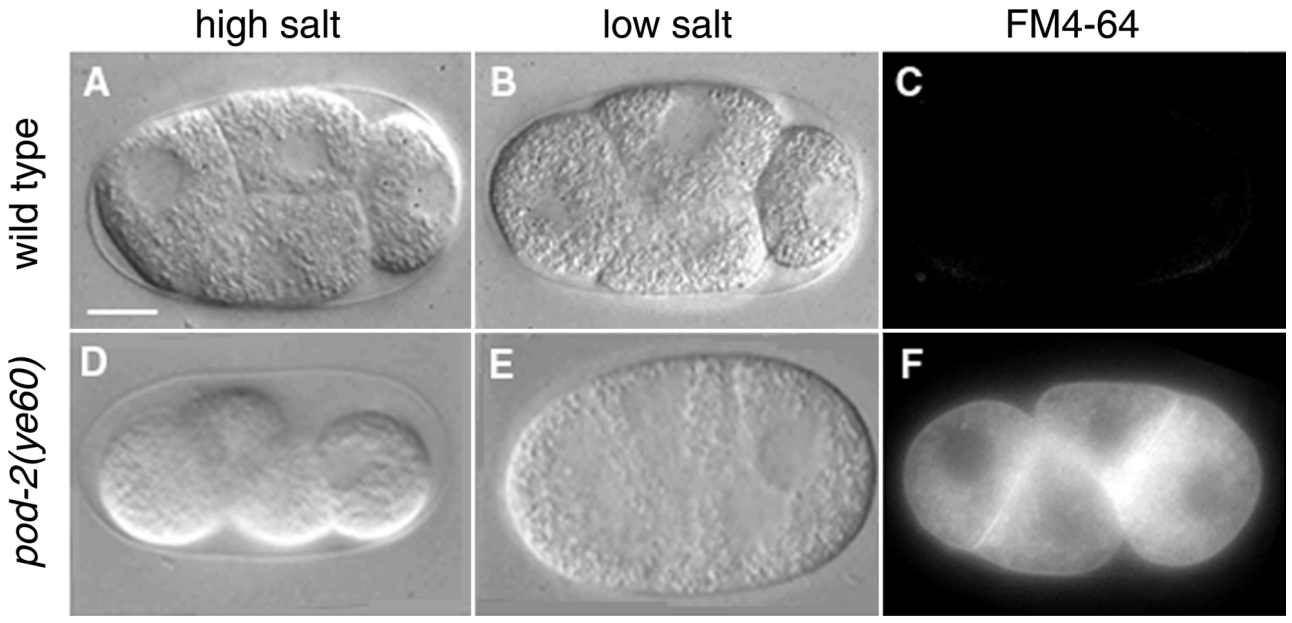

FIG. 3. *pod-2(ye60)* eggshells fail to protect the embryo from osmotic stress and fluorescent dyes. (A, B, D, E) Nomarski images. (C, F) Fluorescent images taken at identical exposures. Anterior is to the left in all images. (A) A wild-type four-cell embryo in 300 mM salt and (B) 0 mM salt (water). The embryos are impermeable to the salt conditions, neither shrink nor swell, and continue to divide normally. (C) Wild-type embryos bathed in FM4-64 are impermeable to the dye and show no fluorescence. (D) *ye60* mutant embryos respond to 300 mM KCl by shrinking in the eggshell; (E) *ye60* mutant embryos respond to water by swelling in the eggshell. (F) *ye60* mutant embryos are permeable to FM4-64 dye.

some II (see below). These data suggest that loss of polarity is a characteristic of loss of gene function. Since *ye60* showed the strongest polarity defects, we chose this allele for most of our detailed phenotypic analyses of the locus.

To test whether the lack of complete penetrance might be due to the weakness of the *ye60* allele and to confirm that *ye60* behaves as a loss-of-function allele, we placed the *ye60* allele in *trans* to the *ccDf11* deficiency that was found to span the locus. The polarity defect was slightly enhanced in *ye60/ccDf11* embryos (Table 1). Thus, *ye60* may not be a complete loss-of-function allele but is probably a strong reduction-of-function allele with regards to polarity. Although only half of all *ye60* mutant embryos show loss of physical polarity, very few complete embryogenesis at the nonpermissive temperature (see below). Most arrest with several hundred nuclei and fail to undergo morphogenesis, indicating that virtually all *ye60* mutant embryos have developmental defects, whether they divide symmetrically at the first cell division or not.

ye60 Is Required Maternally Prior to Fertilization

Lethality associated with *ye60* is temperature-dependent, and the *ye60* mutant is a strict maternal effect lethal mutation. At the nonpermissive temperature of 15°C, <4% of the embryos hatch; in contrast, 95% hatch at the permissive temperature of 24°C (Table 2). Embryonic lethality at 15°C is strictly dependent on the maternal genotype. *ye60/ye60* embryos derived from *ye60/+* mothers are viable, but *ye60/+* progeny derived from *ye60/ye60* mothers are inviable (Table 2). The gene mutated in *ye60* is not required paternally as *ye60/ye60* males mated into wild-type hermaphrodites give rise to viable progeny (Table 2). We refer to embryos derived from homozygous mutant mothers shifted to 15°C as "*ye60* embryos."

