Cell, Vol. 90, 695–705, August 22, 1997, Copyright ©1997 by Cell Press

Wnt Signaling Polarizes an Early C. elegans Blastomere to Distinguish Endoderm from Mesoderm

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Summary

A polarizing signal induces endoderm production by a 4-cell stage blastomere in C. elegans called EMS. We identified 16 mutations in five genes, *mom-1* through *mom-5*, required for EMS to produce endoderm. *mom-1, mom-2*, and *mom-3* are required in the signaling cell, P_2, while *mom-4* is required in EMS. P_2 signaling downregulates an HMG domain protein, POP-1, in one EMS daughter. The sequence of *mom-2* predicts that it encodes a member of the Wnt family of secreted glycoproteins, which in other systems activate HMG domain proteins. Defective mitotic spindle orientations in *mom* mutant embryos indicate that Wnt signaling influences cytoskeletal polarity in blastomeres throughout the early embryo.

Introduction

To generate cellular asymmetry, extrinsic and intrinsic processes can polarize parent cells prior to division, localizing different developmental regulators to their daughters (for reviews, see Horvitz and Herskowitz, 1992; Gonczy and Hyman, 1996). In the nematode Caenorhabditis elegans, an inductive polarization regulates the development of a 4-cell stage embryonic cell, or blastomere, called EMS (Goldstein, 1992, 1993, 1995a). In response to polarization, the two EMS daughters, E and MS, adopt different fates. E makes all of the endoderm, or intestinal cells, in the embryo, while MS produces mesoderm, which in C. elegans includes pharynx and body wall muscle (Sulston et al., 1983).

A signal from the sister of EMS, a blastomere called P_2, polarizes EMS to induce endoderm (Figure 1). This induction can be analyzed in vitro by culturing blastomeres isolated from early embryos (Goldstein, 1992; Edgar, 1995; Shelton and Bowerman, 1996). If EMS is isolated and left to develop alone, it fails to make endoderm and instead produces two MS-like daughters (Goldstein, 1993). When P_2 and EMS are placed in contact sufficiently early, the EMS daughter born next to P_2 makes endoderm, while the other adopts an MS-like fate. EMS polarization may involve reorganization of the cytoskeleton: exposure of 4-cell stage embryos to chemicals that depolymerize either microfilaments or microtubules prevents endoderm development, while similar treatments at the 8-cell stage have no effect (Goldstein, 1995a). Signal(s) from P_2 also orients the mitotic spindle in EMS, indicating that the microtubule cytoskeleton is one target of a P_2 signal (Goldstein, 1995b). Indeed, P_2 signaling might act solely by regulating cytoskeletal polarity in EMS, influencing gene expression in E and MS only indirectly.

The maternal gene *pop-1* also is required to distinguish the fates of E and MS, in an apparently cell autonomous manner (Lin et al., 1995). In *pop-1* mutant embryos E and MS both adopt E-like fates and produce twice the normal number of intestinal cells. *pop-1* encodes a protein with a single high mobility group (HMG) DNA binding domain similar to vertebrate T cell factor 1 (TCF-1) and mouse lymphoid enhancer-binding factor 1 (LEF-1). Recent work indicates that these proteins are targets for activation by the Wnt signal transduction pathway (Behrens et al., 1996; Miller and Moon, 1996; Riese et al., 1996; Brunner et al., 1997; van de Wetering et al., 1997 [but see Merriam et al., 1997]). POP-1 is present in the nuclei of all 4-cell stage blastomeres, but at the 8-cell stage POP-1 levels are higher in MS than in E, suggesting that down-regulation of POP-1 in E permits specification of endoderm (see Figure 1).

We have identified 5 maternally expressed C. elegans genes required for endoderm induction. Mutations in these genes (called *mom* for *mo*re *m*esoderm) result in E and MS both adopting MS-like fates. We show that *mom-2* encodes a Wnt family member, and we suggest that Wnt signaling regulates cytoskeletal polarity in responding cells. *mom-1*, *mom-2*, and *mom-3* are required in P_2 for signaling, while *mom-4* functions autonomously in EMS. POP-1 functions downstream of the *mom* genes, with Wnt signaling from P_2 acting to downregulate this HMG domain protein in E.

Results

Genetic Identification of Five *mom* Loci

To study the induction of cell polarity in EMS, we used a genetic screen to identify recessive, maternal-effect, embryonic-lethal mutants in which both EMS daughters adopt an MS-like fate and produce excess mesoderm at the expense of endoderm. We identified 16 such mutations, defining 5 genes, *mom-1* through *mom-5*. All the mutant *mom* alleles are recessive and fully penetrant for embryonic lethality (Experimental Procedures). For the stronger alleles, most mutant embryos entirely lack intestinal cells and instead make large amounts of pharynx (Table 1, Figure 2A). In addition, *mom* mutant embryos are severely defective in morphogenesis, producing amorphous clumps of differentiated tissues whether or not they make endoderm (Figure 2A). Thus, mutational inactivation of the *mom* genes affects not just the polarization of EMS but also elongation of the embryo into a long, thin worm. The morphogenesis defect can be partially rescued by paternal contribution of a wild-type allele, but the *mom-2* requirement for endoderm specification is strictly maternal (Figure 2A, Experimental Procedures). Although we detect no zygotic phenotypes associated with mutant alleles of *mom-2, mom-4*, and *mom-5*, homozygous *mom-1* and homozygous *mom-3* mutant hermaphrodites without exception have protruding, nonfunctional vulvae and are mildly uncoordinated

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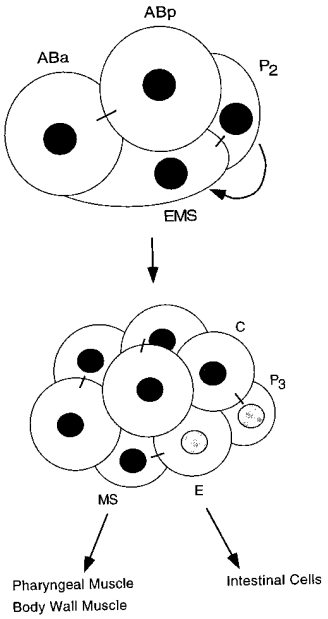

Figure 1. EMS Development Requires Both Autonomously Acting POP-1 and a Signal from P_2

POP-1 protein is found in all nuclei at the 4-cell stage (dark shading). A signal from P_2 to EMS early in the 4-cell stage (curved arrow) polarizes EMS such that the potential to produce endoderm is acquired by only the E daughter of EMS. E contains relatively low levels of POP-1 and makes only intestinal cells, while MS has relatively high levels of POP-1 and produces mesoderm. Sister cells are indicated by short connecting lines.

(C. J. T., A. S., and B. B., unpublished data). Here we focus on the maternal-effect phenotypes caused by mutations in the *mom* genes. We note that because some *mom* genes have zygotic functions, the maternal-effect alleles we have isolated may not be null. Indeed, the different penetrance of the gut phenotypes associated with our different *mom* alleles suggests that most alleles are not null (Table 1). However, double mutant embryos from mothers homozygous for both *mom-2* and *mom-4* have a completely penetrant gut defect (Table 1). Thus, the *mom* gene products, whether they function in a single pathway or in parallel, are essential for the specification of endoderm in early C. elegans embryos.

In addition to their lack of endoderm, *mom-1*, *mom-2*, *mom-3*, and *mom-5* mutant embryos also are defective in orienting the mitotic spindle of an 8-cell stage blastomere called ABar (Table 1, Figure 3). In wild-type embryos, ABar divides along a largely left–right (l/r) axis, roughly orthogonal to the three other AB descendants that all divide along the anterior–posterior (a/p) axis with a pronounced dorsal–ventral (d/v) tilt (Figure 3D). In all but *mom-4* mutants, ABar divides roughly parallel to the other AB descendants (Table 1, Figure 3H). Because the paternal rescue of morphogenesis described above does not correlate with rescue of the ABar spindle orientation (Experimental Procedures), the abnormal axis of the mitotic spindle in ABar cannot fully account for the highly penetrant morphogenesis defect observed in *mom* mutant embryos.

