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The maternal gene *spn-4* encodes a predicted RRM protein required for mitotic spindle orientation and cell fate patterning in early *C. elegans* embryos

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SUMMARY

C. elegans **embryogenesis begins with a stereotyped sequence of asymmetric cell divisions that are largely responsible for establishing the nematode body plan. These early asymmetries are specified after fertilization by the widely conserved, cortically enriched PAR and PKC-3 proteins, which include three kinases and two PDZ domain proteins. During asymmetric cell divisions in the early embryo, centrosome pairs initially are positioned on transverse axes but then rotate to align with the anteroposterior embryonic axis. We show that rotation of the centrosomal/nuclear complex in an embryonic cell called P_1 requires a maternally expressed gene we name *spn-4*. The predicted SPN-4 protein contains a single RNA recognition motif (RRM), and belongs to a small subfamily of RRM proteins that includes one *Drosophila* and two human family members. Remarkably, in mutant embryos lacking *spn-4* function the transversely oriented 'P_1' mitotic spindle appears to re-specify the axis of cell polarity, and the division remains asymmetric. *spn-4* also is required for other developmental processes, including the specification of mesendoderm, the restriction of mesectoderm fate to P_1 descendants, and germline quiescence during embryogenesis. We suggest that SPN-4 post-transcriptionally regulates the expression of multiple developmental regulators. Such SPN-4 targets might then act more specifically to generate a subset of the anterior-posterior asymmetries initially specified after fertilization by the more generally required PAR and PKC-3 proteins.**

Key words: Asymmetric cell division, Cell polarity, Centrosome position, Mesectoderm, Mesendoderm, Microtubules, PAR proteins

INTRODUCTION

The asymmetric partitioning of developmental potential to the daughters of polarized cells is a central theme in development. During embryogenesis in the nematode *Caenorhabditis elegans*, the first mitotic division is asymmetric (Sulston et al., 1983). The zygote, a cell called P_0, divides into a larger anterior daughter, AB, and a smaller posterior daughter, P_1 (Fig. 1). Subsequently, P_1 and three of its immediate descendants undergo a sequence of four additional asymmetric divisions that establish much of the nematode body plan (Bowerman and Shelton, 1999).

Each asymmetric division in the early embryo is preceded by a rotation of the nucleus and its associated pair of centrosome-nucleated microtubule asters. These rotations align mitotic spindle axes with the anteroposterior (AP) axis of cell polarity (Hyman and White, 1987; Schierenberg, 1987; Hyman, 1989), and spindle rotation appears to promote the differential segregation of cytoplasmic determinants that become polarized in distribution along the AP axis before division. For example, cytoplasmic ribonucleoprotein particles called P granules, required for germline fate, are segregated to a single germline precursor during each of the four asymmetric divisions that produce the germline progenitor P_4 (Strome and Wood, 1983; Hird et al., 1996; Kawasaki et al., 1998). Before division in P_0 and P_1, P granules move towards the posterior pole and accumulate at the cortex. The P_0 and P_1 centrosome pairs initially are aligned roughly perpendicular to the AP axis, but during prophase they rotate about 90° (Hyman and White, 1987). Thus, the posterior daughters, P_1 and P_2, inherit nearly all the P granules.

Genetic and molecular studies have shown that, in addition to P granules, many cytoplasmic developmental regulators in the early *C. elegans* embryo become polarized in distribution along the AP axis prior to asymmetric divisions and, thus, are differentially segregated to anterior and posterior daughter cells (Guo and Kemphues, 1995; Etemed-Mogadham and Kemphues, 1995; Boyd et al., 1996; Draper et al., 1996; Guedes and Priess, 1996; Mello et al., 1996; Tabuse et al., 1998; Hung and Kemphues, 1999; Schubert et al., 2000). These polarized

regulators include a group of cortically localized kinases and PDZ domain proteins encoded by the *par* genes and the *pkc-3* gene. The widely conserved PAR and PKC-3 proteins are required for the asymmetric division of P_0, and homologues in other organisms also regulate cell polarity (Doe and Bowerman, 2001). In *C. elegans*, the P_0 zygote normally produces two daughters of unequal size that subsequently divide asynchronously with orthogonally oriented mitotic spindles (Fig. 1). In *par/pkc-3* mutant embryos, both P_0 daughters are equal in size and divide synchronously. At the two-cell stage in *par-2* mutant embryos, both mitotic spindles remain transversely oriented, while in *par-3* mutants, both spindles rotate to become longitudinally oriented. In *par-2; par-3* double mutants, as in *par-3* single mutants, both spindles rotate, indicating that PAR-2 and PAR-3 are not required for spindle rotation itself but restrict it to P_1 (Cheng et al., 1995; Hung and Kemphues, 1999).

Asymmetric cell divisions in the early embryo are alike in that mitotic spindles rotate to lie along the AP axis, but each rotation differs in detail. In P_0, cortically localized components of the dynein microtubule motor complex are required for spindle rotation (Skop and White, 1998; Gönczy et al., 1999). By interacting with astral microtubules throughout the cortex, dynein-mediated forces may align the P_0 spindle axis with the longer AP axis of the oblong zygote. In P_1, spindle rotation requires a more localized capture of astral microtubules by a discrete 'remnant' complex, left after the completion of P_0 cytokinesis and marked by a small, persistent ring of microfilaments (Hyman and White, 1987; Hyman, 1989; Waddle et al., 1994). Upon capture of astral microtubules emanating from one pole, the nucleus and its associated centrosomal asters rotate as a complex, in response to force(s) that pull one centrosome towards the remnant site. While apparently intrinsic mechanisms act in P_0 and P_1, spindle rotation in a daughter of P_1 called EMS requires an inductive Wnt signal (Goldstein, 1995; Schlesinger et al., 1999). Thus, at least in part, distinct mechanisms act during each rotation. Consistent with this view, rotation is abnormal at the two-cell stage in *par* and *pkc-3* mutants (see above), but P_0 rotation appears roughly normal (Rose and Kemphues, 1998).

To identify the mechanisms that position mitotic spindles, we used genetic screens to find embryonic-lethal mutants with defects in mitotic spindle orientation. We isolated three mutant alleles of a *C. elegans* gene we name *spn-4*, for spindle-orientation defective-4. The predicted SPN-4 protein contains a single RNA recognition motif (RRM) and is a member of a small subfamily of conserved worm, fly and mammalian RRM proteins. In *spn-4* mutant embryos, the first mitotic division appears normal, but at the two-cell stage, the posterior mitotic spindle fails to rotate and by metaphase re-specifies the cellular axis of polarity. Maternal expression of *spn-4* also is required for at least three other developmental processes. We therefore suggest that the predicted SPN-4 RRM protein post-transcriptionally influences the expression of multiple factors, each of which acts more specifically to regulate developmental pathways in early *C. elegans* embryos.

MATERIALS AND METHODS

Strains and nematode culture

We cultured *C. elegans* as described (Brenner, 1974). N2 Bristol was used as the wild-type strain. The following alleles and balancer chromosomes were used. Linkage group (LG) III *lon-1(e185)*, *par-3(it71)* and qC1 (LGIII inversion balancer: *dpy-19ts*, *glp-1*). LG IV *him-8(e1489)*. LG V *dpy-11(e224)*, *unc-42(e270)*, nT1 (LG IV/V translocation balancer. let-?), DnT1(LG IV/V translocation balancer, Dominant unc-?(n754) let-?), *spn-4* (*or25*, *or80* and *or191ts*). LG X *lin-2(e1309)*. The following strains were used: KK571 *par-3 lon-1*/qC1 III, DR108 *dpy-11 unc-42 V*, EU4 *lin-2 X*, EU923 *spn-4(or191ts) V*, EU588 *him-8(e1489) IV; spn-4(or191ts)* V, EU769 *+/nT1 IV; spn-4(or25) unc42*/nT1 V, EU370 *+/DnT1 IV; spn-4(or25)*/DnT1, EU772 *+/DnT1 IV; spn-4(or80)*/DnT1 and EU918 *par-3 lon-1*/qC1 III; *spn-4(or191ts)*V. The temperature-sensitive allele of *spn-4(or191ts)* was maintained by growing homozygous animals at 15°C. To observe the mutant phenotype, L4 larvae were raised overnight at 25°C before collecting early embryos from young adult hermaphrodites.

The following strains carrying integrated cell-type-specific green fluorescent protein (GFP) reporter transgenes were used to facilitate identification of specific cell types or specific gene expression.

PD4251: *myo-3::gfp* ccIs4251[*myo-3::*GFP]I, GFP expressed in all body wall muscles and vulval muscles (Liu and Fire, 2000; provided by A. Fire, Carnegie Institute of Washington).

JH227: *pie-1::gfp* axEx73[pJH3.92;pRF4], PIE-1::GFP expressed in germ line precursors (Reese et al., 2000; provided by G. Seydoux, Johns Hopkins University School of Medicine).

OK0029: *ceh-22::gfp* cuIs1[*ceh-22::*GFP;pRF4], GFP expressed in pharyngeal muscle cells (Okkema et al., 1997, provided by P. Okkema, University of Illinois).

DP132: *unc-119::gfp* [pDP#MMUGF12;pRF4], GFP expressed in most or all neuronal cells (D. Pilgrim personal communication; provided by D. Pilgrim, University of Alberta).

JR186: *med-1::gfp* wIs93[pMM280;pRF4], GFP expressed in E and MS and their descendants (Maduro et al., 2001; provided by M. Maduro and J. Rothman, University of California, Santa Barbara).

JR553: *end-1*::*gfp* wIs28[*end-1*::GFP::lacZ;pRF4] (Maduro et al., 2001; provided by M. Maduro and J. Rothman, UCSB).

JM62: *elt-2*::GFP wIs84[*elt-2*::GFP, pFR4] (Maduro et al., 2001; provided by J. McGhee, U. Calgary).

HC14: *vab-7::gfp* qtIs1[*vab-7*::GFP; pRF4], GFP expressed in the posterior great-grand-daughters of C (Cxxp cells; C. Hunter, personal communication, provided by C. Hunter, Harvard University).

Isolation of spn-4 alleles

The *or25* and *or80* alleles were isolated in genetic screens for non-conditional maternal-effect embryonic-lethal mutants (Kemphues et al., 1988), while the *or191ts* allele was isolated in genetic screens for temperature-sensitive embryonic-lethal mutants (Encalada et al., 2000). The *or191ts* allele is 100% embryonic lethal when homozygous mothers are raised at 25°C (n=579). At 15°C, 84% of the embryos hatch (555/661). All three mutant alleles were mapped to center of linkage group V using meiotic segregation and recombination to position the alleles relative to previously identified loci (data not shown). The three alleles failed to complement each other in crosses that generated all possible hetero-allelic outcross progeny (data not shown). Linkage group and three-factor mapping were used to position the *or191ts* allele at approximately +0.1149 on chromosome V. From a strain with the genotype *spn-4(or191ts)/dpy-11 unc-42 V*, 27/29 Unc-nonDpy recombinants picked up *spn-4(or191ts)* while 2/29 did not. Of 48 Dpy-nonUnc recombinants, two picked up *spn-4(or191ts)* and 46 did not. The essential requirements for *spn-4* are strictly maternal, as wild-type males crossed with homozygous *spn-4(or80)* hermaphrodites do not rescue the embryonic-lethality (broods with more than 290 embryos were scored; none of 1047 embryos from three broods hatched). To test for zygotic requirements during embryogenesis, broods of embryos were collected from heterozygous *spn-4(or80)*/+ hermaphrodites at room temperature. Nearly all the embryos hatched (1262/1269). 99 progeny

from or80/+ hermaphrodites were followed from hatching until adulthood. 21/99 grew to adulthood but produced broods of dead embryos, the remaining 78 produced viable progeny. No other phenotypes were observed. We conclude that maternal expression is sufficient for all essential *spn-4* requirements. Homozygous *spn-4(or191ts)* L1 larvae raised at 25°C grow to adulthood and produce broods of dead embryos, suggesting there are no zygotic requirements for *spn-4* during post-embryonic development.

