

SRC-1 and Wnt Signaling Act Together to Specify Endoderm and to Control Cleavage Orientation in Early *C. elegans* Embryos

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correct position prior to division (reviewed in Schuyler and Pellman, 2001; Segal and Bloom, 2001). In these examples, division axes appear to be determined by intrinsic asymmetries within the dividing cells. However, in other cases cell-cell contacts appear to orient or influence the cell division axis. For example, a recent study in *Drosophila* suggests that cell contact between epithelial cells can mask polarity cues that would otherwise direct the spindle complex to orient along the intrinsically polarized basal/apical axis of the epithelium (Lu et al., 2001). Epithelial cells can also respond to tissue polarity signals that orient the development of clusters of epithelial cells with respect to a common tissue axis. Tissue polarity signaling is best characterized at present in *Drosophila*, where Wnt/Wg related components including the seven-membrane-spanning receptor Frizzled and a PDZ domain protein Disheveled have been implicated in controlling planar polarity within the epithelium (reviewed in Adler and Lee, 2001).

C. elegans endoderm induction provides one of the best-studied examples of a cell division that is oriented in response to extrinsic signals. Endoderm induction occurs at the four-cell stage when the posterior most cell, called P2, induces its anterior sister cell, called EMS, to divide A/P and to produce a posterior descendant that gives rise to the entire endoderm of the animal. The nature of P2/EMS signaling has been the subject of classical embryological studies involving the isolation of blastomeres and their reassembly into chimeric embryos. In intact embryos, after EMS is born, its centrosomes migrate to occupy positions on the L/R axis of the embryo and then rotate with the mitotic apparatus to align on the A/P axis. EMS then divides to produce an anterior descendant called MS that produces primarily mesoderm and a posterior descendant called E that produces endoderm. When cultured in isolation, EMS fails to rotate its mitotic apparatus and divides symmetrically to produce two daughters that resemble the anterior mesodermal precursor, MS. If the P2 cell is placed back in contact with the isolated EMS cell, then EMS can orient its spindle toward this contact site and can divide asymmetrically to produce an E-like daughter at the P2 proximal side of the EMS cell (Goldstein, 1995a, 1995b). Thus, P2/EMS signaling induces not only the endoderm fate but can also direct the cleavage orientation of the EMS blastomere.

Genetic studies have identified genes involved in P2/EMS signaling. Several of these genes define components of the conserved Wnt/Wg signaling pathway, including *mom-1* (Porcupine), *mom-2* (Wnt/Wg), *mom-5* (Frizzled), *wrm-1* (β -catenin/Armadillo), *apr-1* (adenomatous polyposis coli, APC), and *pop-1* (TCF/LEF) (Lin et al., 1995; Rocheleau et al., 1997; Thorpe et al., 1997). Genetic studies also indicate that other signaling mechanisms contribute to endoderm specification. These mechanisms include components related to MAP kinase signaling factors, *mom-4* (TAK1, MAP kinase, kinase kinase related) and *lit-1* (Nemo, MAP kinase related) (Ishitani et al., 1999; Meneghini et al., 1999; Rocheleau et al., 1999; Shin et al., 1999). A key target of P2/EMS

Summary

In early *C. elegans* embryos, signaling between a posterior blastomere, P2, and a ventral blastomere, EMS, specifies endoderm and orients the division axis of the EMS cell. Although Wnt signaling contributes to this polarizing interaction, no mutants identified to date abolish P2/EMS signaling. Here, we show that two tyrosine kinase-related genes, *src-1* and *mes-1*, are required for the accumulation of phosphotyrosine between P2 and EMS. Moreover, *src-1* and *mes-1* mutants strongly enhance endoderm and EMS spindle rotation defects associated with Wnt pathway mutants. SRC-1 and MES-1 signal bidirectionally to control cell fate and division orientation in both EMS and P2. Our findings suggest that Wnt and Src signaling function in parallel to control developmental outcomes within a single responding cell.

Introduction

During development, cells often orient their division axes with respect to internal asymmetries or with respect to the tissue or body axis. In several well-studied cases of oriented cell division, the mitotic apparatus appears to recognize cell-intrinsic cues that direct the spindle to orient along a predetermined axis of the cell. Examples include recognition of the budding axis by the mitotic spindle in yeast and recognition of the anterior-posterior (A/P) axis by the mitotic spindle in early germline blastomeres of *C. elegans* embryos. In both cases, astral microtubules emanating from one of the two centrosomes capture and become anchored at a cortex site and then shorten, rotating the mitotic apparatus into the

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signaling is the downregulation of POP-1 protein in the posterior daughter of EMS called E. Certain single mutants as well as certain double mutant combinations among the P2/EMS signaling factors cause POP-1 levels to remain high in the E blastomere and consequently induce E to adopt the fate of its sister blastomere MS (Rocheleau et al., 1997; Thorpe et al., 1997). Several of the genetically defined P2/EMS signaling components exhibit alterations in cleavage axes in the early embryo and exhibit skewed A/P orientation of the EMS division. However, within the intact embryo, no single or multiple mutant combinations completely prevent the A/P orientation of the EMS division axis (Rocheleau et al., 1997; Thorpe et al., 1997; Schlesinger et al., 1999). These findings indicate that as yet unidentified factors must direct endoderm specification and EMS spindle orientation during P2/EMS signaling.