E and MS Both Adopt MS-like Fates in *mom* Mutant Embryos

Terminally differentiated *mom* mutant embryos that lack intestinal cells also appear to make excess pharynx (Figure 2A), suggesting that E might produce an MS-like pattern of cell fate. We tested this possibility in three ways. We used tissue-specific antibodies to determine if E in *mom-2* mutant embryos produces mesodermal cell types normally made by MS, we tested the genetic requirements for ectopic pharyngeal cell production by E, and we analyzed the cell lineage of some E descendants to determine if they adopt cell cycle times and cleavage patterns similar to those made by a wild-type MS blastomere. As described below, our results indicate that E adopts an MS-like fate in *mom-2* mutant embryos.

To examine the fate of E, we first used a laser microbeam focused through a microscope to kill every cell in early *mom-2* mutant embryos except for E. These partial embryos produced differentiated descendants that were fixed and stained with cell type–specific monoclonal antibodies (Table 2, Figure 2B). Similar laser ablation data were obtained for mutant embryos lacking *mom-1, mom-3* and *mom-4* function (A. S., C. J. T., M. Meneghini, and B. B., unpublished data). In control experiments using wild-type embryos, E always produced intestinal cells but not two cell types made by MS: pharyngeal muscle and body wall muscle. For two strong *mom−2* alleles, E in most operated embryos failed to produce any intestinal cells. Instead, when E failed to make gut, it produced four cell types normally made by MS: pharyngeal and body wall muscle cells, pharyngeal gland cells, and pharyngeal marginal cells (Table 2). MS in *mom* mutant embryos also produces pharynx and body wall muscle (Table 2). As no other early blastomere besides MS in wild-type embryos produces both pharynx and body wall muscle (Sulston et al., 1983), E and MS in *mom* mutant embryos both appear to adopt MS-like fates.

If E in *mom-2* embryos develops like MS, then it should require *skn-1* but not *glp-1* to produce pharyngeal muscle cells (Priess et al., 1987; Bowerman et al., 1992). MS makes about half the pharyngeal cells produced during embryogenesis, with the remainder made by two granddaughters of the 4-cell stage blastomere ABa (Sulston et al., 1983). The production of pharyngeal cells by ABa descendants requires an inductive signal from MS at about the 12-cell stage (Priess et al., 1987; Hutter and Schnabel, 1994; Mango et al., 1994a); *glp-1* encodes a putative receptor required for ABa descendants to receive the MS signal (Priess et al., 1987; Austin and Kimble, 1989; Yochem and Greenwald, 1989). Mutations in *glp-1* result in an absence of ABa-derived pharyngeal cells but do not affect the production of pharyngeal cells by MS (Priess et al., 1987). Mutations in *skn-1* result in a loss of all pharyngeal cells, as *skn-1* is required both to specify MS fate and to activate the MS signal that induces ABa descendants to make pharyngeal cells (Bowerman et al., 1992; Mello et al., 1992; Shelton and Bowerman, 1996). By staining fixed, terminally differentiated embryos from double mutant mothers with cell-type specific antibodies (Experimental Procedures), we found that *skn-1; mom-2* embryos produce few or no pharyngeal cells: 17 of 46 double mutant embryos made

Table 1. Penetrance of Endoderm Defect and ABar Cleavage Abnormality in *mom* Mutant Embryos

Gene	Allele	% Embryos Lacking Gut (n)[a]	Fraction of Embryos with Aberrant ABar Cleavage[b]
mom-1	*or10*	85 (215)	10/10
	or46	52 (160)	7/7
	or65	50 (275)	
	or83	46 (153)	
	or70	39 (321)	13/13
mom-2	*or85*	88 (485)	
	or9	77 (226)	
	or48	74 (380)	
	or42	72 (194)	28/31
	or33	50 (210)	
	or77	8 (65)	15/15
mom-3	*or78*	65 (361)	30/30
mom-4	*or39*	40 (100)	0/12
	or49	24 (181)	0/12
	or11	1 (228)	2/17
mom-5	*or57*	5 (167)	9/9
skn-1; mom-2	*zu67; or42*	100 (241)	—
mom-4; mom-2	*or39; or42*	100 (124)	—

[a]To assay production of intestinal cells, embryos were collected from *mom* mutant hermaphrodites and allowed to develop at least 10 hr at 20°C. The presence of intestinal cells was scored using polarizing light microscopy to detect intestine-specific birefringent gut granules.
[b]The orientation of the mitotic spindle of the ABar blastomere was examined in lateral views of 8-cell stage embryos using Nomarski optics. In wild-type embryos, the anterior daughter of ABar contacts the MS blastomere (Figure 3D). ABar cleavage was scored as defective if its posterior daughter contacted MS (Figure 3H).

between 1 and 5 pharyngeal muscle cells while the remainder made none, compared to the 39 made in wild type (Sulston et al., 1983). In contrast, both E and MS from *glp-1; mom-2* double mutant embryos produce pharynx: 9 of 10 embryos in which E was isolated, and 6 of 6 in which MS was isolated, made large numbers of pharyngeal muscle cells. Because E and MS in *mom-2* mutant embryos require *skn-1* but not *glp-1* function to produce pharyngeal cells, we conclude that both EMS daughters adopt an MS-like fate.

To define their fates more precisely, we compared the cell lineages E and MS produce in *mom-2* mutant embryos to the wild-type MS lineage (Figure 2C). C. elegans embryos produce a nearly invariant cell lineage, as determined by scoring the relative positions of daughter cells following each division and observing their eventual fates (Sulston et al., 1983). In wild-type embryos, E and MS become different shortly after their birth: Ea and Ep are the first cells to migrate inside the embryo during gastrulation, and they divide about 15 min later than do MSa and MSp. In *mom-2* embryos, Ea and Ep fail to gastrulate, and they divide nearly synchronously with MSa and MSp. Subsequent divisions of the E descendants also occur with approximately MS-like timing, and several cell deaths that occur in a wild-type MS lineage also occur at the corresponding points in the lineage of E in some *mom-2* mutant embryos. However, we often do not observe characteristic cell deaths in both the E and MS lineages (Figure 2C). We conclude that E and MS in *mom-2* mutant embryos both adopt fates similar but not identical to a wild-type MS.

mom-2 Encodes a Wnt Family Member

We used positional cloning to identify the wild-type *mom-2* gene (Figure 4). After mapping *mom-2* to a small genetic interval, we obtained rescue of the mutant phenotype by germline transformation with the cosmids F52E1 and ZK427. Sequence data from the C. elegans Genome Center predicts a gene, F38E1.7, on ZK427 that would encode a member of the Wnt family of secreted signaling molecules (Experimental Procedures). To determine if F38E1.7 is the *mom-2* locus, we microinjected antisense RNA from one exon of the predicted gene into the syncitial gonad of wild-type animals. All injected animals produced dead embryos with a Mom mutant phenotype (Experimental Procedures). To confirm further the gene identity, we sequenced six mutant alleles of *mom-2*. Each allele contains a single lesion within the coding sequence, except for one mutation at a splice junction (Figure 4). We conclude that *mom-2* is F38E1.7 and that it encodes a member of the Wnt family of secreted signaling glycoproteins. The predicted MOM-2/Wnt protein is 363 amino acids in length and includes 24 highly conserved cysteine residues. BLAST searches indicated that the predicted MOM-2 protein is most closely related to mammalian Wnt-2, sharing approximately 40% amino acid identity with mouse and human Wnt-2. We conclude that the polarization of gut potential in EMS requires the Wnt signal transduction pathway.