Molecular cloning and phylogenetic analysis of spn-4

To clone *spn-4*, we used a combination of germline transformation with wild-type genomic DNA (Mello et al., 1991) and RNA-mediated interference (RNAi; Fire et al., 1998). Germline transformation with a single cosmid, T11B11, rescued the broods of embryos produced by homozygous *spn-4(or191ts)* hermaphrodites at 25°C. The concentration of injected cosmid DNA was 5 μg/ml. A dominant *rol-6* gene was used as marker for transformation by co-injecting the plasmid pRF4 at 100 μg/ml (Mello et al., 1991). Genomic yeast DNA was used as carrier at 100 μg/ml. Eight stable transmitting lines were obtained using cosmid T11B11; in four lines the embryonic lethality of *or191ts* was complemented. We then used RNAi to reduce the function of each predicted gene in T11B11. Microinjecting into the ovaries of wild-type hermaphrodites dsRNA made from a 640 base pair fragment of DNA amplified by PCR from exon 5 of the predicted gene ZC404.8 resulted in embryonic lethality with a *spn-4*-like mutant phenotype. To confirm the gene identity we sequenced ZC404.8 (GenBank Accession Number, CE07598) in the three independently isolated *spn-4* mutant strains. Five fragments of 500-600 bp, overlapping roughly 100 bp, were amplified from genomic DNA using PCR (Williams, 1995). Bands were excised from agarose gels and purified with GeneClean II (Bio 101, La Jolla, CA) and cloned into pGEM-T vector (Promega, Madison, WI). Sequencing was carried out at the University of Oregon DNA sequencing facility, using an ABI 377 Prism automated fluorescent sequencer. Clones from two independent PCR reactions for each allele were sequenced and compared with sequences from *lin-2(e1309)* animals, the parental strain used for mutagenesis. Codon 75 of *or191ts* allele is mutated from GGA to GAA, resulting in a G to E substitution. Codon 91 of the *or25* allele is mutated from GGG to GAG also resulting in a G to E substitution. The sequence of *or80* revealed a 617 bp deletion in the 3′ untranslated region (3′ UTR).

To identify related proteins, we compared sequences in databases using the gapped Blast program (http://www.ncbi.nim.nih.gov). We found two *C. elegans*, one fly, one mouse and two human sequences related to SPN-4. The related proteins exhibit substantial sequence identity within their RRMs, in addition to having conserved residues throughout their length. For phylogenetic analysis, we included the full sequences of SPN-4 and related proteins, and the *Saccharomyces cerevisiae* RRM protein NSR1 was used as the outgroup. Protein sequence alignments and phylogenetic tree construction were done using the MacVector software.

RNA interference

To reduce gene function using RNAi (Fire et al., 1998), dsRNA was made in vitro from cDNA clones and microinjected into wild-type ovaries at a concentration of at least 1 μg/μl. The cDNA clones used and the corresponding genes were the following: *pie-1*, yk613c12; *skn-1*, yk18e11; *pal-1*, yk213g9; *par-3*, yk344b2 (all provided by Yuji Kohara) and *mom-2*, pJC359 (a full-length cDNA cloned into the pGEM-T vector). *spn-4* dsRNA was made from the clone pZG05, a PGEM-T vector containing a 640 bp insert that was amplified from ZC404.8 exon 5, using the T11B11 cosmid as template. RNAi depleted embryos were harvested 16-24 hours post injection.

Immunofluorescence and microscopy

Laser ablations were performed as described (Avery and Horvitz, 1989), using a Zeiss Axioskop equipped with Nomarski DIC optics, a VSL-337 laser (Laser Science) and a MicroPoint fiberoptic laser adaptor (Photonics Instruments). Embryos were collected from a watch glass filled with M9 medium using a mouth pipette, after cutting open young adult hermaphrodites with a scalpel blade. Embryos were transferred to 3% agarose cushions and overlaid with a 22×22 mm glass cover-slip for viewing with Nomarski optics. To calculate the volume of sister blastomeres, the diameter of each blastomere was measured at the end of cytokinesis of the parent cell, spherical shape was assumed for volume calculation (Tabara et al., 1999). Mitotic spindle orientation angles were measured from digital images of intact embryos made using Nomarski DIC optics. Spindle orientations in P_0 and P_1 are shown as the angle made by a line connecting the centrosomes relative to the long (AP) axis of the embryo. Measurements were made just after nuclear envelope breakdown. A similar distribution of spindle orientation defects was observed in embryos mounted within a hanging drop of M9 medium suspended from a glass coverslip mounted over a depression slide. In *spn-4(or80)* mutants, the AB spindle was transversely oriented in 6/8 embryos and was at about 45° in 2/8 embryos; the P_1 spindle was transversely oriented in 2/7 embryos, about 45° in 4/7 and resembled wild type in 1/7 embryos.

Immunostaining was carried out using the following mouse monoclonal antibodies and dilutions: 3NB12 (1:100) to detect a myosin specific for pharyngeal muscle cells (Miller et al., 1983), NE8 4C6.3 (1:100) to detect body wall muscle cells (antibody collection MRC LMB, Cambridge, Kaletta et al., 1997), J126 (1:100) to detect intestinal cells (Mango et al., 1994), Dm1α to detect α-tubulin (Sigma), CS1 to detect MEX-5 (Schubert et al., 2000) and F2A to detect SKN-1 protein (Bowerman et al., 1993). The following rabbit polyclonal antibodies and dilutions were also used: anti-PGL-1 (1:100) to detect P granules (Kawasaki et al., 1998; gift from S. Strome) and anti-PAR-2 (Boyd et al., 1996; gift from K. Kemphues). DNA was stained with DAPI or TOTO (Molecular Probes). Immunostaining with the antibodies J126, 3NB12, NE8 4C6.3 and rabbit anti-PGL-1 was done using the cold methanol/acetone fixation protocol (Bowerman et al., 1993). SKN-1 (Bowerman et al., 1993), PAR-2 (Shelton et al., 1999), MEX-5 (Lin et al., 1995) and α-tubulin (Severson et al., 2000) immunostaining was as described. Birefringent gut granules characteristic of intestinal cells were detected using polarizing optics (Bowerman et al., 1992). Hypodermal cells were identified based on their morphology as observed using Nomarski DIC optics: they have characteristically flattened cell shapes with relatively large smooth nuclei containing a single prominent nucleolus. Digital Nomarski images were acquired using a DAGE MTI VE1000 camera under the control of Scion image software. Digital fluorescence microscopy image acquisition was performed using either a BioRad MRC 1024 confocal microscope, or a Zeiss Axioskop equipped for epifluorescence with a Micromax EBF512 cooled CCD camera (Roper Scientific).

RESULTS

Rotation of the P_1 mitotic spindle requires an RRM protein

To identify genes required for mitotic spindle orientation, we screened mutagenized populations of worms for embryonic-lethal mutants with cell fate patterning and/or cell division defects (Materials and Methods). In one conditional mutant and two non-conditional mutants, the posterior mitotic spindle at the two-cell stage frequently fails to rotate prior to cell division (Fig. 1). These recessive, maternal-effect mutant alleles define a single genetic locus we call *spn-4* (Materials and Methods). The essential requirements for *spn-4* are strictly maternal, and we have not detected any zygotic requirements

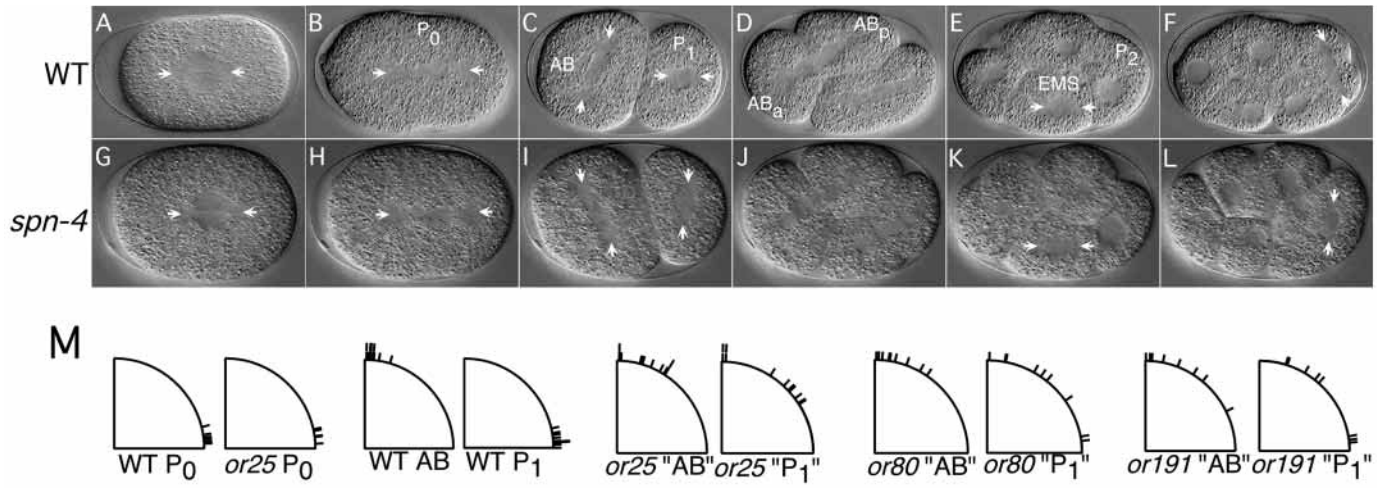

Fig. 1. Nomarski photomicrographs of wild type (A-F) and *spn-4(or80)* (G-L) embryos. Photographs of wild-type and *spn-4* embryos were taken at equivalent time points. Arrows point at centrosomes, and cell names are indicated. (M) Schematic drawings illustrate spindle angles observed at the one-cell and two-cell stages in wild-type and *spn-4* embryos (see Materials and Methods). Each dash at the periphery of a quadrant represents a single scored embryo. In wild type, P_0 spindle angles range from 4° to 14° (n=6), the AB spindle angles range from 74° and 89°, and the P_1 spindle angles from 0° to 12° (n=11). In *spn-4* mutant embryos, the following ranges were observed: *or25* 'P_0' varied from 3°-12° (n=5), *or25* 'AB' and *or25* 'P_1' varied from 58°-89° and 32°-89° (n=11); *or80* 'AB' and *or80* 'P_1' varied from 61°-89° and 7°-89° (n=8); *or191ts* 'AB' and *or191ts* 'P_1' varied from 26°-90° and 7°-72° (n=8).

(Materials and Methods). In all three mutant strains, spindle positioning is normal at the one-cell stage, and although the posterior two-cell stage spindle fails to rotate, embryonic cell divisions remain asymmetric (Fig. 1).

After identifying *spn-4*, we used genetic and molecular methods to determine that this locus corresponds to the predicted gene ZC404.8 (Materials and Methods). We sequenced ZC404.8 in *spn-4* mutants and found mis-sense mutations in the alleles *or25* and *or191ts* (Fig. 2A), and a 617 base pair 3′UTR deletion in *or80* (Materials and Methods). Reducing ZC404.8 function using RNA interference (RNAi; see Materials and Methods) results in a mutant phenotype nearly identical to that caused by our strongest mutant allele. We therefore have used RNAi, in addition to mutant alleles, to define requirements for *spn-4*.

The predicted ZC404.8 open reading frame encodes a 351 amino acid protein with a single RNA recognition motif (Fig. 2A), the most common RNA binding motif known (Haruhiko and Dreyfuss, 1997; Varani and Nagai, 1998). BLAST searches show that SPN-4 is a member of a small subfamily of RRM proteins (Fig. 2; see Discussion). The mis-sense mutations in *spn-4(or25)* and *spn-4(or191ts)* affect highly conserved residues in the RRM (Fig. 2A).

We conclude that the SPN-4 RRM protein may act indirectly to regulate spindle rotation by post-transcriptionally modulating the expression of protein(s) that participate more directly.

spn-4 is required for mitotic spindle rotation in *par-3* mutant embryos

In two-cell stage *spn-4* mutants, both mitotic spindles remain transversely oriented, as in *par-2* mutant embryos (see Introduction). In contrast to *par-2* mutants, though, the first division is unequal in *spn-4* embryos, and the larger anterior daughter divides before the smaller posterior daughter, as in wild type (Fig. 1). Thus, *spn-4* appears to be required more specifically than is *par-2* for rotation in P_1. To further compare the roles of the *par-2* and *spn-4*, we examined spindle orientation in *par-3; spn-4* double mutants. In contrast to *par-3* mutants and *par-2; par-3* double mutants (see Introduction), both mitotic spindles fail to rotate in most *par-3; spn-4* double mutant embryos (Fig. 3). In other respects, the *par-3; spn-4* double mutants resemble *par-3* mutants: the first division is equal and the two daughters divide synchronously (Fig. 3).