To determine whether the gene mutated in *ye60* is required throughout embryonic development or only for a specific period, we performed temperature-shift experiments. We found that this gene is required only prior to fertilization (Fig. 2). *ye60* embryos shifted from permissive to nonpermissive temperature just prior to fertilization are mostly viable. Conversely, *ye60* embryos shifted from nonpermissive to permissive temperature just prior to fertilization are mostly inviable. Since these data were based on extrapolating back in time from two-cell embryos (see Fig. 2 legend and Materials and Methods), we verified these results with one-cell embryos. A total of 0/23 embryos shifted from nonpermissive to permissive temperature at the one-cell stage hatched, and 9/11 embryos shifted from permissive to nonpermissive temperature at the one-cell stage hatched.

Similar genetic analyses were performed for *ye162* and *ye180*. *ye162* and *ye180*, like *ye60*, are required maternally. We also performed temperature-shift experiments and dem-

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TABLE 2

ye60 Is Cold-Sensitive and Shows a Strict Maternal Requirement

Genotype of mother	Genotype of embryos	Temp. (°C)	% Viable embryos	No. embryos scored
+/+	+/+	15	99	1726
ye60/ye60[a]	*ye60/ye60*	15	3.7	1988
ye60/ye60[a]	*ye60/ye60*	24	95	1094
ye60/+[b]	*ye60/+*, *ye60/ye60*, +/+	15	96	707
ye60/ye60[c]	*ye60/ye60*, *ye60/+*	15	2.2	729
+/+[d]	+/+, *ye60/+*	15	97	845

[a] *ye60* hermaphrodites were grown at 24°C, shifted as L4's to the designated temperature, allowed to lay eggs, and then removed.
[b] Test for zygotic requirement. See Materials and Methods.
[c] Test for maternal requirement. See Materials and Methods.
[d] Test for paternal requirement. See Materials and Methods.

onstrated that, like *ye60*, *ye162* is required prior to fertilization for embryonic viability (data not shown; see Discussion).

ye60 Defines a Second Polarity and Osmotic Defective (pod) Gene

Our laboratory recently characterized a new type of mutant that perturbed polarity in the one-cell embryo and also resulted in osmotically sensitive embryos. The gene encoding this mutant was called *pod-1*. We wanted to test whether the *ye60* mutant also gave rise to osmotic defects.

Due to the protective nature of the eggshell, wild-type embryos are unaffected by high or low salt and are impermeable to dyes such as FM4-64 (Figs. 3A–3C; Rappleye *et al.*, 1999). Only when the inner layer of the eggshell is compromised will salt concentration affect embryos and dyes enter (Rappleye *et al.*, 1999). In *pod-1* mutant embryos, the embryos are sensitive to changes in salt concentration and are permeable to dyes, presumably because of a defective eggshell.

ye60 mutant embryos display similar eggshell defects as *pod-1*. *ye60* mutant embryos placed in high or low ionic strength solutions shrink or swell, respectively (Figs. 3D and 3E). Furthermore, *ye60* mutant embryos are permeable to dyes such as FM4-64 (Fig. 3F), further confirming that the eggshell is compromised. *ye60* is only the second mutant characterized that gives rise to polarity and osmotically defective embryos. As with *pod-1*, we confirmed that osmotic stress is not likely the source of *ye60* embryonic polarity defects since another osmotic mutant, *emb-20(g27)*, lineaged in the same medium shows normal polarity (see Materials and Methods). As noted above, *ye162* and *ye180* are, like *ye60*, osmotically sensitive since they were isolated in a directed genetic screen for such mutants.