mom-1, mom-2, and *mom-3* Are Required in P_2 for Endoderm Induction, while *mom-4* Is Required in EMS

To determine which *mom* genes are required in P_2 for signaling and which are required in EMS for responding, we assembled genetically mosaic partial embryos in vitro (Figure 5 and Experimental Procedures). Using blastomeres isolated from wild-type and *mom* mutant embryos, we found that *mom-1, mom-2*, and *mom-3*

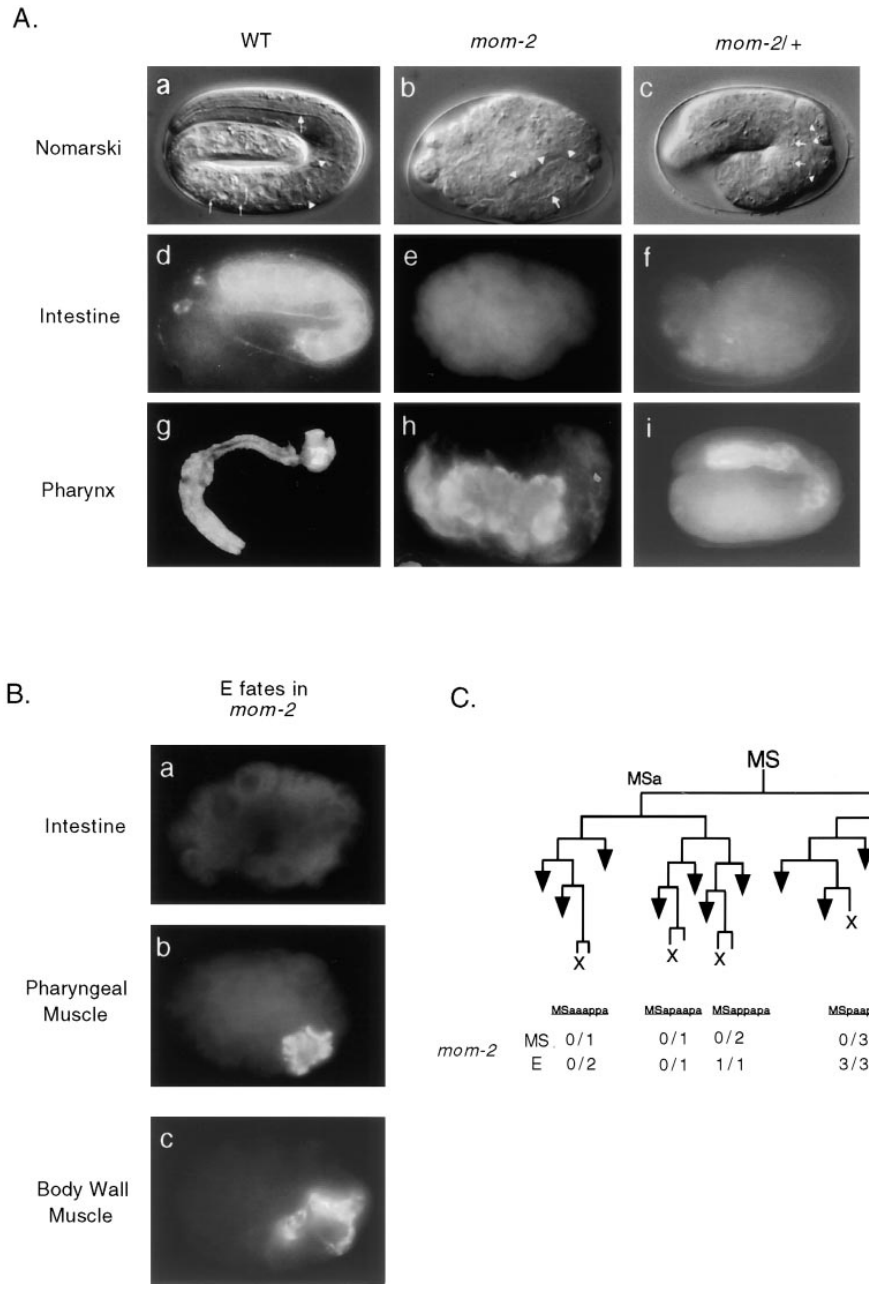

Figure 2. E Adopts an MS-like Identity in *mom-2* Embryos

(A) Immunofluorescence and light micrographs of wild-type (left column), *mom-2* (middle column), and *mom-2*/+ embryos (right column) from *mom-2*/*mom-2* mothers. Embryos were allowed to develop 15 hr (a, b, e, and f), or 8 hr (c and d) at 20°C. (a, b, and c) Living embryos viewed with Nomarski optics. Pharyngeal tissue is surrounded by a prominent basement membrane (arrowheads), and contains a secreted cuticle (wide arrows). Visible in wild-type embryos, but not in *mom-2* or *mom-2*/+ embryos, are intestinal cells with characteristically large nuclei containing a single large nucleolus (thin arrows in [a]). While homozygous *mom-2* embryos fail to undergo any morphogenesis and invariably arrest as unelongated clumps of differentiated tissue (b), *mom-2*/+ embryos, even those lacking intestine, often elongate into short, stubby worms (c and i). Paternal contribution of a wild-type copy of *mom-2* does not rescue the intestine defect (Experimental Procedures). (d, e, and f) Intact embryos stained with J126, a monoclonal antibody (MAb) that recognizes intestinal cells. (g, h, and i) Intact embryos stained with 9.2.1, a MAb that recognizes pharyngeal muscle cells.

(B) Immunofluorescence micrographs of operated *mom-2* embryos in which all blastomeres in the embryos except E were killed with a laser microbeam. Embryos were allowed to develop 8 hours (a and c), or 16 hours (b) at 20°C before fixing and staining. MAb J126 (a) recognizes intestinal cells, MAb 9.2.1 (b) recognizes pharyngeal muscle cells, and MAb 5.6 (c) recognizes body-wall muscle.

(C) Cell deaths scored in E and MS lineages from *mom-2* mutant embryos (below) compared to the wild-type MS lineage (above). Vertical bars represent time; horizontal bars represent cell division. Programmed cell deaths are indicated by Xs. The fraction of embryos in which the corresponding E and MS lineages in *mom-2* mutant embryos produced cell deaths are indicated below each X.

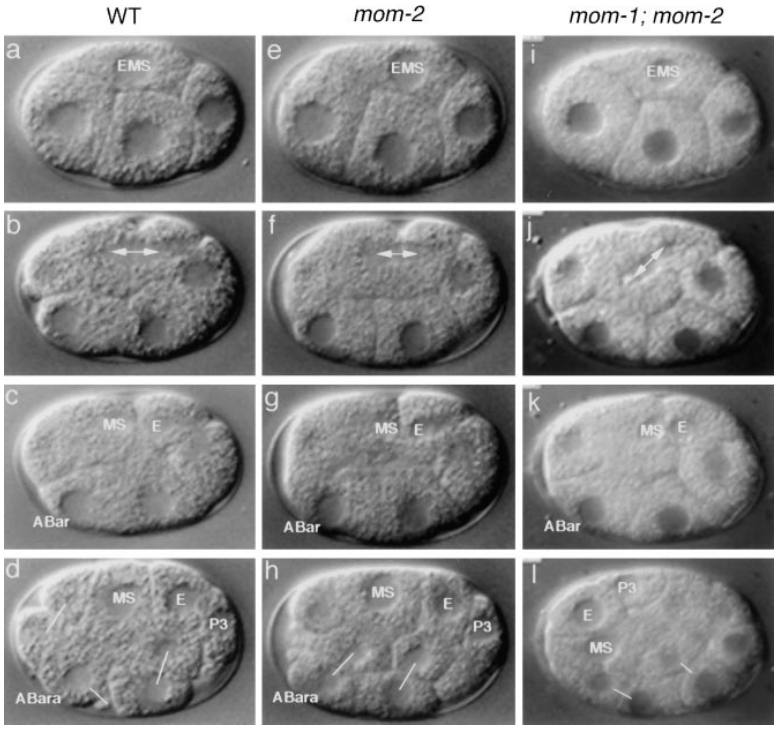

Figure 3. Mitotic Spindle Axes Are Misoriented in *mom-2* and *mom-2; mom-1* Mutant Embryos