We conclude that PAR-2 and PAR-3 have earlier and more general roles in the regulation of anterior-posterior asymmetry, and that SPN-4 acts downstream of, or in parallel to the PAR proteins to more specifically regulate spindle rotation in P_1.

Early embryonic cells still divide asymmetrically in *spn-4* mutant embryos

Although the posterior mitotic spindle at the two-cell stage in a *spn-4* mutant fails to rotate, the cell in which it resides nevertheless divides asymmetrically. In wild-type embryos the volume of the anterior daughter, EMS, is two to three times the volume of the posterior daughter, P_2 (n=11; Materials and Methods), and EMS divides before P_2 (Fig. 1E,F). In *spn-4* mutant embryos the same relative volumes are maintained (*or25* n=6, *or80* n=5, *or191ts* n=6), and the 'P_1' descendants divide asynchronously with roughly normal spindle orientation (Fig. 1). Thus *spn-4* is required for rotation specifically in P_1 and not in other asymmetrically dividing embryonic cells.

We also examined other asymmetries that normally appear during the division of P_1, including the segregation of cytoplasmic P granules to germline precursors. In wild-type embryos, P granules move posteriorly prior to the asymmetric division of germline precursors (see Introduction). In *spn-4* mutant embryos, P-granule segregation in P_0 appears normal. Early in mitosis at the two-cell stage in *spn-4* mutants, during prophase in 'P_1', P granules again appear normal, being enriched at the posterior cortex (Fig. 4E,F; n=12). However, by pro-metaphase, P granules are localized laterally, near one

pole of the transversely oriented posterior mitotic spindle (7/8 embryos). In all two-cell stage *spn-4* embryos fixed during metaphase (n=3; Fig. 4H) or during anaphase or telophase (n=6), P granules were always centered around one spindle pole. In four-cell stage embryos, P granules were present only in the smaller daughter of 'P_1' (Fig. 4I,J; n=18). In embryos allowed to develop in culture medium after removal of their eggshells at the two-cell stage, P granules were present in only a single cell at the four-cell stage in wild-type (n=4) and in *spn-4* mutant embryos (n=3). Therefore constraints imposed by the eggshell on spindle orientation do not appear to influence asymmetric segregation of P granules in *spn-4* embryos.

We next examined the distribution of PAR-2 in *spn-4* mutant embryos. As in wild type, we found that PAR-2 is restricted to the posterior cortex in P_0 (J. E. G., data not shown) and initially in 'P_1' as well (n=6; Fig. 4A,B). However, PAR-2 redistributes when the P_1-like cell enters mitosis, forming by metaphase or anaphase a laterally positioned cortical crescent centered around one spindle pole (n=7; Fig. 4D).

As a final assay for cell polarity in *spn-4* mutant embryos, we examined the distribution of MEX-5, a CCCH finger protein that becomes enriched in the anterior cytoplasm of dividing germline precursors; MEX-5 and a nearly identical protein MEX-6 are required to restrict PIE-1 and other proteins to posterior cytoplasm (Schubert et al., 2000). As in wild type, MEX-5 was present at higher levels in 'AB' than in 'P_1' in all fixed two-cell stage *spn-4* mutant embryos (n=10), and we also detected MEX-5 at higher levels in 'EMS' than in 'P_2' in four-cell stage embryos (n=12).

We conclude that the P_1-like cell still divides asymmetrically and correctly segregates many cell fate determinants in *spn-4* mutants, at least in part because the mitotic spindle can reorient the axis of cell polarity.

spn-4 is required to specify endoderm fate

After determining that the division of the P_1-like cell in *spn-4* mutant embryos remains asymmetric, we further assessed its development by examining the differentiated cell types it produces. In a wild-type embryo, P_1 divides to produce a posterior mesectoderm and germline precursor called P_2, and a ventral mesendoderm precursor called EMS (Sulston et al., 1983; see Fig. 1). To assess EMS fate in *spn-4* mutants, we first examined the production of intestinal cells. In a wild-type embryo, the posterior daughter of EMS, called E, makes all 20 of the

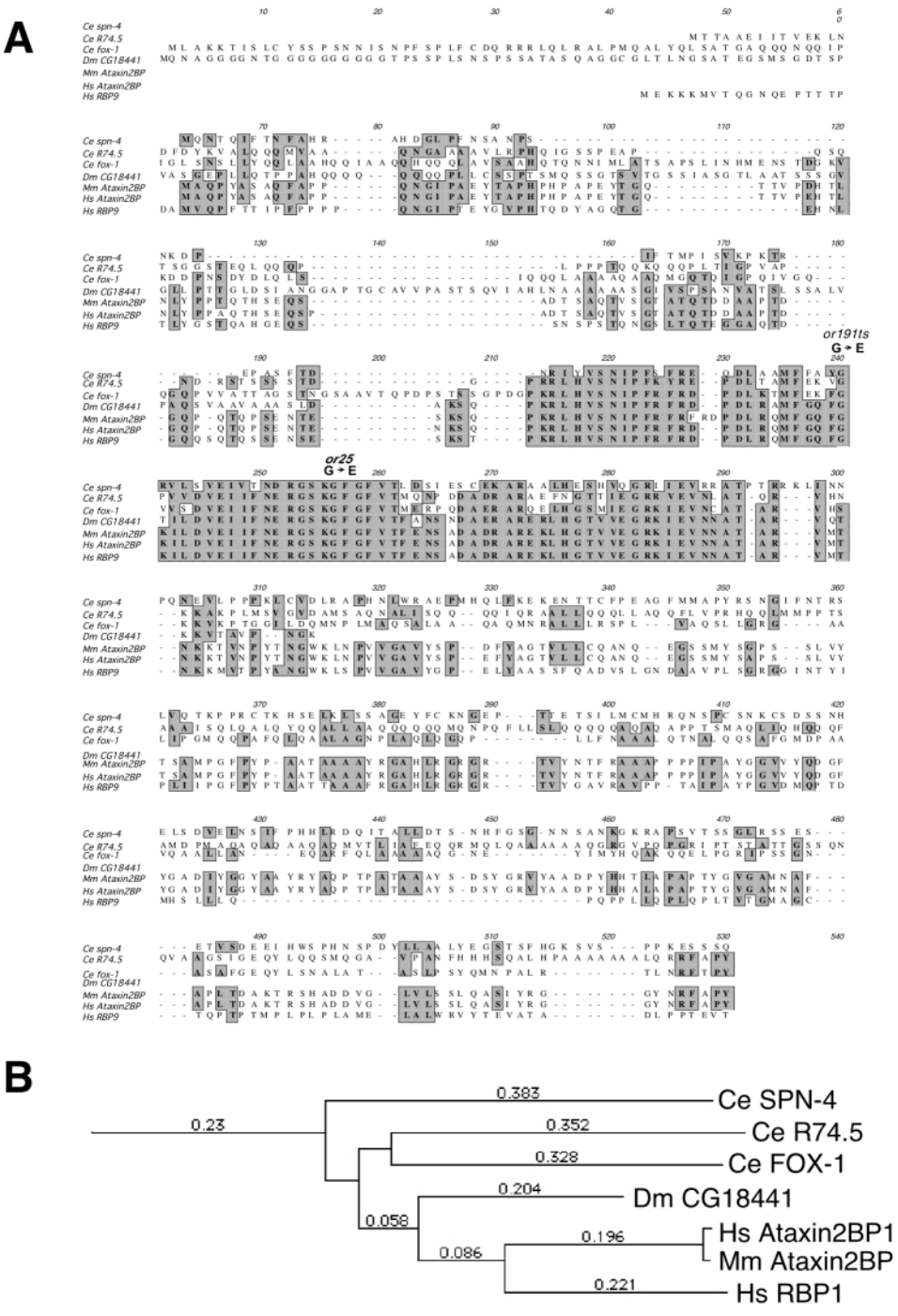

Fig. 2. (A) Sequence alignment of full-length SPN-4 and related *Drosophila*, mouse and human proteins. Grey indicates regions of similarity. The mutations found in *or25* and *or191ts* both result in G to E substitutions at highly conserved positions in the RRM (amino acids 213 to 300). (B) A molecular phylogeny based on the sequence similarities among SPN-4 related proteins. The length of a branch is proportional to the number of amino acid changes; SPN-4 is the most diverged member.

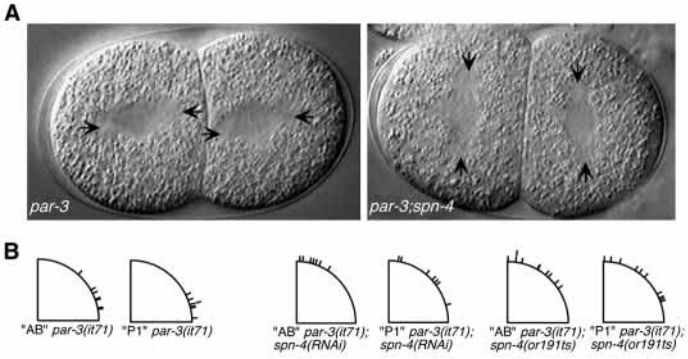

Fig. 3. *spn-4* is required for spindle rotation in two-cell stage *par-3* mutant embryos. (A) Nomarski photomicrographs of *par-3(it71)* and *par-3(it71);spn-4(RNAi)* mutant embryos. Arrows point at the centrosomes. In *par-3* embryos, both cells divide with longitudinally oriented spindles. In *par-3; spn-4* double mutant embryos, both divide with transversely oriented spindles. In both *par-3* and *par-3; spn-4* embryos the two cells are similar in size and divide synchronously. (B) Schematic depiction of mitotic spindle orientations in two-cell stage in *par-3* and *par-3; spn-4* mutant embryos. In *par-3* mutants, spindle angles range from 3° to 48° (n=7). Two allelic combinations of *par-3; spn-4* double mutants were analyzed. In *par-3(it71); spn-4(RNAi)* spindle orientation ranges from 16° to 87° (n=7), in *par-3(it71);spn-4(or191ts)* from 20° to 89° (n=10).

intestinal cells produced in *C. elegans*. Using polarizing optics to score terminally differentiated embryos for the presence or absence of birefringent gut granules, we determined that only 43% of *spn-4(RNAi)* mutant embryos (41/95) and 59% of *spn-4(or80)* embryos (83/140) make intestinal cells. About 80% of *spn-4(or25)* embryos (66/89), and nearly all *spn-4(or191ts)* embryos (81/84) make gut. Similarly, using an antibody that recognizes a surface antigen on intestinal cells, we found that 39% of *spn-4(or80)* embryos (47/121) produce positively staining cells (Fig. 5A; see Materials and Methods). Even though the 'P_1' spindle remains transversely oriented in *spn-4* mutants, one daughter is always larger, and the larger daughter always divides first, at least in part along the AP axis, to produce a posterior E-like daughter (Fig. 1). We used a laser micro-beam to kill this E-like cell in *spn-4(RNAi)* and *spn-4(or80)* mutant embryos and found that no gut granules were made (n=9 and n=13, respectively). Thus endoderm potential is properly segregated to one 'P_1' granddaughter in those *spn-4* embryos that make intestinal cells, in spite of the spindle rotation defect at the two-cell stage.