To determine whether *ye60* might be a mutation in a

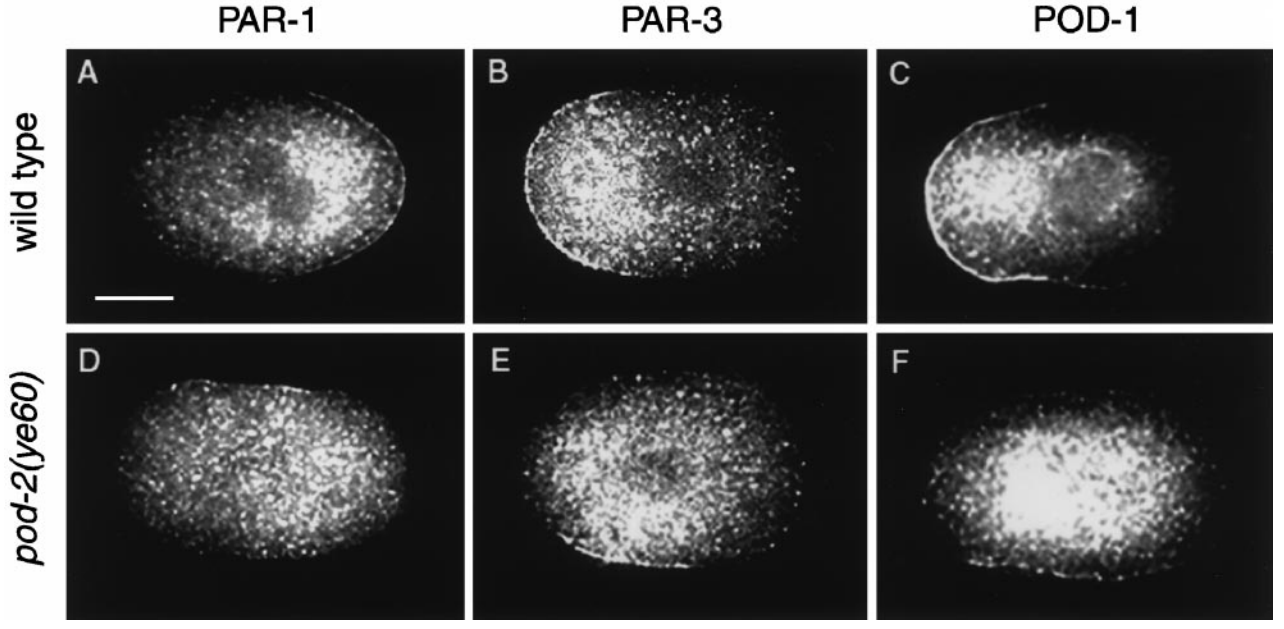

FIG. 4. *pod-2* is required to polarize other proteins required for a-p polarity. One-cell wild-type embryos (A–C) and *ye60* embryos (D–F). Anterior is to the left in all images. In wild-type one-cell embryos, (A) PAR-1 protein localizes to the posterior cortex, (B) PAR-3 localizes to the anterior cortex, and (C) POD-1 is enriched in the anterior cortex. All proteins are also present in the cytoplasm. In many *pod-2* mutant embryos, these proteins are no longer found in the correct location. Often (D) PAR-1, (E) PAR-3, and (F) POD-1 appear enriched along a lateral edge in *ye60* embryos. Embryos in (A) and (D) are at pronuclear joining, (B), (C), (E) and (F) are at prometaphase.

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TABLE 3

pod-1 Null Does Not Enhance Polarity Defects in *pod-2 (ye60)*

Genotype of mother	% Two-cell embryos with loss of polarity[a]
Wild type	0 (n = 32)
pod-1 (ye11)	44 (n = 34)
pod-2 (ye60)	49 (n = 35)
pod-2 (ye60); pod-1 (ye11)	46 (n = 26)
pod-2 (ye60); pod-1 (ye11)[b]	31 (n = 13)

[a] Embryos were fixed by freeze fracture and analyzed by DAPI staining unless otherwise noted.

[b] The first embryonic division was followed live in these embryos, and the cross-sectional area of the cells calculated after division.

known gene, we mapped the allele relative to known genetic and physical markers (see Materials and Methods). *ye60* maps to the left arm of chromosome II between the physical polymorphisms stP100 and pkP2015, a region of the genome with 375 open reading frames and no known polarity or embryonic lethal mutants. Therefore, this mutant defines a new polarity osmotic gene that we call *pod-2*. *ye162-ye180* also map between the same two markers (see Materials and Methods).

The pod Genes Function in a Common Polarity Pathway

That *pod-2* mutant embryos share a number of phenotypes with *pod-1* mutant embryos suggested that these two genes might function in a common pathway. Mutations in either gene similarly result in osmotic defects and in loss of polarity in half of all embryos. Other polarity phenotypes associated with loss of *pod-1* are also found in *pod-2(ye60)*. In 9 out of 18 *ye60* embryos examined, posteriorly directed cytoplasmic flows that occur during early pronuclear migration in the one-cell embryo were qualitatively absent. Consistent with this finding, pseudocleavage furrow formation, thought to be a consequence of these flows, failed to form or formed weakly and anteriorly in all *pod-2(ye60)* embryos examined.

To test the common pathway hypothesis, we compared the phenotype of embryos mutated for each gene individually with embryos mutated for both genes. As previously demonstrated (Rappleye *et al.*, 1999; Table 1), about half the embryos derived from the *pod-1(ye11)* null allele or the *pod-2(ye60)* allele show symmetric cleavage of one-cell embryos (Table 3). If *pod-1* functions in the same pathway as *pod-2*, then elimination of *pod-1* (using our null allele) in a *pod-2* mutant background should not enhance the polarity defect. Indeed, embryos lacking *pod-1* and *pod-2* show the same percent loss of polarity as lacking either gene alone (Table 3), demonstrating that the two genes function in a common pathway.