All embryos are shown in lateral views with anterior to the left and ventral up; maternal genotypes are indicated at the top of each column. (A, E, and I) Wild-type, *mom-2*, and *mom-2; mom-1* embryos at the 4-cell stage. (B, F, and J) EMS cleavage. In wild-type and *mom-2* embryos, the EMS spindle aligns on the a/p axis, while in some *mom-2; mom-1* embryos, it is tilted along the d/v axis, as shown here. (C, G, and K) 8-cell stage. E and MS are positioned normally in the *mom-2; mom-1* embryo following cytokinesis. (D, H, and L) 12-cell stage. In wild-type embryos, ABar divides along a mostly l/r axis, transverse to that of the other AB descendants; the division axes of ABal and ABpr are marked for comparison. The anterior daughter of ABar, ABara, touches MS. In *mom-2* mutant embryos, ABar divides parallel to the other AB descendants and ABarp instead of ABara contacts MS. In a small fraction of *mom-2; mom-1* embryos (2 of 21 embryos scored, an example of which is shown here), we observe dramatic rearrangements of blastomeres, beginning at the 8-cell stage. In both cases, posterior blastomeres moved anteriorly, such that E and MS became the anteriormost cells in the embryo. Other *mom-2; mom-1* embryos showed the same ABar cleavage axis defect observed in *mom-2* mutant embryos. Surprisingly, double mutant embryos made intestinal cells more often than either single mutant: 52% of *mom-2; mom-1* embryos (n=197) compared to 15% for *or10* and 28% for *or42* embryos (Table 1). Laser ablation experiments with double mutant embryos indicate that E but not MS produces endoderm: Of 14, 7 isolated E's made intestinal cells, compared to 0 of 15 isolated MS blastomeres.

are required in P_2, while *mom-4* is required in EMS. Because of the low penetrance of the endoderm phenotype in *mom-5* mutant embryos (Table 1), we have not been able to determine which blastomere requires *mom-5* function. The development of P_2 appears normal in *mom-1, mom-2*, and *mom-3* mutant embryos (Experimental Procedures), suggesting that the wild-type genes function specifically in P_2 signaling. Finally, our identification of *mom-2* as a Wnt gene is consistent with its function being required in P_2 for signaling.

mom-4 and the Interpretation of Cell Polarity in EMS

Our blastomere mosaic analysis indicates that *mom-4* is required in EMS to respond to the P_2 signal (Table 3). To determine how *mom-4* functions with respect to the establishment or interpretation of cell polarity, we examined *mom-4* function in *pie-1* mutant embryos. Mutations in *pie-1* cause P_2 to develop like EMS, producing excess pharynx and intestine (Mello et al., 1992). Unlike EMS, P_2 in *pie-1* embryos does not require a signal from

Table 2. Cell Types Produced by E and MS in *mom-2* Mutant Embryos

Genotype	Blastomere Isolated	Intestinal Cells	Body Wall Muscle	Pharyngeal Muscle	Pharyngeal Gland Cells	Pharyngeal Marginal Cells
Wild type	E	13/13	0/7	0/10	—	—
mom-2(or42)	E	9/170	17/17	15/15	10/10	16/17
mom-2(or9)	E	2/16	—	8/10	—	—
Wild type	MS	0/8	11/11	12/12	8/8	9/9
mom-2(or42)	MS	0/64	6/6	8/10	13/14	12/13
mom-2(or9)	MS	0/9	—	6/6	—	—

All blastomeres in the embryo except MS or E were killed using a laser microbeam. ABa, ABp, and P2 were killed at the 4-cell stage, then either MS or E was killed following division of EMS. The operated embryos were allowed to develop overnight and then scored for the presence of intestinal cells using polarizing optics. Operated embryos not making gut were fixed and stained with MAbs to detect the tissues indicated (see Experimental Procedures). When E was isolated by laser ablation at the 8-cell stage, 9 of 22 operated embryos made intestinal cells. The nine that made intestine were double stained with J126 and 3NB12; one made both pharyngeal and intestinal cells while the remainder made only intestine.

Table 3. P2 and EMS Blastomere Mosaics

Blastomeres Recombined		Fraction of Recombinant Embryos Making Gut
P2	EMS	
WT	WT	46/47
WT	*mom-1*	7/7
mom-1	WT	2/7
WT	*mom-2*	10/12
mom-2	WT	1/8
WT	*mom-3*	11/11
mom-3	WT	4/12
WT	*mom-4*	2/10
mom-4	WT	7/7

P2 and EMS blastomeres were isolated from wild-type and *mom* mutant embryos and recombined to form genetically mosaic partial embryos. The genotypes of the recombined blastomeres are shown in the left column. The mutant alleles used were: *mom-1(or10), mom-2(or42), mom-3(or78)*, and *mom-4(or39)*. The fraction of partial embryos making intestinal cells in each experiment is shown in the right column.

any neighboring blastomere to produce intestinal cells; in isolation it still divides into one E-like and one MS-like daughter (Goldstein, 1995a). The ability of a *pie-1* mutant P_2 to produce endoderm autonomously raises the possibility that an intrinsic polarity within P_2 is sufficient to specify endoderm.

Several lines of evidence indicate that P_2 possesses an intrinsic polarity that might specify endoderm in *pie-1* mutant embryos. First, P_2 isolated from wild-type or *pie-1* embryos always divides asymmetrically, producing one larger and one smaller daughter, called C and P_3 in wild type. Moreover, the smaller P_3 daughter inherits cytoplasmic particles called P granules, which are segregated to P_3 as P_2 divides (Strome and Wood, 1983; Hird et al., 1996). Finally, although it is not known to have any function in P_2, the HMG domain protein POP-1 accumulates asymmetrically in the daughters of P_2, with C having higher levels than P_3 (Lin et al., 1995; Figure 1). In *pie-1* mutant embryos, C nearly always becomes MS-like, while P_3 always adopts an E-like fate, consistent with lower levels of POP-1 permitting the specification of endoderm in both E and P_3 in *pie-1* mutant embryos. To summarize, in *pie-1* embryos P_2's intrinsic polarity may be sufficient to specify E fate in P_3, suggesting that the intrinsic polarity of P_2 and the induced polarity of EMS share common properties.

To position *mom-4* relative to the establishment of cell polarity during endoderm induction, we constructed a *mom-4; pie-1* strain and asked if *mom-4* is required for P_2 to produce endoderm in *pie-1* mutant embryos (Table 4). By two criteria, P_2 in *mom-4; pie-1* double mutant embryos remains polarized. P_2 divides asymmetrically (24 of 24 embryos observed by light microscopy), and P granules are segregated to P_3 (11 of 11 embryos; see Experimental Procedures). While intact *pie-1* embryos make extra intestinal cells, *mom-4; pie-1* double mutant embryos usually make no endoderm (C. J. T. and B. B., unpublished data). Using laser ablation experiments to isolate the daughters of P_2 and EMS in double mutant embryos, we found that both E and P_3 usually failed to produce intestinal cells (Table 4). Because *mom-4* is required for P_3 to make intestinal cells in *pie-1* mutant embryos, even though P_2 remains polarized, *mom-4* may be required to interpret, not to establish, polarity in a wild-type EMS blastomere.