Finally, in an attempt to further reduce gene function, we injected *spn-4* dsRNA into homozygous *spn-4(or80)* hermaphrodites, but this did not change the fraction of embryos that made gut granules (38/86 *or80RNAi* embryos, or 42%) relative to *spn-4(RNAi)* embryos (43%; see above). We conclude that the large 3′UTR deletion in *spn-4(or80)* strongly reduces gene function, and RNA interference may completely eliminate it.

spn-4 is required for the specification of pharyngeal mesoderm

We next examined the fate of the anterior daughter of EMS, called MS, which normally produces the posterior part of the pharynx, a neuromuscular feeding organ in the head of a nematode. Two 12-cell stage anterior embryonic cells called ABara and ABalp produce the rest of the pharynx in a wild-type embryo (Sulston et al., 1983), in response to signal(s) from MS (Mango et al., 1994). We used a monoclonal antibody that recognizes a pharyngeal myosin to score the production of pharyngeal muscle cells in fixed, terminally differentiated *spn-4* mutant embryos (see Materials and Methods). We found that 83% of *spn-4(or80)* mutant embryos (77/93) fail to produce any pharyngeal muscle cells (Fig. 5A), while the remainder make only two to five cells, instead of the 39 made by a wild-type embryo (Sulston et al., 1983). Similarly, using a transgenic strain that expresses green fluorescent protein (GFP) in pharyngeal muscle cells (Materials and Methods), we found

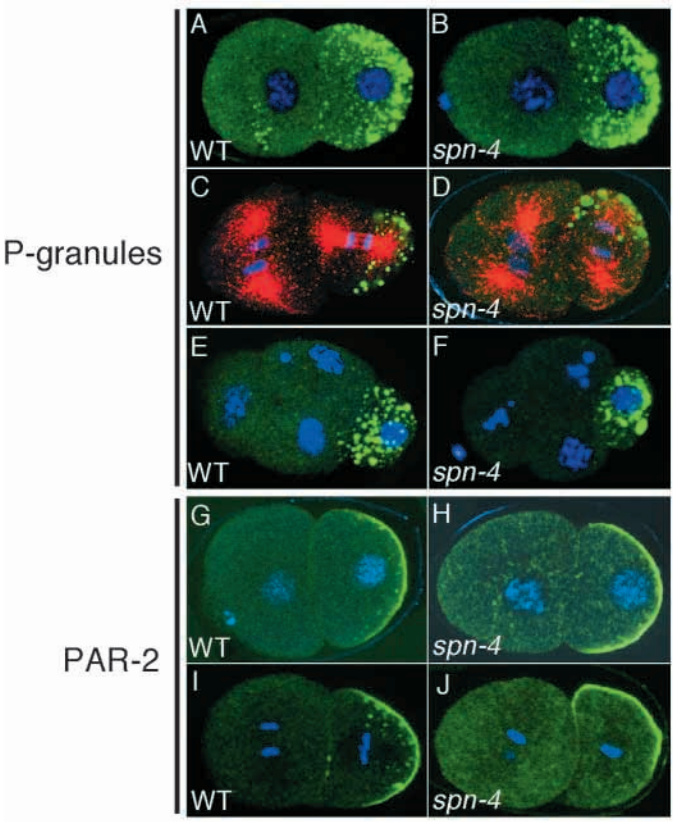

Fig. 4. Confocal photomicrographs of PAR-2 and P-granule immunofluorescence in wild type and *spn-4* mutant embryos. P granules (green) are segregated to one daughter in the first two cell divisions in fixed wild-type (A,C,E) and *spn-4(or80)* embryos (B,D,F). In wild-type embryos and *spn-4(or80)* embryos, P granules are restricted to the posterior cortex during prophase in P_1 (A,B). P granules are shifted laterally by anaphase in a *spn-4(or80)* mutant embryo (D). Note the linear arrays of P granules, particularly in the wild-type embryo (C). Thus, P granules may associate with astral microtubules, although most MTs appear not to have survived fixation in these embryos. During P_1 prophase in wild-type and *spn-4(or80)* embryos, PAR-2 (green) is restricted to the posterior cortex of P_1 (G,H). By metaphase, PAR-2 remains posterior in wild type (I). In *spn-4(or80)* embryos, PAR-2 shifts laterally to form a crescent centered around one pole of the 'P_1' spindle (J). Microtubules (C,D) are red, DNA is blue.

that 73% of *spn-4(RNAi)* mutant embryos (91/124) fail to make any pharyngeal muscle, and the remainder (33/124) make only a few GFP-positive cells.

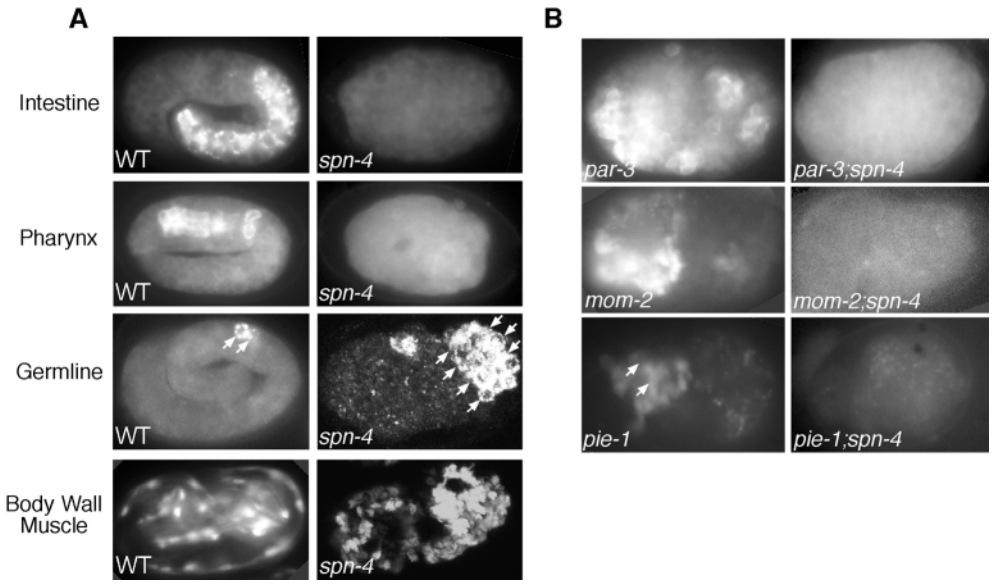

Fig. 5. (A) *spn-4* embryos fail to produce intestinal and pharyngeal muscle cells, but make extra body wall muscle and germline. Fluorescence micrographs of terminally differentiated wild-type (left column) and *spn-4* mutant embryos (right column). Intestinal cells were detected with the monoclonal antibody (mAb) J126; pharyngeal muscle cells with the mAb 3NB12; germline cells with polyclonal antibodies that recognize the P-granule component PGL-1; body wall muscle cells with a myo-3::GFP transgenic line. Micrographs of germline and body wall muscle cells in *spn-4* embryos were obtained using confocal microscopy, the remaining micrographs were obtained using epifluorescence. See the text for numbers of embryos examined.
(B) *spn-4* is required for specifying pharyngeal muscle in *par-3*, *mom-2* and *pie-1* mutants. In *par-3(it71)* and *par-3(it71);spn-4(or191ts)* embryos, pharyngeal muscle cells were detected with the mAb 3NB12; in *mom-2* and *pie-1* RNAi mutant embryos pharyngeal muscle cells were detected using the GFP reporter *ceh-22::GFP*. In each case, only background staining is detected in the double mutant embryos. All micrographs obtained using epifluorescence microscopy. See the text for numbers of embryos examined.

In summary, most *spn-4* mutant embryos fail to produce both MS-derived and AB-derived pharyngeal muscle cells, while a smaller fraction of *spn-4* mutants fail to make E-derived intestinal cells.

spn-4 is required for the ectopic specification of pharynx in mutants that fail to restrict SKN-1 function to EMS

The loss mesendoderm in *spn-4* embryos resembles the mutant phenotype observed in embryos lacking SKN-1, a maternally expressed transcription factor required for EMS to produce pharyngeal and intestinal cells (Bowerman et al., 1992) and for MS to induce pharyngeal cell production by AB descendants (Shelton and Bowerman, 1996). Other maternally expressed proteins limit SKN-1 function to EMS, and SKN-1 is required for the ectopic production of pharyngeal cells and intestinal cells in all mutants that fail to limit mesendoderm fate to EMS (Bowerman, 1998). To further compare their requirements, we asked if *spn*-4, like *skn-1*, is required for the ectopic specification of pharyngeal and intestinal cells in *par-3*, *pie-1* and *mom-2* mutants (Fig. 5B). In *par-3* mutant embryos, several AP fate asymmetries are lost (Kemphues et al., 1988; Etemad-Moghadam and Kemphues, 1995), and all four-cell stage blastomeres can produce SKN-1-dependent pharyngeal mesoderm (Bowerman et al., 1997). We found that while *par-3(it71)* embryos nearly always produce large numbers of pharyngeal muscle (61/66 embryos), most *par-3(it71); spn-4(or191ts)* embryos fail to produce any pharyngeal muscle (75/87; Fig. 5B; see Materials and Methods). In *pie-1* mutant embryos, both EMS and its sister P_2 adopt EMS-like fates, producing twice the normal number of pharyngeal and intestinal cells. PIE-1 is a putative transcriptional repressor that is localized to germline precursors, including P_2, where it prevents SKN-1 from specifying an EMS-like fate (Mello et al., 1992; Mello et al., 1996; see Fig. 6). We can detect large numbers of intestinal and pharyngeal cells in nearly all differentiated *pie-1(RNAi)* mutants (35/35 and 33/35, respectively), while most *pie-1(RNAi); spn-4(RNAi)* double mutant embryos fail to produce any intestinal or pharyngeal cells (59/87 and 75/87, respectively; Fig. 5B). In *mom-2* mutant embryos, both EMS daughters adopt MS-like fates and produce extra pharynx at the expense of intestine (Thorpe et al., 1997; Rocheleau et al., 1997). However, no pharyngeal muscle cells are made by most *mom-2(RNAi); spn-4(RNAi)* double mutant embryos (139/157 or 89%, Fig. 5B). We conclude that like the transcription factor SKN-1, the SPN-4 RRM protein is required to specify mesendoderm fate in *C. elegans*.

Mesendoderm is replaced by PAL-1-dependent mesectoderm in *spn-4* mutants

In *skn-1* mutants, the two daughters of 'EMS' develop like C, a daughter of P_2 that produces mesectoderm (Bowerman et al., 1992). We therefore asked if 'EMS' in *spn-4(RNAi)* embryos also produces C-like daughters. In a wild-type embryo, C produces 13 epidermal cells, 32 body wall muscle cells and two neurons (Sulston et al., 1983). To examine EMS fate in *spn-4(RNAi)* embryos, we used a laser micro-beam to kill all four-cell stage blastomeres except 'EMS' in embryos from one of two transgenic strains. One strain expresses GFP specifically in body wall muscle cells and the other expresses GFP in neurons (Materials and Methods). Most isolated *spn-4* mutant 'EMS' blastomeres (13/18) produced at least 10-20 body wall muscle cells, although 3/18 failed to produce any GFP-positive cells. In 9/9 operated *spn-4* embryos, 'EMS' produced 1-3 GFP-positive neuronal cells. In all cases, the isolated precursors appeared to produce numerous differentiated descendants with morphologies characteristic of epidermal cells (data not shown; Materials and Methods). These cell fate transformations are consistent with EMS, or its daughters,

adopting C-like fate(s) in *spn-4* mutant embryos. To further test this hypothesis, we examined the activity of a maternally expressed homeodomain protein called PAL-1 that is required to specify C fate (Hunter and Kenyon, 1996). Normally, PAL-1 activates the expression of another homeodomain protein called VAB-7, which is first detectable in half of the great grand-daughters of C (Ahringer, 1997). We used a laser microbeam to kill embryonic cells in *spn-4(RNAi)* and wild-type embryos, using a strain that expresses a *vab-7::GFP* transgene (Materials and Methods). As expected, we detected no VAB-7::GFP expression after killing P_2 in wild-type embryos (9/9). However, after killing all cells but 'EMS' in *spn-4(RNAi)* embryos, roughly half of the descendants of this cell express VAB-7::GFP (9/9). No VAB-7::GFP-positive cells were detected in 28/29 *pal-1(RNAi);spn-4(RNAi)* double mutant embryos. We conclude that in *spn-4* mutant embryos, as in *skn-1* mutants, the daughters of EMS adopt C-like fates as a result of ectopic PAL-1 activity in EMS and its descendants.