Although *pod-1* and *pod-2* share similar polarity and eggshell phenotypes, *pod-2* embryos do not display some of the nonpolarity defects associated with *pod-1* embryos. *pod-2(ye60)* embryos do not form abnormal endocytically derived compartments and do not form hyaline zones. To determine whether the lack of these phenotypes might be due to incomplete penetrance of the *ye60* allele, we examined embryos derived from hemizygous *ye60/ccDf11* mothers. Neither hyaline zones nor circular granule-free zones are apparent in these embryos. However, 4 of 18 *ye60/ccDf11* embryos displayed defects in cell division similar to those in the *pod-1* mutant, in which the cell membrane between some sister cells disappeared. In another embryo, a cleavage furrow failed to form, but the nuclei still divided.

pod-2 Is Required to Polarize PAR and POD-1 Proteins

Six *par* proteins have been identified that are required for polarizing the one-cell *C. elegans* embryo. Homologues of two of these, PAR-1 and PAR-3, have been shown to play important roles in polarizing *Drosophila* cells and possibly mammalian cells as well, and a homologue of a third, PAR-6, has been implicated in polarizing the *Xenopus* embryo (Boehm *et al.*, 1997; Izumi *et al.*, 1998; Shulman *et al.*, 2000; Wodarz *et al.*, 1999).

The PAR-1 and PAR-3 proteins themselves are localized in a polarized fashion in the wild-type *C. elegans* embryo (Etemad-Moghadam *et al.*, 1995; Guo and Kemphues, 1995).

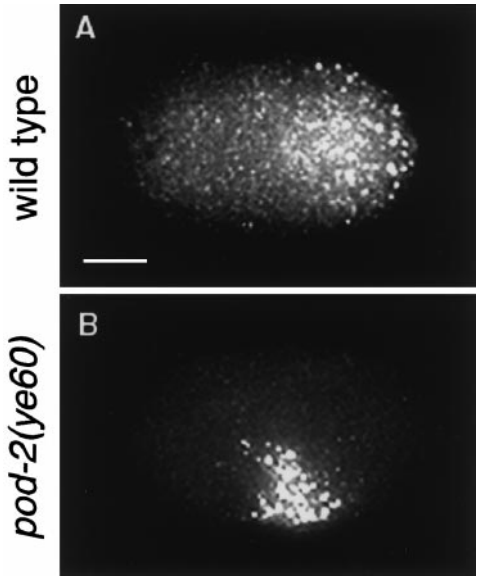

FIG. 5. *pod-2* is required to segregate germline (P) granules. Anterior is to the left. (A) In a metaphase wild-type one-cell embryo, P granules are found in the posterior cytoplasm. (B) In this metaphase *ye60* mutant embryo, P granules are pushed to one side near and slightly posterior of the middle.

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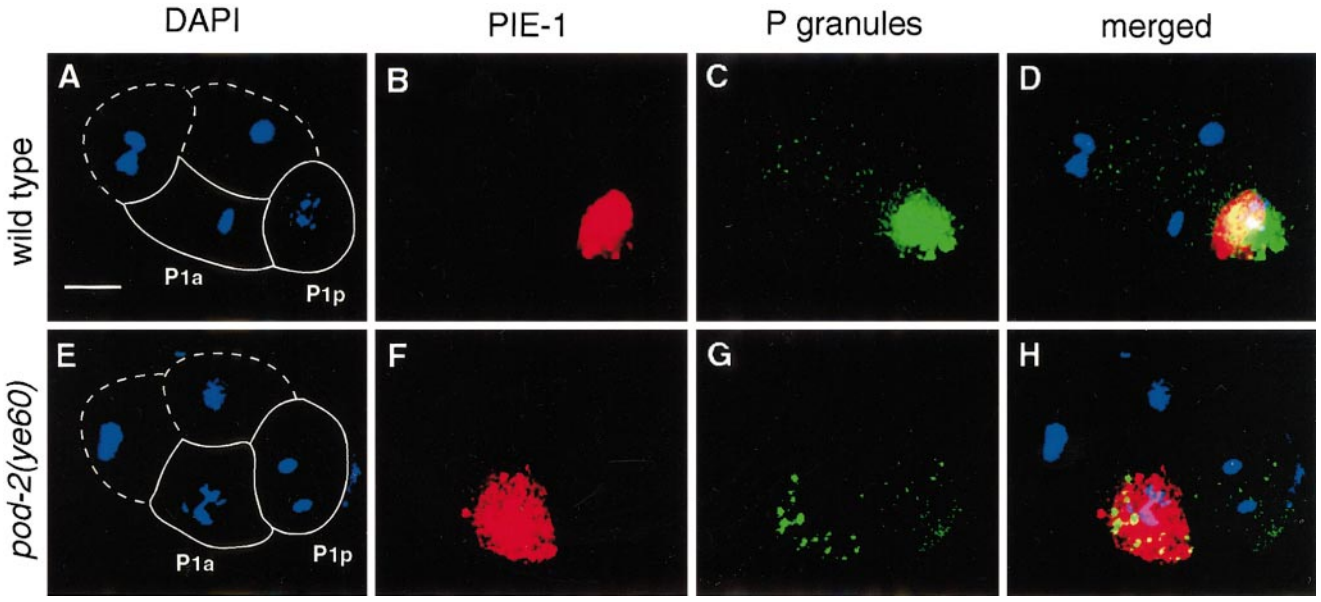