An alternative explanation for the lack of gut in *pie-1; mom-4* double mutant embryos is that autocrine signaling might polarize P_2 in a *pie-1* mutant. If so, *mom-4* might play a role in transducing an autocrine signal. To determine if P_2 uses autocrine, *mom*-dependent signaling to specify endoderm in *pie-1* mutant embryos, we constructed double mutants of *pie-1* with *mom-1*, *mom-2*, and *mom-3*. In all cases, EMS but not P_2 fails to produce endoderm (Table 4). We conclude that the production of endoderm by P_2 in *pie-1* mutant embryos is largely independent of Wnt signaling, and that *mom-4* may be unique among the *mom* genes in operating downstream of, or parallel to, the establishment of cell polarity during endoderm induction. However, because both P_2 and EMS fail to make gut in 5% of intact *pie-1*; *mom-2* mutant embryos (10 of 191) and in 1% of *pie-1*; *mom-3* embryos (4 of 471), *Wnt* signaling might have a minor role in polarizing P_2.

Wnt-Dependent P_2 Signaling Restricts POP-1 Function to MS

As a final approach to positioning the *mom* genes in a pathway of endoderm specification, we examined interactions of the *mom* genes with *pop-1*. Whereas the two

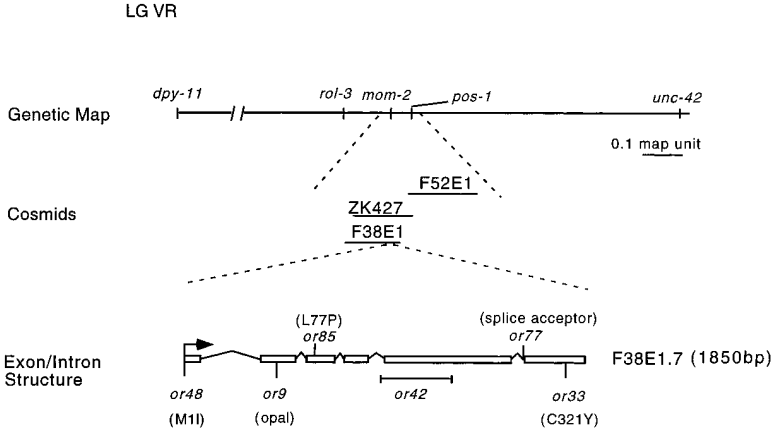

Figure 4. Molecular Cloning of *mom-2*

(A) *mom-2* maps near *pos-1*, on LGV. *mom-2* mutants are rescued by a mix of cosmids ZK427 and F52E1, but not F52E1 alone. Injection of antisense RNA to exon 5 of F38E1.7, a predicted gene on the cosmid F38E1, which overlaps extensively with ZK427, into the gonads of wild-type animals results in a phenotype indistinguishable from that of *mom-2* mutant embryos. All six alleles of *mom-2* have lesions in this gene, as depicted above and below the exon/intron diagram.

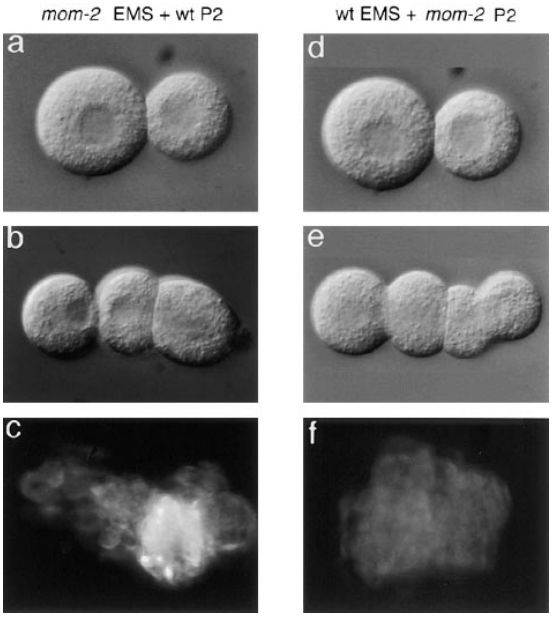

Figure 5. *mom-2* Is Required in P_2 and Not in EMS for the Induction of Endoderm in C. elegans

P_2 and EMS blastomeres were isolated from wild-type and *mom-2* embryos, then recombined in culture medium to form genetically mosaic partial embryos (see Experimental Procedures). Partial embryos were allowed to develop for 20 hr and intestinal cell production assayed using polarizing optics. (A and D) Recombined blastomeres viewed using Nomarski optics. The partial embryo in (A) is composed of a wild-type P_2 blastomere (smaller cell) and a *mom-2* EMS. The partial embryo in (D) was made by recombining a *mom-2* P_2 with a wild-type EMS. (B and E) EMS division in the same partial embryos. P_2 has been previously shown to orient the EMS mitotic spindle such that it "points" at P_2. In both partial embryos shown here, the EMS spindle axis is oriented correctly. (C and F) The differentiated descendants of these partial embryos viewed using polarized light optics to detect gut granules, present only in (C).

daughters of EMS adopt MS-like fates in *mom* mutant embryos, mutational inactivation of *pop-1* results in both EMS daughters adopting E-like fates (Lin et al., 1995). POP-1 is present at higher levels in the nucleus of MS than in the nucleus of E in most wild-type embryos, suggesting that down-regulation of nuclear POP-1 in E permits the production of endoderm (Lin et al., 1995). Because inactivation of the P_2 signal in *mom* mutants causes E to become like MS, P_2 signaling might be required to downregulate POP-1 in E. Consistent with this model, *pop-1; mom-2* and *pop-1 mom-4* double mutant embryos phenotypically resemble *pop-1* single mutants (Figure 6A). We also found that most *mom-2* mutant embryos show equal levels of POP-1 in MS and E (Figure 6B). Therefore, one function of *mom-2/Wnt* signaling is to reduce the nuclear levels of POP-1 in the posterior daughter of EMS. We conclude that *pop-1* may function downstream of all *mom* genes and that down-regulation of POP-1 in E results from polarization of EMS by a Wnt signal from P_2.

mom-2; mom-1 Double Mutant Embryos Exhibit Widespread Defects in Mitotic Spindle Orientation and Blastomere Positioning

The misorientation of the ABar mitotic spindle in *mom* mutant embryos (Table 1) and the observation that a signal from P_2 orients the mitotic spindle in EMS (Goldstein, 1995b) prompted us to consider if the *mom* genes might be required for proper orientation of mitotic spindles in other blastomeres. Normally, the EMS spindle sets up along the l/r axis and then rotates to lie along the a/p axis, pointing toward P_2 (Hyman and White, 1987). We examined the orientation of the EMS spindle in *mom* mutant embryos and observed subtle misalignment of the mitotic spindle in some *mom-1*, *mom-3*, and *mom-5* mutant embryos (A. S., C. J. T., and B. B., unpublished data).