In wild-type four-cell stage embryos, both SKN-1 and PAL-1 are present at high levels in the nucleus of EMS, but only SKN-1 appears active (Bowerman et al., 1993; Hunter and Kenyon, 1996). Because PAL-1 is active in EMS in *spn-4* mutant embryos, we asked if the normal role of SPN-4 might be to negatively regulate PAL-1 in EMS and thereby permit the specification of mesendoderm by SKN-1. We reasoned that if SPN-4 blocks PAL-1 function in EMS, then depleting PAL-1 function in *spn-4* mutant embryos should restore the production of SKN-1-dependent pharyngeal cells. We therefore stained fixed *spn-4(or80); pal-1(RNAi)* double mutants with an antibody that recognizes pharyngeal muscle (see Materials and Methods). We found that, as in *spn-4(or80)* single mutants, most *spn-4(or80); pal-1(RNAi)* double mutants fail to produce any pharyngeal muscle (81/91 embryos), while the remainder (10/91) make only one to five cells.

We conclude that the failure to specify pharyngeal cell fates in *spn-4* mutant embryos does not result from the ectopic activity of PAL-1 in EMS, and that SPN-4 does not simply prevent PAL-1 from functioning in EMS.

SPN-4 is not required to restrict the function of PIE-1 to P_2, or for SKN-1 to activate MED-1 expression in EMS

To account for the lack of mesendoderm in *spn-4* mutant embryos, we considered three additional explanations: (1) mis-localization of PIE-1, a negative regulator of SKN-1, might prevent mesendoderm specification; (2) SKN-1 might not accumulate to sufficiently high levels to specify EMS fate; (3) *spn-4* might be required to specify EMS fate independent of SKN-1 and PIE-1 function. In wild-type four-cell stage embryos, SKN-1 accumulates to high levels in both P_2 and EMS, but not in the anterior blastomeres ABa and ABp (Bowerman et al., 1993; see Fig. 6). At the same time, PIE-1 accumulates to high levels only in P_2, where it blocks SKN-1 function (Mello et al., 1996; Seydoux et al., 1996; see Fig. 6). Because the mitotic spindle in 'P_1' fails to rotate in *spn-4* mutant embryos, we initially hypothesized that PIE-1 might be mis-localized to both 'P_1' daughters and thus prevent SKN-1 from specifying mesendoderm fate in either cell. However, as we observed with P granules and with PAR-2 (see above), the P_1-like cell in a *spn-4* mutant embryo still divides asymmetrically, and nuclear PIE-1::GFP is detectable only in its smaller daughter (n=12; Fig. 6D). Because PIE-1 localization appears normal in *spn-4* mutant embryos, and because *pie-1; spn-4* double mutants fail to produce mesendoderm (Fig. 5B), we conclude that mis-localization of PIE-1 function cannot account for the lack of mesendoderm in *spn-4* mutants.

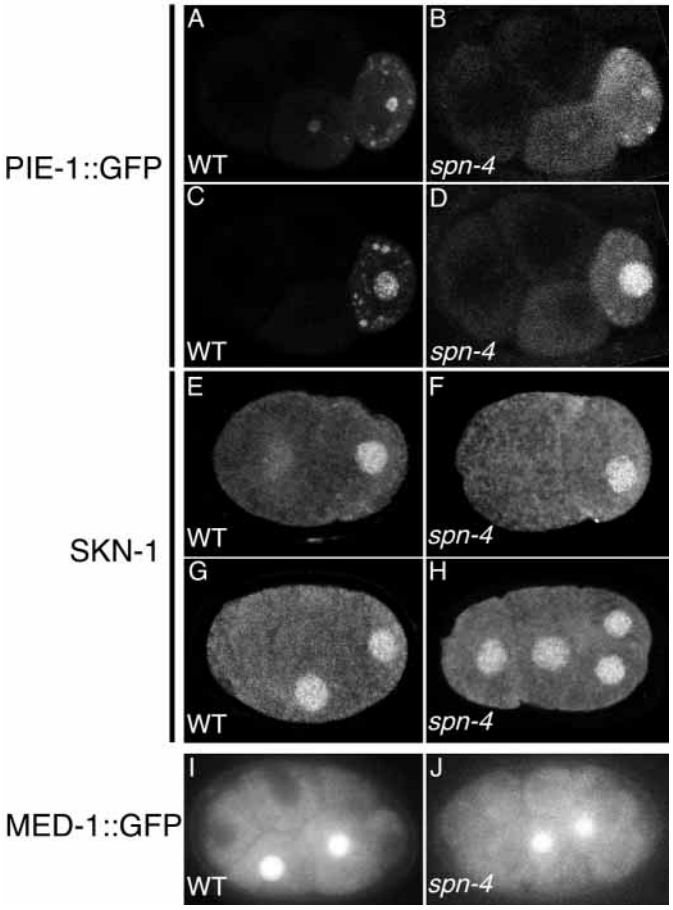

Fig. 6. PIE-1, SKN-1 and MED-1 expression in *spn-4* embryos. PIE-1::GFP is segregated to the posterior daughter of P_1 at the 4-cell stage in live wild-type embryos (A,C) and in live *spn-4(RNAi)* embryos (B,D). PIE-1::GFP localizes to centrosomes during mitosis (A,B) and to nuclei in older four-cell stage embryos (C,D). In wild-type embryos, SKN-1 protein was detected with a mAb at high levels in P_1 and its daughters (E,G). In fixed 2-cell stage *spn-4(or80)* mutant embryos, SKN-1 is present at high levels in P_1 in some embryos (F; see text). At the four-four cell stage, SKN-1 is present only in P_1 descendants in fixed wild-type embryos (G). SKN-1 is present at high levels throughout a fixed *spn-4(or80)* embryo (H). In live wild-type embryos, MED-1::GFP is expressed zygotically in the two daughters of EMS. In live *spn-4(RNAi)* embryos MED-1::GFP in the two daughters of the anterior daughter of P_1 (F). PIE-1 and SKN-1 micrographs were obtained using confocal microscopy, MED-1::GFP micrographs were obtained using epifluorescence microscopy.

We next asked if SPN-4 is required for the normal expression of SKN-1, using a monoclonal antibody to stain fixed *spn-4* mutant embryos (Fig. 6F,H; see Materials and Methods). In 5/10 two-cell stage *spn-4* embryos, SKN-1 appeared normal, with detectable levels confined to 'P_1'. However, SKN-1 is undetectable in some two-cell stage *spn-4*

mutant embryos (5/10), while we always detect SKN-1 in P_1 at the two-cell stage in wild-type (n=10). To our surprise, high levels of SKN-1 are present throughout four-cell stage *spn-4* mutants (8/9 embryos), instead of being confined to the two daughters of 'P_1' (1/9), as in wild type (9/9 embryos). In summary, the accumulation of SKN-1 in 'P_1' may be delayed in *spn-4* embryos, but *spn-4* also is required to prevent high levels of SKN-1 from accumulating in AB daughters at the four-cell stage.

Because SKN-1 levels in EMS appear normal in *spn-4* mutant embryos, we asked if SKN-1 can activate the zygotic transcription of a downstream target gene called *med-1*, which encodes a GATA transcription factor required zygotically for mesendoderm fate (Maduro et al., 2001). MED-1 expression requires both SKN-1 and SKN-1-binding sites in the *med-1* promoter, and ectopic expression of SKN-1 is sufficient to drive ectopic expression of a MED-1::GFP translational fusion (Maduro et al., 2001). To determine if SPN-4 and SKN-1 act in the same pathway, or in different pathways required for mesendoderm fate, we examined the expression of MED-1::GFP in *spn-4(RNAi)* embryos. Intriguingly, we found that MED-1::GFP expression is normal in *spn-4(RNAi)* embryos, appearing at the four-cell stage in EMS and remaining on in EMS descendants (15/15 embryos; Fig. 6J). We also examined the expression of END-1 and ELT-2, two additional GATA transcription factors required for endoderm fate that are expressed in E descendants later in embryogenesis and act downstream of MED-1 (Zhu et al., 1997; Zhu et al., 1998; Fukushige et al., 1998**)**. Using two transgenic strains that express GFP driven by the *end-1* and *elt-2* promoters (Material and Methods), we detected no *end-1* driven GFP expression in all 19 *spn-4(RNAi)* embryos analyzed. Similarly, we detected *elt-2* driven GFP expression in only 19/101 *spn-4(RNAi)* embryos (data not shown). Although *spn-4(RNAi)* mutant embryos produce intestinal cells much more frequently than we detect *end-1* or *elt-2* driven GFP expression, all GFP-positive embryos did produce intestinal cells (data not shown). These results suggest that SPN-4 acts in parallel to SKN-1, in pathways that may converge downstream of MED-1 but upstream of END-1 and ELT-2.

As a final test of how *spn-4* and *skn-1* might interact to promote mesendoderm fate, we examined the production of intestinal cells in *spn-4(RNAi); skn-1(RNAi)* double mutants. Reducing or eliminating either *spn-4* or *skn-1* function results in partially penetrant losses of endoderm: 57% of *spn-4(RNAi)* embryos, 80% of *skn-1(zu67)* and 70% (26/38) of *skn-1(RNAi)* mutant embryos fail to make gut (Bowerman et al., 1992; data in this paper). All *spn-4(RNAi); skn-1(RNAi)* double mutants fail to produce birefringent gut granules (n=66). The increased penetrance of the endoderm defect in the double mutant is consistent with SPN-4 and SKN-1 acting in distinct, parallel pathways to promote the specification of endoderm fate.

The germline proliferates abnormally in *spn-4* mutant embryos

To further characterize the P_1-like cell in *spn-4* mutant embryos, we next scored the cell types produced by its P_2-like daughter. In a wild-type embryo, P_2 divides asymmetrically to produce another germline precursor called P_3 and a larger somatic daughter called C. P_3 divides into the body wall muscle precursor D and the germline progenitor P_4, while C produces skin and body wall muscle and two neurons (Sulston et al., 1983). In *spn-4(RNAi)* embryos, isolated P_3 and C blastomeres produced body wall muscle cells (5/5 and 9/9 operated embryos, respectively), and 5/5 isolated 'P_2' blastomeres produced a normal pattern of VAB-7::GFP expression, as in wild-type embryos.

Although the descendants of P_2 produce roughly normal patterns of cell fate, *spn-4* mutant embryos appear to make extra germline (Fig. 5A). In nearly all terminally differentiated *spn-4(or80)* mutant embryos (61/62), we detected at least six to eight P-granule-positive cells, in contrast to the two produced by a wild-type embryo (Sulston et al., 1983). To determine the origin of the excess germline, we examined P granules segregation in more detail. In wild-type embryos, P granules are sequentially segregated to the germline progenitor P_4, and then to both P_4 daughters, Z_2 and Z_3 (Fig. 7), which then remain quiescent until larval development (Sulston et al, 1983). In *spn-4* embryos, P granules are still segregated to P_3 -like (8/8) and P_4-like (13/13) cells. The P_4-like cell then divides prematurely to produce two P granule-positive cells (7/7; Fig. 7). These results suggest that the extra germline we detect in terminally differentiated *spn-4* mutants (Fig. 5A) reflects a loss of germline quiescence during embryogenesis, rather than an ectopic specification of germline fate. We also examined expression of the germline protein PIE-1 in more detail. When the P_2-like daughter in a *spn-4* mutant divides, PIE-1::GFP is segregated to a single daughter, as in wild type (5/5 embryos). However, when this P_3-like cell divides, PIE-1::GFP is not detectable in either daughter in most embryos (11/13). In 2/13 embryos, PIE-1::GFP was detected only transiently in one of the two 'P_3' daughters. Normally, PIE-1 is detectable in P_4 until it divides and is detected transiently in both P_4 daughters (Mello et al., 1996; Seydoux and Strome, 1999).

In summary, development of the P_2-like cell in a *spn-4* mutant embryo is largely normal, except for a premature decline in PIE-1 levels and an apparent loss of germline quiescence.