FIG. 6. In *ye60* embryos, germline potential can segregate to the wrong daughter of P_1. Anterior is to the left in all panels. P_1a refers to the anterior daughter of P_1 (normally called EMS); P_1p refers to the posterior daughter of P_1 (normally called P_2). (A–D) A wild-type four-cell embryo triply stained for DAPI (A), PIE-1 (B), P granules (C), and merged image (D). Note that PIE-1 and P granules are found only in P_1p and P_1p is delayed in the cell cycle relative to P_1a (prometaphase versus metaphase). (E–H) A *ye60* mutant four-cell embryo triply stained for DAPI (E), PIE-1 (F), P granules (G), and merged image (H). Note that PIE-1 and P granules are now found in P_1a and that P_1a is delayed in the cell cycle relative to the posterior daughter (prometaphase versus anaphase).

PAR-1 protein is normally found in a cortical cap at the posterior of the one-cell embryo (Fig. 4A; 38/45 embryos examined). Conversely, PAR-3 protein is normally in a cortical cap at the anterior of the one-cell embryo (Fig. 4B; 25/25 embryos examined). Both proteins are also found in the cytoplasm. Although not yet formally demonstrated, it is assumed that the asymmetric localization of these proteins is important for their function in establishing polarity in the early embryo.

To determine whether *pod-2* plays a role in the polarization of these proteins, we localized PAR-1 and PAR-3 in *ye60* mutant embryos using antibodies. The localization of both proteins is perturbed in the mutant. Out of 45 *ye60* mutant one-cell embryos stained with PAR-1 antibody, 62% showed either mislocalization of PAR-1 to a lateral cortical edge of the embryo (Fig. 4D; 9/45), no cortical (but still cytoplasmic) staining (15/45), or lack of staining altogether (4/45). The other 38% (17/45) of the embryos showed normal PAR-1 localization. PAR-3 protein localization was perturbed in 55% of *ye60* embryos and displayed localization to a lateral cortical edge (9/31 embryos; Fig. 4E), uniform cortical localization (3/35 embryos), or no cortical staining (5/31 embryos). The remaining 45% (14/31) of the embryos showed normal PAR-3 localization.

Since POD-1 protein is also asymmetrically localized in the one-cell embryo (Rappleye *et al.*, 1999), we tested whether the polarization of POD-1 protein depends on *pod-2*. In wild-type one-cell embryos, POD-1 is enriched in the anterior cortex (Fig. 4C; 15/19 embryos examined). In 58% of *pod-2(ye60)* embryos, POD-1 is abnormally localized, either to a lateral cortical edge (10/31 embryos; Fig. 4F), uniformly around the cortex (4/31 embryos), or posteriorly enriched (4/31 embryos). In the remaining 42% of the embryos (13/31), POD-1 showed normal localization.

pod-2 Is Required for the Proper Subcellular Localization of Germline Components

Germline granules are a standard marker for studying developmental asymmetry in the early *C. elegans* embryo. In wild-type embryos, germline granules (or "P" granules) are segregated to the very posterior of the one-cell embryo by the middle of the first cell cycle when the two pronuclei meet (Fig. 5A). In this way, only the posterior daughter that gives rise to germline inherits the granules.

We examined P granule distribution in *pod-2(ye60)* mutant embryos. In roughly half of these embryos (11/23), P granule distribution was similar to wild type. In a few (3/23), the staining for P granules was not concentrated in the posterior but faint and diffuse throughout the cell. The remainder showed an interesting defect. In nine of the one-cell embryos examined past pronuclear meeting, P granules were found near the middle of the embryo, either exclusively or in addition to in the posterior, and were often pushed to one side (Fig. 5B).