To test more stringently if the *mom* genes are required generally for mitotic spindle orientations in the early embryo, we constructed a *mom-2(or42)*; *mom-1(or10)* double mutant strain, combining two of our strongest *mom* alleles (Experimental Procedures, Table 1). We observed partially penetrant but widespread defects in mitotic spindle orientation and blastomere positioning in *mom-2; mom-1* double mutants (Figure 3). For example, in many *mom-2; mom-1* embryos, the mitotic spindle in EMS is misaligned (10 of 22 EMS cleavages scored). EMS often divided more along a l/r or a d/v axis (Figure 3l). In addition to the relatively frequent EMS spindle orientation defects, a small fraction of *mom-2;*

Table 4. P3 and E Fate in *pie-1; mom* Double Mutant Embryos

Genotype	Blastomere Isolated	Fraction of Operated Embryos Making Gut
pie-1(zu127)	P3	14/16
	E	13/13
mom-1(or10); pie-1(zu127)	P3	3/3
	E	0/2
mom-2(or42); pie-1(zu127)	P3	7/7
	E	6/42
mom-3(or78); pie-1(zu127)	P3	7/7
	E	3/18
mom-4(or39); pie-1(zu127)	P3	0/13
	E	2/10

All blastomeres in the embryo except P3 or E were killed using a laser microbeam. The operated embryos were allowed to develop overnight, and the production of intestinal cells was assayed using polarizing optics to detect gut granules.

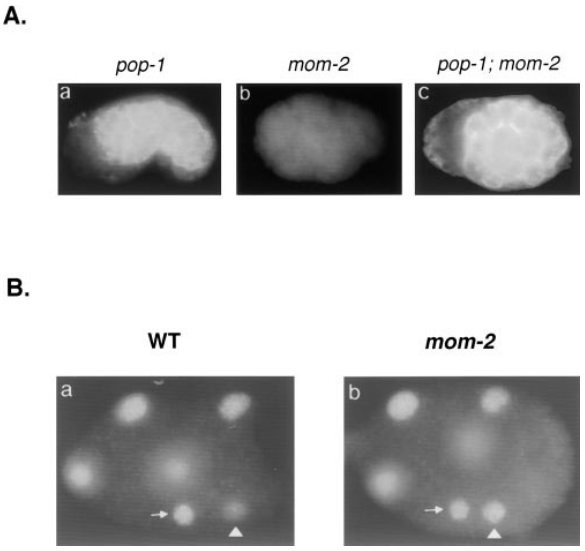

Figure 6. *mom-2* Acts to Negatively Regulate *pop-1* in E

(A) Immunofluorescence micrographs of *pop-1*, *mom-2*, and *pop-1; mom-2* embryos fixed and stained with J126 to detect intestinal cells. *pop-1* embryos produce twice the normal amount of endoderm (a), while most *mom-2(or42)* embryos fail to make any (b). *pop-1; mom-2* embryos make twice the normal amount (c); laser ablation experiments show that both MS and E make gut in *pop-1; mom-2* double mutant embryos, as in *pop-1* alone (see text). When *pop-1* function was eliminated in homozygous *mom-4* mutant mothers by RNA-mediated gene silencing (see Experimental Procedures), mutant embryos produced intestinal cells comparable in number to those made by *pop-1* mutant embryos (n = 43).

(B) Immunofluorescence micrographs of wild-type and *mom-2(or42)* embryos, stained with POP-1 antiserum. The embryos are oriented with the anterior end to the left, and the ventral surface toward the lower part of the photo. In the wild-type embryo, MS (indicated by the arrow) contains higher levels of POP-1 than does E (arrowhead). We observed this pattern in 19 of 24 wild-type embryos, similar to the frequency reported in Lin et al., 1995. In 39 of 53 *mom-2(or42)* embryos, an example of which is shown here, E and MS accumulate equal levels of POP-1. In both of the 7-cell stage embryos shown here, no staining is visible in P_2, which is in mitosis. Equal levels of staining are visible in the four AB descendants.

mom-1 embryos exhibited widespread abnormalities in mitotic spindle orientation and blastomere positioning. In some embryos, C, a daughter of P_2, divided d/v instead of a/p, and 16-cell stage AB descendants divided with random orientations (data not shown). In a few cases, posterior and ventral blastomeres moved to the anterior end of the embryo, displacing the cells normally there (compare Figures 3D and 3I). No defects were observed in the orientation of the mitotic spindles in the germline precursors P_0, P_1, P_2, and P_3 (Figure 3K, data not shown), lending support to the notion that germline precursors possess an intrinsic polarity (Schierenberg, 1987). Whether the abnormal positioning of blastomeres in some *mom-2; mom-1* embryos results indirectly from spindle orientation defects or is caused by changes in cell adhesion or migration is not clear. These data indicate that Wnt signaling influences cell polarity in somatic blastomeres throughout the early embryo and suggest that Wnt signaling regulates cytoskeletal polarity.

Discussion

We isolated mutations in 5 maternally expressed genes, *mom-1* through *mom-5*, each required for induction of endoderm during embryogenesis in C. elegans. One gene required for endoderm induction, *mom-2*, is predicted to encode a member of the widely conserved Wnt family of secreted glycoproteins. These signaling proteins polarize cell fates across tissues and in some cases are known to polarize individual cells (Nusse and Varmus, 1992; Parr and McMahon, 1994; Herman et al., 1995; Miller and Moon, 1996). *mom-2/Wnt* signaling specifies endoderm by posttranscriptionally down-regulating the HMG domain protein POP-1 in the posterior daughter of an early blastomere. Widespread defects in mitotic spindle orientation in *mom-2*; *mom-1* double mutant embryos suggest that Wnt signaling influences the cytoskeleton to regulate polarity in blastomeres throughout the early embryo.

Wnt Signaling Specifies Endoderm in C. elegans Embryos

Our genetic and molecular analyses of *mom-2* indicate that Wnt signaling from P_2 at the 4-cell stage polarizes the ventralmost blastomere EMS, thereby distinguishing endoderm from mesoderm. The Wnt signal transduction pathway has been conserved throughout animal evolution, being found in metazoan organisms as diverse as humans, fish, frogs, insects, mice, and nematodes (Nusse and Varmus, 1992; Parr and McMahon, 1994; Herman et al., 1995). Intriguingly, we have shown that three genes, *mom-1, mom-2*, and *mom-3*, are required in P_2 for signaling; only two genes, *porcupine* and *wingless*, are known to be required in signaling cells for Wnt pathway function in Drosophila (Kadowaki et al., 1996). Homozygous *mom-1* and *mom-3* hermaphrodites, in addition to producing *mom* mutant embryos, have protruding vulvae, exhibit a highly penetrant egg-laying defect, are mildly uncoordinated and often rupture at the vulva upon reaching adulthood (C. J. T., A. S., and B. B., unpublished data). As *mom-2* animals do not show these zygotic defects, *mom-1* and *mom-3* may regulate the function of other C. elegans Wnt(s) during larval development.

mom-2/Wnt Signaling Down-Regulates the HMG Domain Protein POP-1

Our analysis of endoderm induction demonstrates that Wnt-mediated signaling downregulates POP-1 in the E daughter of EMS. For example, the opposite phenotypes of *pop-1* and *mom-2* mutants and the finding that *pop-1; mom-2* double mutants resemble *pop-1* single mutants suggest that *mom-2* acts upstream to regulate *pop-1* negatively. Furthermore, in wild-type embryos POP-1 is present at high levels in the nucleus of MS but not of E, and only E makes endoderm (Lin et al., 1995). In *mom-2* mutants, nuclear POP-1 levels are high in both EMS daughters, and both adopt MS-like fates. Thus, Wnt signaling from P_2 polarizes EMS such that nuclear levels of POP-1 are down-regulated in E, permitting endoderm fate. As *pop-1* mRNA is maternally provided,

this regulation must occur posttranscriptionally. It is not known whether the asymmetric accumulation of POP-1 involves an active localization of preexisting POP-1 to MS, a degradation of POP-1 in E, or preferential translation of *pop-1* mRNA in MS. We note that POP-1 levels could be regulated solely by polarizing the cytoskeleton of EMS to localize factors that act posttranscriptionally to restrict high levels of nuclear POP-1 to MS.