Anterior blastomeres in *spn-4* mutant embryos ectopically produce PAL-1-dependent body wall muscle

In addition to lacking intestine and pharynx and making extra germline, large numbers of body wall muscle cells are present throughout most terminally differentiated *spn-4* mutant embryos (Fig. 5A). As 80/81 body wall muscle cells are made by P_1 in a wild-type embryo (Sulston et al., 1983), the distribution of body wall muscle in *spn-4* embryos suggest**s** that both two-cell stage blastomeres might be making body wall muscle. To determine the origin of the muscle cells in *spn-4* mutants, we used a laser micro-beam to isolate 'P_1' and 'AB' in *spn-4(RNAi)* embryos from a transgenic line that expresses GFP in body wall muscle (Materials and Methods). In most operated embryos, both posterior (11/11) and anterior (8/11) two-cell stage blastomeres produced at least 10-20 GFP-positive cells. Thus anterior blastomeres in *spn-4* mutant embryos produce ectopic body wall muscle, indicating that *spn-4* is required to restrict muscle specification to descendants of P_1.

Ectopic specification of body wall muscle in AB descendants also occurs in mutants that fail to restrict either

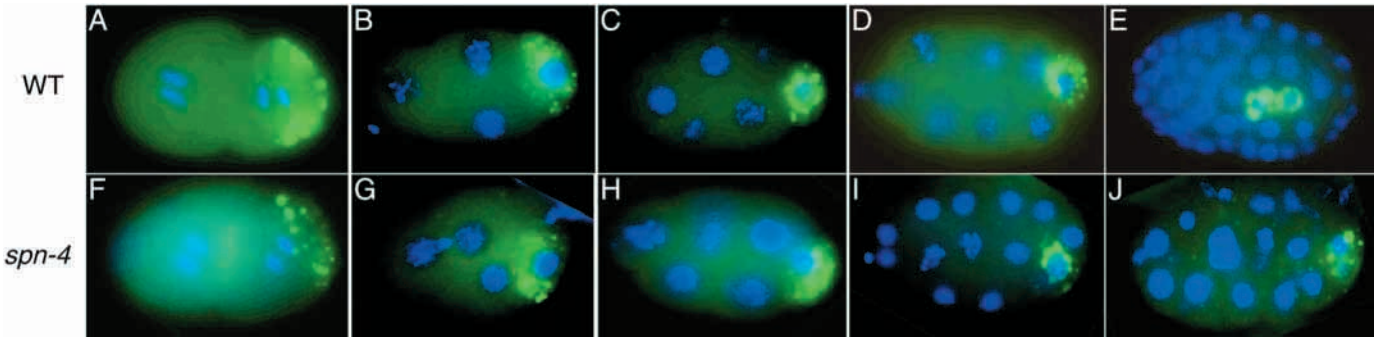

Fig. 7. P granules distribution in *spn-4* mutant embryos. In wild-type embryos P granules are segregated to the germline precursors P_1 (A), P_2 (B), P_3 (C), P_4 (D) and finally the two daughters of P_4, Z_2 and Z_3 (E). In *spn-4(or80)* embryos, P granules also are segregated to a single daughter during each of the first four divisions that normally produce the germline progenitor P_4 (F-J). However, in most terminally differentiated *spn-4(or80)* embryos, at least six positively staining cells are detected (see Fig. 5A). During anaphase in two-cell stage embryos, P granules localize at the cortex near one pole of the posterior mitotic spindle in both wild-type embryos (A) and *spn-4(or80)* mutant embryos (F). Note the linear arrays of P-granules radiating out from the spindle pole in (A) and especially in (F), as in Fig. 4 (C,D). All images were obtained using epifluorescence microscopy.

SKN-1 or PAL-1 to P_1 descendants (Bowerman, 1998). In *mex-1* mutant embryos, SKN-1 is present at high levels in all four-cell stage blastomeres, and 'AB' descendants produce ectopic, SKN-1-dependent pharyngeal and body wall muscle (Mello et al., 1992; Bowerman et al., 1993). In *mex-3* mutant embryos, PAL-1 is present at high levels in all four-cell stage blastomeres, and 'AB' descendants produce ectopic, PAL-1-dependent skin and body wall muscle (Draper et al., 1996; Hunter and Kenyon, 1996). Because *spn-4* mutant embryos fail to produce pharyngeal and intestinal cells, it seemed unlikely that mis-localization of SKN-1 (Fig. 6) could account for the ectopic production of body wall muscle by AB descendants. As expected, when we used a laser micro-beam to isolate 'AB' in *skn-1(RNAi); spn-4(RNAi)* double mutants, 9/10 operated embryos still produced numerous GFP-positive body wall muscle cells. By contrast, after staining fixed terminally differentiated embryos with a cell type-specific antibody (Materials and Methods), we found that 35% (28/80) of *spn-4(or80); pal-1(RNAi)* double mutant embryos failed to produce any body wall muscle cells. Thus body wall muscle production in *spn-4* mutant embryos appears largely dependent on PAL-1. Consistent with PAL-1 functioning ectopically in AB descendants, we also detected expression of a PAL-1-dependent VAB-7::GFP fusion protein in descendants of 'AB' in 9/9 operated *spn-4(RNAi)* embryos in which 'P_1' was killed with a laser micro-beam.

To summarize, in addition to resembling *skn-1* mutant embryos in lacking mesendoderm, *spn-4* mutants also resemble *mex-3* mutants in failing to restrict PAL-1 activity, and hence mesectoderm fate, to P_1 descendants.

DISCUSSION

Our analysis of the *spn-4* locus suggests that the encoded RRM protein influences multiple developmental pathways early in *C. elegans* embryogenesis. The posterior two-cell stage mitotic spindle fails to align with the AP axis, mesendoderm is usually absent, mesectoderm is specified ectopically throughout the embryo, developmental regulators are mis-localized, and the germline proliferates abnormally. These defects may reflect requirements for *spn-4* in multiple, independent processes. For example, the spindle rotation defect does not appear responsible for the loss of mesendoderm, given the normal segregation of PIE-1 and other germline factors, the general requirement for SPN-4 in other mutants that produce mesendoderm ectopically and the more normal development of mesendoderm in other spindle orientation-defective mutants (Zwaal et al., 1996; Rose and Kemphues, 1998). To influence these different developmental processes, we suspect that SPN-4 regulates multiple more specifically acting regulatory factors.

SPN-4 belongs to a small subfamily of RRM proteins that has three members in *C. elegans*, one in *Drosophila* and two in humans. One of the three family members in *C. elegans* is FOX-1, which acts post-transcriptionally to prevent expression of XOL-1, a key regulator of dose compensation and sex determination in *C. elegans* (Nicoll et al., 1997). Thus it seems likely that SPN-4 also acts post-transcriptionally, presumably by binding and regulating the expression of target RNAs. Although no analysis of the single *Drosophila* locus has been reported, one of the two human family members, A2BP1, binds Ataxin 2. Glutamine-expansion mutations in Ataxin 2 are associated with spinocerebellar ataxia (Shibata et al., 2000).

The role of *spn-4* in mitotic spindle rotation

Although *spn-4* appears to regulate multiple developmental processes, to our knowledge *spn-4* mutants exhibit the most specific defect in P_1 spindle rotation yet described. We do observe some anterior spindle orientation defects at the two-cell stage, particularly in *spn-4(or191ts)* embryos, curiously the weakest allele with respect to the endoderm defect. However, the posterior rotation defect is substantially more severe and more highly penetrant, particularly in *spn-4(or25)* and *spn-4(or80)* embryos. Other maternal genes involved in spindle rotation at the two-cell stage appear to act less directly or less specifically. The PAR and PKC-3 proteins spatially restrict but are not required for mitotic spindle rotation in two-cell stage embryos (Rose and Kemphues, 1998; see Introduction). Mutational inactivation of three additional genes (*let-99*, *ooc-3* and *ooc-5*), and RNAi depletion of heterotrimeric G protein subunits and a protein called RIC-8, each result in spindle orientation defects at the two-cell stage

(Zwaal et al., 1996; Rose and Kemphues, 1997; Basham and Rose, 1999; Gotta and Ahringer, 2000; Miller and Rand, 2000; Pichler et al., 2000). In *let-99* mutant embryos, the 'AB' spindle orientation defects are more severe and more penetrant than those in *spn-4* mutants, and P_0 spindle rotation is partially defective (Rose and Kemphues, 1997). Furthermore, PAR-2 distribution is normal in *let-99* mutant embryos, while P granules are mis-localized to both 'P_1' daughters. The *let-99* gene encodes a novel protein. Embryos lacking heterotrimeric G protein subunits show defects in centrosome migration and in P_0 spindle positioning, in addition to spindle orientation defects in both P_1 and in AB descendants (Zwaal et al., 1996; Gotta and Ahringer, 2000; Miller and Rand, 2000). Both *ooc-3* and *ooc-5* single mutants exhibit a highly penetrant failure in 'P_1' spindle rotation, but P granules are mis-localized to both daughters and PAR-2 is depleted or absent (Basham and Rose, 1999; Pichler et al., 2000). Moreover, *ooc-3* and *ooc-5* mutants also have defects in oogenesis and in P_0 spindle rotation, and in *ooc-3; ooc-5* double mutants, both two-cell stage mitotic spindles are frequently mis-oriented. The *ooc-3* gene encodes a novel protein that localizes to the endoplasmic reticulum, suggesting a possible link between protein trafficking and spindle rotation (Pichler et al., 2000). In summary, *spn-4* mutants exhibit a relatively specific defect in P_1 spindle rotation, and thus far only in *spn-4* mutants does the mitotic spindle appear capable of re-orienting the axis of cell polarity.

Rotation of the nucleus and centrosomes in P_1 appears to require the capture of astral microtubules by factors present at the remnant site left upon the completion of cytokinesis when P_0 divides (Hyman, 1989; Waddle et al., 1994). Capture of either pole is thought to be sufficient for proper rotation (Hyman, 1989), and the minus-end-directed microtubule motor protein dynein, and associated dynactin components, localize to this site (Gönczy et al., 1999; Skop and White, 1998). In a wild-type embryo, rotation in P_1 is preceded by anterior movement of the centrosomal/nuclear complex towards the remnant site. In *spn-4* mutants, anterior movement of the nucleus appears normal (data not shown), but spindle rotation fails or is only partially complete. Thus, *spn-4* may be required not for the initial capture of astral microtubules but later and more specifically for rotation itself. Similarly, in two-cell stage *ooc-3* and *ooc-5* mutant embryos, the posterior nucleus moves anteriorly but fails to rotate (Basham and Rose, 1999). These findings indicate that the capture and rotation machinery may be complex and involve different kinds of force application and movement.

The mitotic spindle and cell polarity

Even when spindle rotation fails completely, the division of the posterior blastomere in two-cell stage *spn-4* embryos remains highly asymmetric. The smaller daughter develops much like a wild-type P_2, except for abnormal proliferation of the germline. As in a wild-type embryo, the EMS-like cell in *spn-4* embryos divides along the a-p axis, before its P_2-like sister, although it usually fails to produce the pharyngeal and intestinal cells made by EMS in a wild-type embryo. When *spn-4* embryos do make endoderm, the posterior daughter of the EMS-like cell produces it, as in wild type. The surprising lack of polarity defects in *spn-4* mutant embryos suggests that spindle rotation is not essential for the proper segregation of many cell fate determinants during the asymmetric division of P_1.

To explain the persistence of asymmetry during the division of 'P_1' in *spn-4* embryos, we suggest that the poles of the mitotic spindle become polarized and, in the absence of rotation, re-specify the cellular axis of polarity. We propose this model based on our observations that both P granules and PAR-2 redistribute by late in the two-cell stage to form a more transverse axis of polarity, and on our results showing that the asymmetric segregation of PIE-1 and MEX-5 appear normal in *spn-4* mutants. Previous studies have shown that two distinct mechanisms contribute to P granule localization. P granules move towards the cortex at one pole of asymmetrically dividing cells, and P granules left behind are unstable (Hird et al., 1996). In addition to directional movement and degradation contributing to polarity prior to division, we suggest that the mitotic spindle in P_1 maintains polarity during cell division. Such a role for the P_1 spindle may be particularly important when, owing to constraints imposed by the eggshell, the P_1 and AB spindles adopt variably skewed axes as they elongate during anaphase (see Fig. 1). Intriguingly, P granules in wild-type and *spn-4* mutant embryos frequently appear to form linear arrays that project out from one P_1 spindle pole (Figs 4, 7). Based on inhibitor studies, P-granule localization to the posterior cortex in P_0 does not appear to require microtubules (Strome and Wood, 1983; Hill and Strome, 1988), although proper P-granule localization in P_2 does (Hird et al., 1996). Based on our results, we suggest that astral microtubules might be important for maintaining an axis of polarity in P_1.