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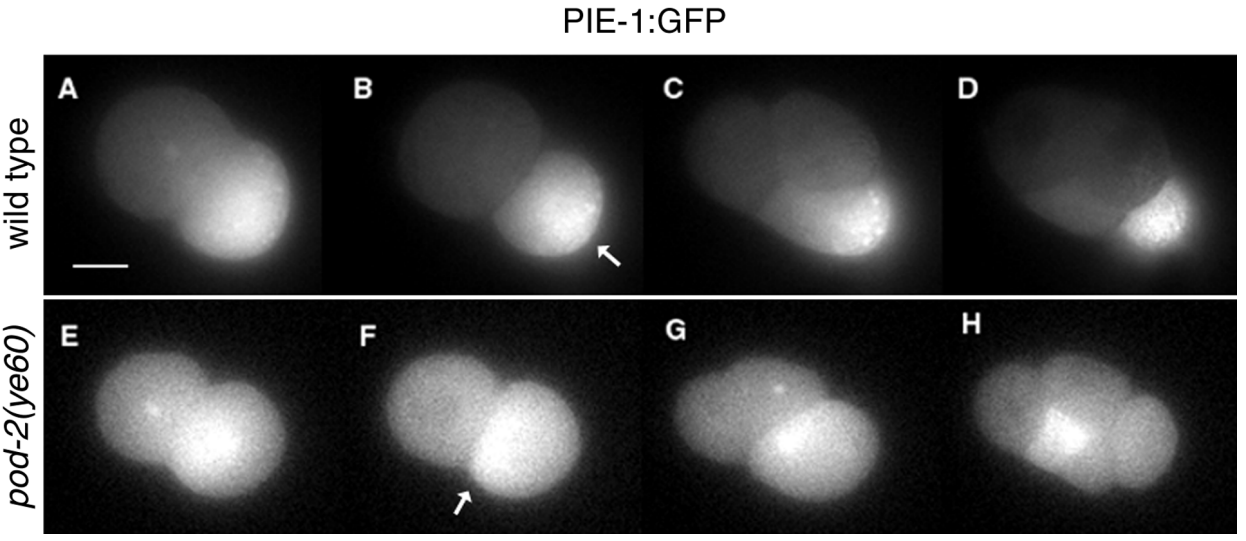

FIG. 7. PIE-1 missegregation defect visualized in live embryos. PIE-1: GFP in (A–D) a live wild-type embryo and (E–H) a live *ye60* embryo. Anterior is to the left in all panels. (A) In a wild-type one-cell embryo during division, PIE-1 is enriched in the posterior of the embryo. (B) After division, PIE-1 becomes enriched in the posterior of P_1 (arrow) such that when P_1 divides (C), PIE-1 is exclusively found in the posterior daughter of P_1, P_2 (D). (E) In this dividing *ye60* mutant one-cell embryo, PIE-1 is enriched in an area that will subsequently be in the anterior of the P_1 cell (F; arrow). When P_1 divides (G), the anterior daughter of P_1 inherits PIE-1 (H).

This lateral localization of germline granules has not been previously described and prompted us to look carefully at the localization of P granules and germline determinants in four-cell embryos to determine how they were being partitioned. We fixed and triply stained four-cell wild-type and *ye60* embryos with DAPI, PIE-1 antibody, and P granule antibody. Using DAPI, the P_2 cell can be distinguished from the other three cells of the wild-type four-cell embryo by its delayed cell cycle timing (Fig. 6A). Furthermore, in addition to P granules, the P_2 cell is the only cell in wild type to contain the PIE-1 protein (Figs. 6B and 6C). PIE-1 protein, which is distinct from P granules, is a CCCH-type finger-containing protein required for repressing transcription and somatic development in germline precursor cells such as P_2 (Mello *et al.*, 1992, 1996; Seydoux and Dunn, 1997; Tenenhaus *et al.*, 1998). A merged image of a triply stained embryo is shown in Fig. 6D.

When P granules and PIE-1 were coexamined in *ye60* four-cell embryos, we found that, in 9/30 embryos, both markers were abnormally localized to a single cell that corresponded to the normal location of the anterior daughter of the P_1 cell, EMS (Figs. 6F and 6G). This phenotype occurs at a similar frequency whether the four-cell embryos are completely symmetric (i.e., *par*-like as judged by the synchrony of the four cells) or relatively wild type. In cases where there was asynchrony in the four-cell embryo, the cell cycle of the anterior daughter of P_1 was also the most delayed (Fig. 6E; triple staining shown in Fig. 6H), providing further evidence that this cell was behaving like P_2. In the remaining embryos, both P granules and PIE-1 were spread out among all the cells of the four-cell embryo (3/30 embryos), localized to a single cell in an ambiguous location (3/30 embryos), or localized normally (15/30 embryos). In most cases, PIE-1 and P granules showed identical localizations, although in a few embryos P granules were found at lower levels in other cells as well. This novel germ cell phenotype and the abnormal distribution of P granules in one-cell embryos suggested that, in the *ye60* mutant, components that determine the germline are as a unit incorrectly localized early on and then eventually inherited by the wrong cell. Alternatively, it was possible that in four-cell embryos containing germline components where EMS should be, the components were initially properly segregated but that during the division of P_1, the forming P_2 daughter cell "flipped" into EMS's anterior position.