The negative regulation of *pop-1* is surprising because POP-1 is related to transcription factors such as LEF-1 and TCF-1 that are activated, not down-regulated, by Wnt signaling (Lin and Priess, 1995; Miller and Moon, 1996; but see Merriam et al., 1997). In both Xenopus and Drosophila, LEF-1/TCF-1-like proteins bind β-catenin and appear to translocate with it to the nucleus (Behrens et al., 1996; Riese et al., 1997; van de Wetering et al., 1997). Indeed, LEF-1's ability to induce axis duplication and to activate target genes requires β-catenin, suggesting that a complex of the two proteins transcriptionally activates target genes. Furthermore, mutational analysis of the Drosophila TCF homolog *pangolin* indicates that *wingless* signaling activates *pangolin* (Brunner et al., 1997; Riese et al., 1997). We conclude that although Wnt signaling activates HMG domain transcription factors in other systems, it can regulate an HMG domain protein negatively during endoderm induction in C. elegans. POP-1 is similar to Pangolin and LEF-1 in the region required for interacting with β-catenin (Riese et al., 1997), raising the interesting possibility that such interactions might be capable of both activating and repressing the function of HMG-domain transcription factors.

Wnt Signaling Regulates Cytoskeletal Polarity throughout the Early Embryo

The partially penetrant spindle orientation defects observed in *mom-2; mom-1* double mutant embryos suggest that Wnt signaling regulates polarity in somatic blastomeres throughout the early embryo. Consistent with a role for the *mom* genes in polarizing the fates of blastomeres other than EMS, defects were observed also in the cell fate patterns produced by the 4-cell stage blastomere ABp in *mom-1*, *mom-2* and *mom-3* embryos (C. J. T., A. S., and B. B., unpublished data). Moreover, misorientation of the mitotic spindle in ABar is the mostly highly penetrant defect we have observed in *mom-1*, *mom-2*, *mom-3*, and *mom-5* single mutant embryos. It is possible that the MOM-2 secreted by P_2 is a diffusible ligand that polarizes blastomeres throughout the embryo. Alternatively, *mom-2* signaling from P_2 might be required indirectly for orienting the ABar mitotic spindle, or MOM-2 might be expressed by cells other than P_2. Finally, *mom-1; mom-2* mutant embryos show extensive abnormalities in orienting mitotic spindles in somatic blastomeres throughout the early embryo, while *mom-1* and *mom-2* single mutants do not. One possible explanation for these observations is that *mom-2* acts primarily to polarize gut potential, while a second Wnt functions primarily to orient mitotic spindles. Such functional distinctions among Wnts may exist in Xenopus, as ectopic expression of *Xwnt-5A* causes defects in cell–cell adhesion, while ectopic *XWnt8* causes duplication of the d/v axis (Du et al., 1995).

In other systems, Wnt signaling is thought to function, at least in part, by regulating gene transcription in responding nuclei. In C. elegans, the *mom* genes may control both endoderm induction and mitotic spindle orientation solely by influencing polarity of the cytoskeleton. In such a model, Wnt signaling causes a differential regulation of POP-1 in E and MS due to their inheritance of qualitatively distinct cytoplasms. Additional studies in C. elegans promise to shed new light on how Wnt signaling can influence the cytoskeleton in addition to regulating gene expression.

Experimental Procedures

Strains and Alleles

N2 Bristol was used as the wild-type strain; the basic methods of C. elegans culture, mutagenesis, and genetics were as described (Brenner, 1974). The mutations and balancer chromosomes used are listed by chromosome as follows: LGI: *dpy-5(e61)*, *him-1(e879), mom-4(or11, or39, or49)*, *mom-5(or57)*, *pop-1(zu189)*, *hT1(I;V), hT2(I;III)*; szT1(I;X). LGII: *mom-3(or78), rol-6(e187), mnC1(inversion balancer: dpy-10; unc-52).* LGIII: *dpy-18(e364), glp-1(e2141ts), pie-1(zu127), qC1(inversion balancer: dpy-19, glp-1, mog-1).* LGIV: *skn-1(zu67), DnT1(IV;V), nT1(IV;V).* LGV: *dpy-11(e224), mom-2(or9, or33, or42, or48, or77, or85), pos-1(zu148), rol-3(e754), unc-23(e324), unc-42(e270), unc-76(e911). LGX: dpy-6(e14), lin-2(e1309), lon-2(e678), mom-1(or10, or46, or65, or70, or83), unc-6(n102).* The double mutant strains used in this study have the following genotypes: *dpy-5 mom-4(or39)/hT1; mom-2(or42)/hT1*; *dpy-11 mom-2(or42)/++; unc-6 mom-1(or10)/++*, *pop-1 dpy-5/hT1; mom-2(or42) unc-42/hT1*, *dpy-18 pie-1/qC1; mom-2(or42) unc-42/Dnt1*, *skn-1/nT1; mom-2(or42) unc-42/Dnt1,* and *glp-1(ts); mom-2(or42)/DnT1*. Standard linkage group and three-factor analysis were used to map the *mom* genes: *mom-1* maps near the center of LGX (more precise data available via Acedb), mom-2 is located on the right arm of LGV (see below), *mom-3* maps on the extreme right arm of LGII, and *mom-4* and *mom-5* are on the right arm of LGI.

Isolation of *mom* Alleles

The *mom-1* allele *or46* and the *mom-5* allele *or57* were isolated in a screen for Tc1 transposon-induced mutations, as described elsewhere (Mello et al., 1994). All other alleles of *mom* genes were isolated by a screen for maternal effect embryonic lethal mutations after methanesulfonic acid ethyl ester mutagenesis (Kemphues et al., 1988). *mom-2* is required maternally, as no viable self-progeny were produced by hermaphrodites homozygous for *mom-2(or42)* (0%, $n > 5000$). *mom-2* is not required zygotically; all embryos produced by a *mom-2(or42)/+* hermaphrodite are viable (100%, $n = 1086$). However, paternal contribution of a wild-type *mom-2* allele can weakly rescue the morphogenesis defect. Of 638 *mom-2/+* embryos obtained by mating wild-type males into purged *mom-2(or42)* hermaphrodites, 8 hatched, and 5 others elongated substantially but did not hatch. All of the hatchers died as very young larvae. Visual inspection of unhatched embryos indicated that morphogenesis of the pharynx was more normal in the *mom-2/+* embryos: 30 of 48 embryos scored had a well-elongated pharynx with a buccal cavity, compared with 5 of 98 *mom-2/mom-2* embryos. The penetrance of the gut defect in unhatched *mom-2/+* embryos was only slightly changed: 35% made gut, compared to 28% for *mom-2/mom-2* (Table 1), suggesting that only the morphogenesis defect was rescued. In 61 of 63 *mom-2/+* embryos scored, ABar divided along an aberrant axis as it does in *mom-2/mom-2* embryos. The two embryos in which ABar divided along its normal axis showed no signs of elongation, while another embryo in which ABar divided abnormally showed substantial morphogenesis, elongating to nearly the two-fold stage. Thus, the rescue of the elongation defect observed in *mom-2/+* embryos does not appear to correlate with rescue of the ABar cleavage axis defect.