The apparent ability of the P_1 mitotic spindle in *spn-4* mutants to re-specify an axis of cell polarity is surprising because previous experiments have suggested that random capture of either spindle pole by the cytokinetic remnant at the anterior cortex appears sufficient to align the P_1 spindle along the AP axis (Hyman, 1989). For example, the remnant site is capable of capturing the opposite spindle pole after the original connection to one pole is severed with a laser micro-beam, although the developmental consequences of such manipulations were not reported (Hyman, 1989). If spindle poles become polarized such that cytoplasmic factors are directed to one pole, either polarization occurs after the normal time of astral microtubule capture and spindle rotation, or capture is not random. A more detailed genetic and molecular analysis of this process should improve our understanding of these issues.

Precedent for mitotic spindles having a role in cell polarity has come from studies of asymmetrically dividing *Drosophila* neuroblasts (Doe and Bowerman, 2001). Mutations in genes that regulate neuroblast polarity, such as Inscuteable and Pins, cause cortical proteins to be mis-localized early in neuroblast cell division. However, by late in mitosis, these cortical proteins become localized to one pole and the neuroblasts divide asymmetrically, a phenomenon referred to as 'telophase rescue' (Peng et al., 2000). Thus in both *Drosophila* neuroblasts and in the early *C. elegans* embryo, mitotic spindles may be capable of maintaining and even re-directing axes of cell polarity. In neither case, however, is it known whether the spindle poles are in fact responsible for re-specifying the axis of polarity. For example, in *Drosophila* neuroblasts it is possible that the cortical proteins become polarized and then orient the mitotic spindle (Peng et al., 2000). To address this issue more conclusively in *C. elegans*, we are currently analyzing microtubule requirements using GFP

translational fusions to visualize the microtubule cytoskeleton and P granules in live embryos.

spn-4 as one mediator of asymmetries more generally specified by the PAR proteins

SPN-4 appears to mediate the establishment of a subset of the asymmetries specified more generally by the earlier acting, cortically localized PAR and PKC-3 proteins. Consistent with this model, *spn-4* mutant embryos retain most of the cell size, cell cycle and cell fate asymmetries observed in wild-type embryos, while *par* mutant embryos suffer widespread, nearly complete losses of these early asymmetries. Moreover, *spn-4* is required for mitotic spindle rotation at the two-cell stage in *par-3* mutant embryos, and PAR-2 is localized normally in one-cell and pre-prometaphase two-cell stage *spn-4* mutant embryos. These results indicate that SPN-4 acts either downstream of, or in parallel to PAR-2 and PAR-3 to regulate P_1 mitotic spindle rotation, but not all asymmetries specified by the PAR proteins.

Our view that SPN-4 mediates a subset of the asymmetries specified by the PAR proteins is similar to the role proposed for MEX-5 and MEX-6, two nearly identical and partially redundant proteins that each contain two CCCH finger motifs (Schubert et al., 2000). MEX-5 is present at high levels in the anterior cytoplasm of asymmetrically dividing germline precursors. PAR-1 at the posterior cortex restricts MEX-5 to the anterior cytoplasm in P_0, while MEX-5 restricts a group of three additional CCCH regulatory proteins to the posterior cytoplasm. The mechanism by which MEX-5 and MEX-6 act is unknown, although mammalian CCCH fingers have been implicated in RNA binding (Bai and Tolias, 1996; Barabino et al., 1997; Lai et al., 2000). Perhaps SPN-4 regulates different target RNAs through a post-transcriptional mechanism distinct from that used by MEX-5 and MEX-6. Consistent with SPN-4 and MEX-5/6 acting in distinct pathways, MEX-5 localization is unaffected by mutations in SPN-4.

SPN-4 and mesectoderm specification in *C. elegans*

Beyond its role in spindle positioning, *spn-4* is required for proper cell fate patterning in the early embryo. One such pathway affected by mutational inactivation of *spn-4* is the specification of mesectoderm by the homeodomain protein PAL-1 (Hunter and Kenyon, 1996). PAL-1 is present at high levels in P_1 descendants, but not in AB descendants. The KH domain protein MEX-3 is present at high levels in anterior blastomeres and appears to restrict translation of PAL-1 to posterior blastomeres, perhaps by binding 3′UTR sequences in *pal-1* mRNA through two KH motifs (Draper et al., 1996; Hunter and Kenyon, 1996). In *mex-3* mutant embryos, PAL-1 is present at high levels in the descendants of both P_1 and AB, and the AB descendants ectopically produce PAL-1-dependent body wall muscle. Intriguingly, SPN-4 may influence PAL-1 through MEX-3, as SPN-4 has been identified as a MEX-3-interacting protein (N. Huang and C. Hunter, personal communication). Thus SPN-4 may have important protein partners in addition to presumed RNA targets.

Although *spn-4* and *mex-3* both restrict PAL-1 function to P_1 descendants, *spn-4* and *mex-3* mutants exhibit different germline phenotypes. In *mex-3* mutant embryos, P_3 divides equally and both daughters, instead of just one, adopt a germline fate, producing extra germline progenitors (Draper et al., 1996). In *spn-4* mutants, a single germline progenitor appears to be produced but its two daughters apparently proliferate instead of remaining quiescent during the rest of embryogenesis. Perhaps the premature decline in PIE-1 levels we detect in the *spn-4* germline lineage is responsible for the loss of germline quiescence.

Multiple maternal inputs are required to specify mesendoderm in *C. elegans*

SPN-4 also influences at least one other early developmental pathway, the specification of mesendoderm at the four-cell stage in EMS. In this process, SPN-4 appears to act independently of the transcription factor SKN-1, shown previously to be required for mesendoderm fate specification. Like *skn-1*, *spn-4* is required for the specification of mesoderm and endoderm in mutants that produce these cell types ectopically. Moreover, SKN-1 is expressed at roughly normal levels in *spn-4* mutant embryos, and *med-1*, a target gene regulated by SKN-1, is activated in EMS. Curiously, even though SKN-1 is present at high levels in all four-cell stage blastomeres, MED-1 expression in *spn-4* mutant embryos is limited to EMS descendants. Presumably SPN-4 regulates the expression of other factors that either contribute directly to the specification of mesendoderm, or somehow provide a permissive environment for the specification of mesendoderm fate by the transcription factors SKN-1 and MED-1.

The maternal contributions to the specification of mesendoderm in *C. elegans* appear complex. In addition to SKN-1 and SPN-4, a CCCH finger protein called POS-1 also is required to specify the fate of the mesendoderm precursor EMS (Tabara et al., 1999). Intriguingly, SPN-4 is the same as the POS-1 binding protein, PIP-1 (K. Ogura and Y. Kohara, personal communication). Thus, SPN-4 and POS-1 may act in one pathway that contributes to mesendoderm fate specification, or they might integrate independent inputs. In addition to SPN-4, SKN-1 and POS-1, the specification of endoderm also requires a Wnt signal (Rocheleau et al., 1997; Thorpe et al., 1997) and the input of a MAP kinase-related pathway (Meneghini et al., 1999; Rocheleau et al., 1999). How these different maternal inputs interact remains largely unknown. The identification of SPN-4 targets, and the targets of other maternal regulators, should help clarify our understanding of mesendoderm development in *C. elegans*.

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REFERENCES

Ahringer, J. (1997). Maternal control of a zygotic patterning gene in *Caenorhabditis elegans*. *Development* **124**, 3865-3869.

Avery, L. and Horvitz, H. R. (1989). Pharyngeal pumping continues after laser killing of the pharyngeal nervous system of *C. elegans*. *Neuron* **3**, 473-485.

Bai, C. and Tolias, P.P. (1996). Cleavage of RNA hairpins mediated by a developmentally regulated CCCH zinc finger protein. *Mol. Cell. Biol.* **16**, 6661-6667.

Barabino, S.M., Hubner, W., Jenny, A., Minvielle-Sebastia, L. and Keller, W. (1997). The 30-kD subunit of mammalian cleavage and polyadenylation specificity factor and its yeast homolog are RNA-binding zinc finger proteins. *Genes Dev*. **11**, 1703-1716

Basham, S. E. and Rose, L. S. (1999). Mutations in *ooc-5* and *ooc-3* disrupt oocyte formation and the reestablishment of asymmetric PAR protein localization in two-cell *Caenorhabditis elegans* embryos. *Dev. Biol.* **215**, 253-263.

Bowerman, B. (1998). Maternal control of pattern formation in early *Caenorhabditis elegans* embryos. *Curr. Top. Dev. Biol.* **39**, 73-117.

Bowerman, B., Eaton, B. A. and Priess, J. R. (1992). *skn-1*, a maternally expressed gene required to specify the fate of ventral blastomeres in the early *C. elegans* embryo. *Cell* **68**, 1061-1075.

Bowerman, B., Draper, B. W., Mello, C. C. and Priess, J. R. (1993). The maternal gene *skn-1* encodes a protein that is distributed unequally in early *C. elegans* embryos. *Cell* **74**, 443-452.

Bowerman, B., Ingram, M. K. and Hunter, C. P. (1997). The maternal *par* genes and the segregation of cell fate specification activities in early *Caenorhabditis elegans* embryos. *Development* **124**, 3815-3826.

Bowerman, B. and Shelton, C. A. (1999). Cell polarity in the early *Caenorhabditis elegans* embryo. *Curr. Opin. Genet. Dev.* **9**, 390-395.

Boyd, L., Guo, S., Levitan, D., Stinchcomb, D. T. and Kemphues, K. J. (1996). PAR-2 is asymmetrically distributed and promotes association of P granules and PAR-1 with the cortex in *C. elegans* embryos. *Development* **122**, 3075-3084.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans. Genetics* **77**, 71-94.

Cheng, N. N., Kirby, C. M. and Kemphues, K. J. (1995). Control of cleavage spindle orientation in *Caenorhabditis elegans*: the role of the genes *par-2* and *par-3*. *Genetics* **139**, 549-559.

Doe, C. Q. and Bowerman B. (2001). Asymmetric cell division: fly neuroblast meets worm zygote. *Curr. Opin. Cell Biol.* **13**, 68-75.

Draper, B. W., Mello, C. C., Bowerman, B., Hardin, J. and Priess, J. R. (1996). The maternal gene *mex-3* encodes a KH domain protein and regulates blastomere identity in early *C. elegans* embryos. *Cell* **87**, 205-216.

Encalada, S. E., Martin, P. R., Phillips, J. B., Lyczak, R., Hamill, D. R., Swan, K. A. and Bowerman, B. (2000). DNA replication defects delay cell division and disrupt cell polarity in early *Caenorhabditis elegans* embryos. *Dev. Biol.* **228**, 225-238.

Etemad-Moghadam, Guo, S. and Kemphues, K. J. (1995). Asymmetrically distributed PAR-3 protein contributes to cell polarity and spindle alignment in early *C. elegans* embryos. *Cell* **83**, 743-752.

Fire, A., Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806-811.

Fukushige T., Hawkins, M. G. and McGhee, J. D. (1998). The GATA-factor ELT-2 is essential for formation of the *Caenorhabditis elegans* intestine. *Dev. Biol.* **198**, 286-302.

Goldstein, B. (1995). Cell contacts orient some cell division axes in the *Caenorhabditis elegans* embryo. *J. Cell Biol.* **129**, 1071-1080.

Gönczy, P., Pichler, S., Kirkham, M. and Hyman, A. A. (1999). Cytoplasmic dynein is required for distinct aspects of MTOC positioning, including centrosome separation, in the one cell stage *Caenorhabditis elegans* embryo. *J. Cell Biol.* **147**, 135-150.

Gotta, M. and Ahringer, J. (2000). Distinct roles for Gα and Gβγ in regulating spindle position and orientation in *Caenorhabditis elegans* embryos. *Nat. Cell Biol* **3**, 297-300.

Guedes, S. and Priess, J. R. (1996). The *C. elegans* MEX-1 protein is present in germline blastomeres and is a P granule component. *Development* **124**, 731-739.

Guo, S and Kemphues, K. J. (1995). *par-1*, a gene required for establishing polarity in *C. elegans* embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. *Cell* **81**, 611-620.