To determine how this unusual defect comes about, we followed the segregation of germline identity, specifically PIE-1:GFP, in live embryos. Similar to PIE-1 staining in fixed embryos, PIE-1:GFP becomes concentrated in wild type in the posterior-most region of late-staged one-cell embryos (Fig. 7A). After the first division, PIE-1 is localized to the posterior region of P_1 (Figs. 7B and 7C) such that the protein is inherited by P_2, the posterior daughter of P_1 (Fig. 7D). We followed PIE-1:GFP localization from the one- or two-cell stage to the four-cell stage in *ye60* embryos. In seven of these embryos, PIE-1 was segregated to the anterior daughter of P_1 (Figs. 7E–7H). In all seven, PIE-1:GFP was enriched in the earliest stages of P_1's cell cycle in the anterior of P_1 (1/7; Fig. 7F), in an anterior lateral region of P_1 (4/7), or along a lateral edge of P_1 (2/7) such that when P_1

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divided, PIE-1 was inherited by the anterior daughter (Fig. 7H). No abnormal shuffling of cell positions was seen. For the five of these seven embryos in which we caught the lineage during the one-cell stage, PIE-1 was concentrated in a central, central-lateral, or posterior-lateral region of the one-cell embryo such that when the cell divided, it was concentrated in the anterior, anterior-lateral, or lateral region of P_1 (Fig. 7E). Hence, the defect can be traced back to the one-cell stage and is caused by abnormal PIE-1 localization and not unusual cell movements.

DISCUSSION

pod-2, with pod-1, Defines a New, Important Class of Embryonic Mutants

The *pod-2* gene is required for correctly establishing a-p polarity in the *C. elegans* embryo. In the *pod-2(ye60)* mutant, both physical asymmetries in the one- and two-cell embryo and the asymmetric localizations of developmental proteins such as PAR-1, PAR-3, POD-1, and P granules are perturbed. Since PAR-3 and POD-1 themselves appear to act early during the establishment of a-p polarity, *pod-2* is also likely to function early. We also isolated two temperature-sensitive alleles, *ye162* and *ye180*, that also very likely mutate the *pod-2* gene since they map to the identical region of chromosome II and display the same set of phenotypes as *ye60*. It is alternatively possible that two different *pod* genes map to this same small interval, although we believe this to be unlikely. The isolation of three alleles with polarity defects, the fact that all alleles are recessive, and the fact that *ye60* in *trans* to deficiency shows similar polarity defects as *ye60* homozygotes, indicate that the wild-type function of the *pod-2* gene is required to establish polarity in the one-cell embryo.

ye60, *ye162*, and *ye180* mutant embryos also show osmotic defects, a phenotype recently studied in the *pod-1* mutant. This result suggests that the *pod-2* gene is required for proper formation or functioning of the inner eggshell, a layer formed by secretion after fertilization (Rappleye *et al.*, 1999). The fact that the great majority of osmotically sensitive embryonic mutants do not show loss of a-p polarity (Rappleye *et al.*, 1999; C.A.R. and R.V.A., manuscript in preparation) indicates that osmotic sensitivity or stress alone is not sufficient to cause the polarity defects in the *ye60* mutant.

Our data demonstrate that *ye60* is encoded by a new *pod* gene, *pod-2*, and that both of the known *pod* genes, *pod-1* and *pod-2*, function in the same polarity pathway. This latter conclusion is based on the findings that mutations in either gene perturb polarity in about 50% of the embryos and that the combination of a *pod-1(null)* and the *pod-2(ye60)* mutation in the same embryo does not enhance this phenotype. These data coincidentally provide strong evidence that the establishment of a-p polarity in the early *C. elegans* embryo requires multiple pathways. Since some *par* mutants perturb a-p polarity in 100% of the embryos (Kemphues *et al.*, 1988), the fact that loss of the *pod-1* and *pod-2* pathway eliminates polarity in only 50% of the embryos is best explained by the presence of another pathway that parallels *pod-1* and *pod-2* and that provides the remaining polarity in their absence. Although *pod-1* and *pod-2* share many phenotypes in common, some of the nonpolarity phenotypes associated with *pod-1* are not present in *pod-2*. We hypothesize that either a null allele of *pod-2* would be required to uncover these defects or *pod-2* is involved with only a subset of functions regulated by *pod-1*. For example, perhaps *pod-2* is involved in a subset of membrane trafficking or actin-modifying functions hypothesized to be regulated by *pod-1* (Rappleye *et al.*, 1999).

That both polarity mutants isolated in our laboratory, using screening techniques not previously tried, are Pod suggests that there are other *pod* genes in *C. elegans*, and that this class of genes is not at saturation. The *pod* genes probably went unnoticed in genetic screens until now because the other phenotypes associated with the mutants complicate their lineaging and require careful salt and pressure conditions to allow the embryos to progress.