Genetic Analysis

mom-2(or42) was mapped to the right arm of LGV, near *rol-3*, using standard two- and three-factor genetic analysis. The other alleles were shown to be alleles of *mom-2* by linkage group mapping and complementation tests. Rol, nonUnc and Unc, nonRol recombinants were picked from a *rol-3 mom-2(or42)/dpy-11 unc-42* strain. Of 71 Rol, nonUnc recombinants, 6 were *mom-2(or42)*, and 8 of 64 Unc, nonRol recombinants were *mom-2(or42)*. This data places *mom-2* approximately 0.08 map units to the right of *rol-3*. Unc, nonPos-1 and Dpy, nonMom-2 recombinants were examined from a *dpy-11 mom-2(or42)/pos-1 unc-42* strain. Of Unc, nonPos-1, 50 picked up *mom-2(or42)*, and 52 of 52 Dpy, nonMom-2 animals picked up *pos-1*, placing *mom-2* extremely close to *pos-1. mom-2(or42)* was outcrossed 10 times to N2, and both chromosomal arms were crossed off to ensure that no other mutations were responsible for the phenotypes described.

Molecular Analysis of *mom-2* and RNA-Mediated Gene Silencing

Cosmid rescue of *mom-2(or42)* was done as described (Mello et al., 1991). Cosmids were injected singly or in groups with a marker *rol-6* plasmid into a *mom-2 unc-23/dpy-11 unc-42* strain. Heterozygous F1 transformants were scored for transmission of the array. From such lines, rolling *unc-23* animals were scored for rescue of the *mom-2* phenotype. A mixture of cosmids F52E1 and ZK427 (injected at 5 ng/μl each) rescued the *mom-2* phenotype. All Unc rollers showed similar levels of rescue. While ~100% of embryos from *mom-2(or42)* mothers fail to hatch (n > 5000 embryos), in a typical rescued brood a few embryos (~2% of the brood) did hatch, and several of the unhatched embryos showed significant rescue of the elongation defect. Some hatched animals grew to adulthood—those that still had the array showed continued rescue, while those that did not have the array laid broods of inviable *mom* embryos. Injection of cosmid F52E1 alone at 10 ng/μl did not rescue *mom-2*.

Cosmid F38E1, sequenced by the C. elegans Genome Project, overlaps with cosmid ZK427. Using PCR, we cloned exon 5 from the predicted gene F38E1.7 into pBluescript SK(+) (Stratagene, La Jolla, CA). Antisense RNA was synthesized from this construct following the method of Guo and Kemphues (1995), then injected into the gonads of wild-type hermaphrodites. We injected ten animals and transferred them to fresh plates daily. Unhatched embryos were removed from plates roughly 24 hr after fertilization and transferred to agar pads for microscopy. For all ten injected worms, some embryonic lethality was visible on day 1 following injection, with the unhatched embryos showing a morphogenesis defect but no gut defect. By day 2, >95% of embryos failed to hatch and showed a strong *mom* phenotype indistinguishable from *mom-2(or42)* embryos. Similar procedures were used to microinject *pop-1* RNA into eight homozygous *mom-4(or39)* mutant mothers to produce *pop-1 mom-4* double mutant embryos. All born more than 12 hr after injection, 5–10 embryos/mother were examined for gut production by scoring morphology and the presence of birefringent gut granules. *pop-1* RNA was made in vitro using an RT–PCR cDNA product corresponding to a full-length *pop-1* message with a T7 promoter site present in the amplifying primers.

We used PCR to clone genomic *mom-2* DNA from wild type, *lin-2* (the strain in which we did the mutagenesis), and all six of our *mom-2* alleles. Separate PCR reactions were used for sequencing each strand, starting 500 bp upstream of the initiation codon, and ending 100 bp downstream of the stop codon. All sequencing was done at the University of Oregon DNA Sequencing Facility, using an ABI 377 Prism automated fluorescent sequencer. Using RT-PCR (according to manufacturer's directions, GIBCO–BRL Life Technologies), we cloned a nearly full-length cDNA from wild-type embryonic RNA. Both strands were sequenced; comparison to the wild-type genomic sequence allowed us to determine the gene structure shown in Figure 4.

Microscopy and Laser Ablations

Laser ablations, embryo fixation, and antibody staining were performed as previously described (Avery and Horvitz, 1989; Bowerman et al., 1992). The monoclonal antibodies 5.6 and 9.2.1 (Miller et al., 1983) were used to detect the presence of body wall muscle and pharyngeal muscle cells, respectively. An intermediate filament antibody, TIB-131 (Pruss et al., 1981), was used to detect pharyngeal marginal cells. Intestinal cells were detected using either polarizing light microscopy in living embryos to score birefringent gut granules or using the monoclonal Ab J126 (Mango et al., 1994b). P granules were stained with MAb OIC1D4 from S. Strome. All P-granule staining was done on mixed stage populations of embryos, which were costained with DAPI to facilitate identification of early embryos in which the blastomere(s) containing P granules could be unambiguously determined. Staining of embryos with POP-1 antiserum and time-lapse lineage analysis were performed as described (Lin et al., 1995; Draper et al. 1996). The lineage data presented in Figure 2 is based on analysis of 3 *mom-2(or42)* embryos in which E did not make intestine.

To determine if P_2 develops normally in *mom* mutant embryos, we examined the localization of germline-associated P granules in P_2 and its descendants. At the 4-cell stage, normally only P_2 contains P granules. When P_2 divides, the P granules are segregated to P_3, the next germline precursor, and then into the germline daughter of P_3, P_4. In *mom-1(or10)*, *mom-2(or42), mom-3(or78), mom-4(or39)*, and *mom-5(or57)* mutant embryos, P granules are localized normally at all stages (data not shown). Further, terminally differentiated *mom* mutant embryos produce two germ cells that are normal in appearance (data not shown). To further examine the fate of P_2, we isolated P_2 descendants in *mom-2(or42)* embryos by laser ablation and then counted the number of muscle cells made by each after fixing and staining with MAb 5.6. In wild-type embryos, two granddaughters of P_2, Ca and Cp, each make 16 body wall muscle cells, while a third granddaughter, D, makes 20 (Sulston et al., 1983). We observed similar numbers of muscle cells produced by these blastomeres in *mom-2* embryos (Ca and Cp each made 14–20 muscle cells, n = 11; D made 18–20, n = 8). By these criteria, P_2 develops normally in *mom* mutant embryos.

Blastomere Isolation and Culture

Blastomeres were isolated from early embryos as described (Edgar, 1995; Shelton and Bowerman, 1996). Wild-type and *mom* mutant animals were cut open in separate watch glasses; 1-cell stage embryos were selected and processed in parallel. The P_1 blastomere was separated from AB early in the 2-cell stage. The two daughters of P_1, EMS and P_2, were separated as quickly as possible to prevent any signaling from occurring. At this time, P_2 and EMS blastomeres of different genotypes were placed in contact with each other using a stream of culture medium. The partial embryos were cultured overnight in a humidity chamber and then scored for the production of intestinal cells using polarizing optics.

Acknowledgments

The authors thank Bruce Draper, Susan Mango, members of the Bowerman laboratory, Vivian Siegel, and anonymous reviewers for helpful comments on this manuscript. We are grateful to Russell Hill for providing the *pos-1* strain; to Rueyling Lin for providing the *pop-1* strain and POP-1 antiserum; to David Miller for providing MAbs 9.2.1 and 5.6; to Susan Strome for MAbs to P granules; and to Will Downs, Jim Priess, Christian Rocheleau, and Craig Mello for valuable discussions and for communication of data prior to publication. Several strains were obtained from the C. elegans Genetic Center, funded by the NIH National Center for Research Resources (NCRR). Special thanks to Aaron Severson for advances in worm microinjection technique; to Chris Shelton for patient guidance with blastomere manipulations; and to Chuck Carey, Craig Miller, David Rivers, and Ari Sainz for constructing double mutant strains. C. J. T. and A. S. were supported by training grants from the National Institutes of Health (HD07348 and GM07413); J. C. C. and B. B. were supported by grants from the American Cancer Society (DB-61) and the National Institutes of Health (GM49869).

Received May 16, 1997; revised July 2, 1997.

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