Haruhiko, S. and Dreyfuss, G. (1997). RNA-binding proteins as regulators of gene espression. *Curr. Opin. Genet. Dev.* **7**, 345-353.

Hill, D. P. and Strome, S. (1988). An anlysis of the role of microfilaments in the establishment and maintenance of asymmetry in *Caenorhabditis elegans* zygotes. *Dev. Biol.* **125**, 75-84.

Hird, S. N., Paulsen, J. E. and Strome, S. (1996). Segregation of germ granules in living *Caenorhabditis elegans* embryos: cell-type-specific mechanisms for cytoplasmic localization. *Development* **122**, 1303-1312.

Hung, T.-J. and Kemphues, K. J. (1999). PAR-6 is a conserved PDZ domain-containing protein that colocalizes with PAR-3 in *Caenorhabditis elegans* embryos. *Development* **126**, 127-135.

Hunter, C. P. and Kenyon, C. (1996). Spatial and temporal controls target *pal-1* blastomere-specification activity to a single blastomere lineage in *C. elegans* embryos. *Cell* **87**, 217-226.

Hyman, A. A. (1989). Centrosome movement in the early divisions of *Caenorhabditis elegans*: a cortical site determining centrosome position. *J. Cell Biol.* **109**, 1185-1194.

Hyman, A. A. and White, J. G. (1987). Determination of cell division axes in the early embryogenesis of *Caenorhabiditis elegans*. *J. Cell Biol.* **105**, 2123-2135.

Kaletta, T., Schnabel, H. and Schnabel, R. (1997). Binary specification of the embryonic lineage in *Caenorhabditis elegans*. *Nature* **390**, 294-298.

Kawasaki, I., Shim, Y.-H., Kirchner, J., Kaminker, J., Wood, W. B. and Strome, S. (1998). PGL-1, a predicted RNA-binding component of germ granules, is essential for fertility in *C. elegans*. *Cell* **94**, 635-645.

Kemphues, K. J., Priess, J. R., Morton, D. G. and Cheng, N. S. (1988). Identification of genes required for cytoplasmic localization in early *C. elegans* embryos. *Cell* **52**, 311-320.

Lai, W. S., Carballo, E., Thorn, J. M., Kennington, E. A. and Blackshear, P. J. (2000). Interactions of CCCH zinc finger proteins with mRNA. Binding of tristetraprolin-related zinc finger proteins to Au-rich elements and destabilization of mRNA. *J. Biol. Chem.* **275**, 17827-17837.

Lin, R., Thompson, S. and Priess, J. R. (1995). *pop-1* encodes an HMG box protein required for the specification of a mesoderm precursor in early *C. elegans* embryos. *Cell* **83**, 599-609.

Liu, J. and Fire, A. (2000). Overlapping roles of two Hox genes and the *exd* ortholog *ceh-20* in diversification of the *C. elegans* postembryonic mesoderm. *Development* **127**, 5179-5190.

Maduro, M. F., Meneghini, M. D., Bowerman, B., Broitman-Maduro, G. and Rothman, J. H. (2001). Restriction of mesendoderm to a single blastomere by the combined action of SKN-1 and a GSK-3ß homolog is mediated by MED-1 and -2 in *C. elegans*. *Mol. Cell* **7**, 475-485.

Mango, S. E., Thorpe, C. J., Martin, P. R., Chamberlain, S. H. and Bowerman, B. (1994). Two maternal genes, *apx-1* and *pie-1*, are required to distinguish the fates of equivalent blastomeres in early *C. elegans* embryos. *Development* **120**, 2305-2315.

Mello, C. C., Kramer, J. M., Stinchcomb, D. and Ambros, V. (1991). Efficient gene transfer in *C.elegans*: extrachromosomal maintenance and integration of transforming sequences. *EMBO J.* **10**, 3959-3970.

Mello, C. C., Draper, B. W., Krause, M., Weintraub, H. and Priess, J. R. (1992). The *pie-1* and *mex-1* genes and maternal control of blastomere identity in early *C. elegans* embryos. *Cell* **70**, 163-176.

Mello, C. C., Schubert, C., Draper, B., Zhang, W., Lobel, R. and Priess, J. R. (1996). The PIE-1 protein and germline specification in *C. elegans* embryos. *Nature* **382**, 710-712.

Meneghini, M. D., Ishitani, T., Carter, J. C., Hisamoto, N., Ninomiya-Tsuji, J., Thorpe, C. J., Hamill, D. R., Matsumoto, K. and Bowerman, B. (1999). MAP kinase and Wnt pathways converge to downregulate an HMG-domain repressor in *Caenorhabditis elegans*. *Nature* **399**, 793-798.

Miller, D. M. I., Ortiz, I., Berliner, G. C. and Epstein, H. F. (1983). Differential localization of two myosins within nematode thick filaments. *Cell* **34**, 477-790.

Miller, K. G. and Rand, J. B. (2000). A role for RIC-8 (Synembryn) and GOA-1 ($G_o\alpha$) in regulating a subset of centrosome movements during early embryogenesis in *Caenorhabditis elegans*. *Genetics* **156**, 1649-1660.

Nicoll, M., Akerib, C. C. and Meyer, B. J. (1997). X-chromosome-counting mechanisms that determine nematode sex. *Nature* **388**, 200-204.

Okkema, P. G., Ha, E., Haun, C., Chen, W. and Fire, A. (1997). The *Caenorhabditis elegans* NK-2 homeobox gene *ceh-22* activates pharyngeal muscle gene expression in combination with *pha-1* and is required for normal pharyngeal development. *Development* **124**, 3965-3973.

Peng, C. Y., Manning, L. Albertson, R. and Doe, C. Q. (2000). The tumour-suppressor genes *lgl* and *dlg* regulate basal protein targeting in *Drosophila* neuroblasts. *Nature* **408**, 596-600.

Pichler, S., Gönczy, P., Schnabel, H., Pozniakowski, A., Ashford, A., Schnabel, R. and Hyman, A. A. (2000). OOC-3, a novel putative transmembrane protein required for establishment of cortical domains and spindle orientation in the P_1 blastomere of *C. elegans* embryos. **127**, 2063-2073.

Reese, K. J., Dunn, M. A., Waddle, J. A. and Seydoux, G. (2000). Asymmetric segregation of PIE-1 in *C. elegans* is mediated by two

complementary mechanisms that act through separate PIE-1 protein domains. *Mol. Cell* **6**, 445-455.

Rocheleau, C. E., Downs, W. D., Lin, R., Wittmann, C., Bei, Y., Cha, Y. H., Ali, M., Priess, J. R. and Mello, C. C. (1997). Wnt signaling and an APC-related gene specify endoderm in early *C. elegans* embryos. *Cell* **90**, 707-716.

Rocheleau, C. E., Yasuda, J., Shin, T. H., Lin, R., Sawa, H., Okano, H., Priess, J. R., Davis, R. J. and Mello, C. C. (1999). WRM-1 activates the LIT-1 protein kinase to transduce anterior/posterior polarity signals in *C. elegans*. *Cell* **97**, 717-726.

Rose, L. S. and Kemphues, K. J. (1997). The *let-99* gene is required for proper spindle orientation during cleavage of the *C. elegans* embryo. *Development* **125**, 1337-1346.

Rose, L. S. and Kemphues, K. J. (1998). Early patterning of the *C. elegans* embryo. *Ann. Rev. Genet.* **32**, 521-545.

Schierenberg, E. (1987). Reversal of cellular polarity and early cell-cell interaction in the embryo of *Caenorhabditis elegans*. *Dev. Biol.* **122**, 452-463.

Schlesinger, A., Shelton, C. A., Maloof, J. N., Meneghini, M. and Bowerman, B. (1999). Wnt pathway components orient a mitotic spindle in the early *C. elegans* embryo without requiring gene transcription in the responding cell. *Genes Dev.* **13**, 2028-2038.

Schubert, C. M., Lin, R., de Vries, C. J., Plasterk, R. H. and Priess, J. R. (2000). MEX-5 and MEX-6 function to establish soma/germline asymmetry in early *C. elegans* embryos. *Mol. Cell* **5**, 671-682.

Severson, A. F., Hamill, D. R., Carter, J. C., Schumacher, J. and Bowerman, B. (2000). The Aurora-related kinase AIR-2 recruits ZEN-4/CeMKLP1 to the mitotic spindle at metaphase and is required for cytokinesis. *Curr. Biol.* **10**, 1162-1171.

Seydoux, G. and Strome, S. (1999). Launching the germline in *Caenorhabditis elegans*: regulation of gene expression in early germ cells. *Development* **126**, 3275-3283.

Seydoux, G., Mello, C. C., Pettitt, J., Wood, W. B., Priess, J. R. and Fire, A. (1996). Repression of gene expression in the embryonic germ lineage of *C. elegans*. *Nature* **382**, 713-716.

Shelton, C. A. and Bowerman, B. (1996). Time-dependent responses to *glp-1*-mediated inductions in early *C. elegans* embryos. *Development* **122**, 2043-2050.

Shelton, C. A., Carter, J. C., Ellis, G. C. and Bowerman, B. (1999). The nonmuscle myosin regulatory light chain gene mlc-4 is required for cytokinesis, anterior-posterior polarity, and body morphology during *Caenorhabditis elegans* embryogenesis. *J. Cell Biol.* **146**, 439-451.

Shibata H., Huynh, D. P. and Pulst, S. M. (2000). A novel protein with RNA-binding motifs interacts with ataxin-2. *Hum. Mol. Genetics* **9**, 1303-1313.

Skop, A. R. and White, J. G. (1998). The dynactin complex is required for cleavage plane specification in early *Caenorhabditis elegans* embryos. *Curr. Biol.* **8**, 1110-1116.

Strome, S. and Wood, W. B. (1983). Generation of asymmetry and segregation of germ-line granules in early *C. elegans* embryos. *Cell* **35**, 15-25.

Sulston, J. E., Schierenberg, E., White, J. G. and Thomson, J. N. (1983). The embryonic cell lineage of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **100**, 64-119.

Tabara, H., Hill, R. J., Mello, C. C., Priess, J. R. and Kohara, Y. (1999). *pos-1* encodes a cytoplasmic zinc-finger protein essential for germline specification in *C. elegans*. *Development* **126**, 1-11.

Thorpe, C. J., Schlesinger, A., Carter, J. C. and Bowerman, B. (1997). Wnt signaling polarizes an early *C. elegans* blastomere to distinguish endoderm from mesoderm. *Cell* **90**, 695-705.

Tabuse, Y., Izumi Y., Piano, F., Kemphues K. J., Miwa J. and Ohno, S. (1998). Atypical protein kinase C cooperates with PAR-3 to establish embryonic polarity in *Caenorhabditis elegans*. *Development* **125**, 3607-3614.

Varani, G. and Nagai, K. (1998). RNA recognition by RNP proteins during RNA processing. *Annu. Rev. Biophys. Biomol. Struct.* **27**, 407-445.

Waddle, J. A., Cooper, J. A. and Waterston, R. H. (1994). Transient localized accumulation of actin in *Caenorhabditis elegans* blastomeres with oriented asymmetric divisions. *Development* **120**, 2317-2328.

Williams, B. D. (1995). Genetic mapping with polymorphic sequence-tagged sites. In *Methods in Cell Biology.* Caenorhabditis elegans*: Modern Biological Analysis of an Organism*. Vol. 48, pp. 81-97. London: Academic Press.

Zhu, J., Hill, R. J., Heid, P. J., Fukuyama, M., Sugimoto, A., Priess, J. R. and Rothman, J. H. (1997). *end-1* encodes an apparent GATA factor that specifies the endoderm precursor in *Caenorhabditis elegans* embryos. *Genes Dev.* **11**, 2883-2896.

Zhu, J., Fukushite, T., McGhee, J. D. and Rothman, J. H. (1998). Reprogramming of early embryonic blastomeres into endodermal progenitors by a *Caenorhabditis elegans* GATA factor. *Genes Dev.* **12**, 3809-3814.

Zwaal, R. R., Ahringer, J., van Luenen, H. G., Rushforth, A., Anderson, P. and Plasterk, R. H. (1996). G proteins are required for spatial orientation of early cell cleavages in *C. elegans* embryos. *Cell* **86**, 619-629.