A Potential Role for Polarity Generation in the Oocyte

Based on *ye60* temperature-shift studies, the requirement for *pod-2* in embryonic development is between 0 and 6 h before fertilization. We also found that *ye162* is required at least 3 h prior to fertilization for embryonic viability. There are two simple explanations for these results. Either *pod-2* function is required before fertilization (and the *pod-2* alleles are conditional for function) or *pod-2* function is required after fertilization (and the alleles are conditional for production or accumulation of the protein during oogenesis). Interestingly, similar temperature-shift results were obtained by using conditional alleles of the polarity genes *par-2* and *par-4* (Cheng *et al.*, 1995; Morton *et al.*, 1992). In the case of *par-4*, it was concluded that *par-4* activity is primarily required during late oogenesis. In the case of *par-2*, it was concluded that the gene acts from oogenesis up until interphase of the two-cell embryo. More recently, it has been shown that the conditional allele used in the *par-4* experiments makes stable PAR-4 protein at the nonpermissive temperature (Watts *et al.*, 2000), demonstrating that this allele does not completely inhibit synthesis or stability.

One interpretation of these *pod-2*, *par-2*, and *par-4* data is that these proteins function prior to fertilization, and that the process of polarizing the a-p axis begins in the oocyte. If so, we must reconcile this finding with the fact that the posterior pole of the embryo is determined by sperm entry irregardless of which end it enters the oocyte (Goldstein and Hird, 1996). We speculate that sperm entry might localize signals activated, but not prelocalized, in the oocyte, or that sperm entry might reinforce polarity established in the oocyte. For example, it is possible that these polarity proteins activate a process in the oocyte essential for

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polarity that is asymmetrically localized to the appropriate location following sperm entry. Alternatively, it is possible that these proteins polarize the oocyte, but that this polarity, normally reinforced by sperm entry, can also be reset by the sperm. Such a redundant mechanism would help ensure the fidelity of the polarization process that establishes a major body axis in a very short period of time. Another interpretation of the data is that the PAR-2, PAR-4, and POD-2 proteins function solely after fertilization, and that the conditional alleles tested are all affecting their synthesis or stability.

pod-2 Has a Distinct Role in the Localization of Germ Cell Components and Perhaps in Localizing a Cue that Specifies the Location of the a-p Axis

In 30% of *pod-2* mutant embryos, germline components (PIE-1 and P granules) and cell cycle timing components are segregated into the wrong daughter of P_1, a phenotype never previously described in over 30 mutants analyzed, including 5 *par* mutants (Kemphues *et al.*, 1988; Tenenhaus *et al.*, 1998). To understand the source of this defect, we studied the dynamic localization of PIE-1 in live *ye60* embryos. These studies suggested that the PIE-1 localization defect in the four-cell embryo could be traced back to a misplacement of PIE-1 in the late one-cell embryo. Taken together with our P granule, PIE-1, and DAPI studies in fixed embryos, many of the components that determine germ cell identity are segregating as a unit to a single, but improper, location in one-cell *pod-2* mutant embryos.

Our data suggest that *pod-2* may be needed either to properly localize a spatial cue in the one-cell embryo at the ends of the a-p axis or to properly move components towards that cue. In the *pod-2* mutant, for example, a cue that localizes germline components to the posterior of the one-cell embryo might be mispositioned along a posterior lateral edge such that these components move to a lateral-posterior position and eventually are inherited by the anterior daughter of P_1 (as was seen). Consistent with a mispositioning of an a-p cue, we found that PAR-1, PAR-3, and POD-1 proteins are often localized along a lateral edge of the embryo, as if the polarity cue was positioned laterally instead of at the anterior or posterior pole. To investigate this further, we costained *pod-2(ye60)* embryos with P granule and POD-1 antibodies (data not shown). We found a strong correlation between lateral POD-1 and P granule localization—in 13/14 embryos in which POD-1 was laterally localized, P granules were also laterally localized. Most of the time (9/13), the granules and POD-1 were on opposite sides. That other polarity mutants do not show this type of skewed polarity phenotype suggests that *pod-2*'s role in this process may be more direct than other polarity genes.

pod-2, with *pod-1*, represents a new and important class of embryonic mutants. This class of genes promises to provide essential insights towards understanding the physical mechanism used to generate embryonic polarity since mutations in these genes affect not only polarity but elements of cellular structure as well. In the case of *pod-2*, these elements include formation/function of the eggshell and proper cell division. Given that both *pod* genes function in a pathway and are required for polarity and other structures not directly coupled to polarity, we hypothesize that they perturb a common physical process with multiple functions in the cell. It seems likely that understanding the function of these *pod* genes also will have wide implications as a number of the genes involved in polarization of the *C. elegans* embryo play important roles in cell polarization in other organisms as well.

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