The *mes-1* gene encodes a probable transmembrane protein with overall structural similarity to receptor tyrosine kinase and was previously described as a factor required for proper asymmetry and cell fate specification in embryonic germlineage (Berkowitz and Strome, 2000). Null mutations in *mes-1* cause a maternal-effect sterile phenotype in which the progeny of homozygous mothers are viable but mature without germcells. Most cell types are specified properly in *mes-1* sterile animals, but the germline cell named P4 adopts the fate of its sister cell, a muscle precursor, called D and produces ectopic muscle at the expense of the germline (Strome et al., 1995). Interestingly, MES-1 protein is localized intensely at the contact site between the germline blastomere and intestinal precursor at each early developmental stage, starting from the four-cell stage where MES-1 is localized at the contact site between P2 and EMS (Berkowitz and Strome, 2000).

Here, we show that an intense phosphotyrosine signal that depends on *mes-1*(+) activity is correlated with MES-1 protein localization. We show that MES-1 is required in both P2 and EMS and appears to act through a second gene, *src-1*, a homolog of the vertebrate protooncogene c-Src^{pp60} (Takeya and Hanafusa, 1983). We describe a probable null mutant of *src-1* that exhibits a fully penetrant maternal-effect embryonic lethal phenotype. The *src-1* and *mes-1* mutants exhibit similar germline defects and have a nearly identical set of genetic interactions with Wnt/Wg pathway components. We show that double mutants between *mes-1* or *src-1* and each of several Wnt/Wg signaling components exhibit a complete loss of P2/EMS signaling, including a loss of the A/P division orientation in the EMS cell. Finally, we show that *mes-1* functions in both EMS and P2 to direct MES-1 protein localization at EMS/P2 junction and to specify A/P cleavage orientation in the EMS cell, while *src-1* is required cell autonomously in EMS for the induction of the EMS A/P division axis. Our findings suggest that a homotypic interaction between MES-1-expressing cells, P2 and EMS, induces a SRC-1-mediated phosphotyrosine signaling pathway that functions in parallel with Wnt/Wg signaling to specify endoderm and to orient the division axis of EMS in early *C. elegans* embryos.

Results

SRC-1 Is Required for Embryonic Body Morphogenesis and Germline Asymmetries

We identified a *C. elegans* homolog of the vertebrate protooncogene c-SRC^{pp60} (reviewed in Schwartzberg, 1998), designated *src-1*. The *src-1* gene is one of two *C. elegans* genes that contain all of the hallmarks of Src family tyrosine kinases (Figure 1; see Experimental Procedures). The second gene, *F49B2.5*, has not been studied previously but has no phenotype by RNAi (Figure 1B and data not shown). We isolated a deletion allele of *src-1*, *cj293*. This deletion removes 4.5 kilobases (kb) of genomic sequence including sequences encoding the SH2 domain as well as the catalytic site of the kinase, resulting in a frameshifted and truncated protein of 137 amino acids (Figures 1A and 1B). This probable null allele of *src-1* causes a recessive maternal effect embryonic lethal phenotype. Consistent with the idea that this embryonic lethal phenotype represents a loss-of-function phenotype for *src-1*, we found that RNAi targeting *src-1* induced an identical embryonic lethal phenotype (see below). Homozygous *src-1(cj293)* hermaphrodites are themselves viable but produce inviable embryos. These *src-1(cj293)*-arrested embryos contain well-differentiated tissues including hypodermis, muscle, pharynx, and intestine, but these embryos fail to undergo body morphogenesis (Figures 2A–2F; see Experimental Procedures).

During early development, *src-1* embryos exhibit normal initial asymmetries in blastomere size and division timing and exhibit a wild-type pattern of cell division axes until the six-cell stage (data not shown). All of the *src-1* mutant embryos examined specified endoderm, and laser ablation studies indicated that endoderm was specified correctly in posterior descendant of the EMS blastomere, the E blastomere (Table 1, and data not shown). Approximately 15% of *src-1(cj293)* embryos exhibited a L/R rather than A/P EMS division resulting in L/R daughters that both contact the P2 cell (Table 1). Approximately 60% of *src-1* embryos exhibited a skewed A/P-L/R division (Figure 2H). In these skewed EMS divisions, although the spindle began to elongate at a L/R angle, it ultimately aligned with the A/P axis to produce daughters that occupy A/P positions that were within the norm for wild-type (data not shown). This skewed division axis phenotype was similar to a defect observed in many Wnt pathway mutants (Schlesinger et al., 1999).

In addition to the defects in the EMS division axis, *src-1* mutant embryos exhibit alterations in the divisions of the germline blastomeres P2, P3, and P4. For example, the P3 cell which normally is smaller than, and divides after, its sister cell, named C, was instead equal to C in size in 5 of 37 embryos examined and divided at the same time as C in one of eight embryos examined. Similarly, P4 was equal in size to its somatic sister cell, D, in 19 of 37 embryos examined and five out of eight embryos exhibited a precocious P4 division. Consistent with transformation from a P4 to a D cell fate, we observed muscle differentiation in 18 out of 20 P4 cells isolated by laser ablation (data not shown; see Experimental Procedures). We stained *src-1* embryos with the

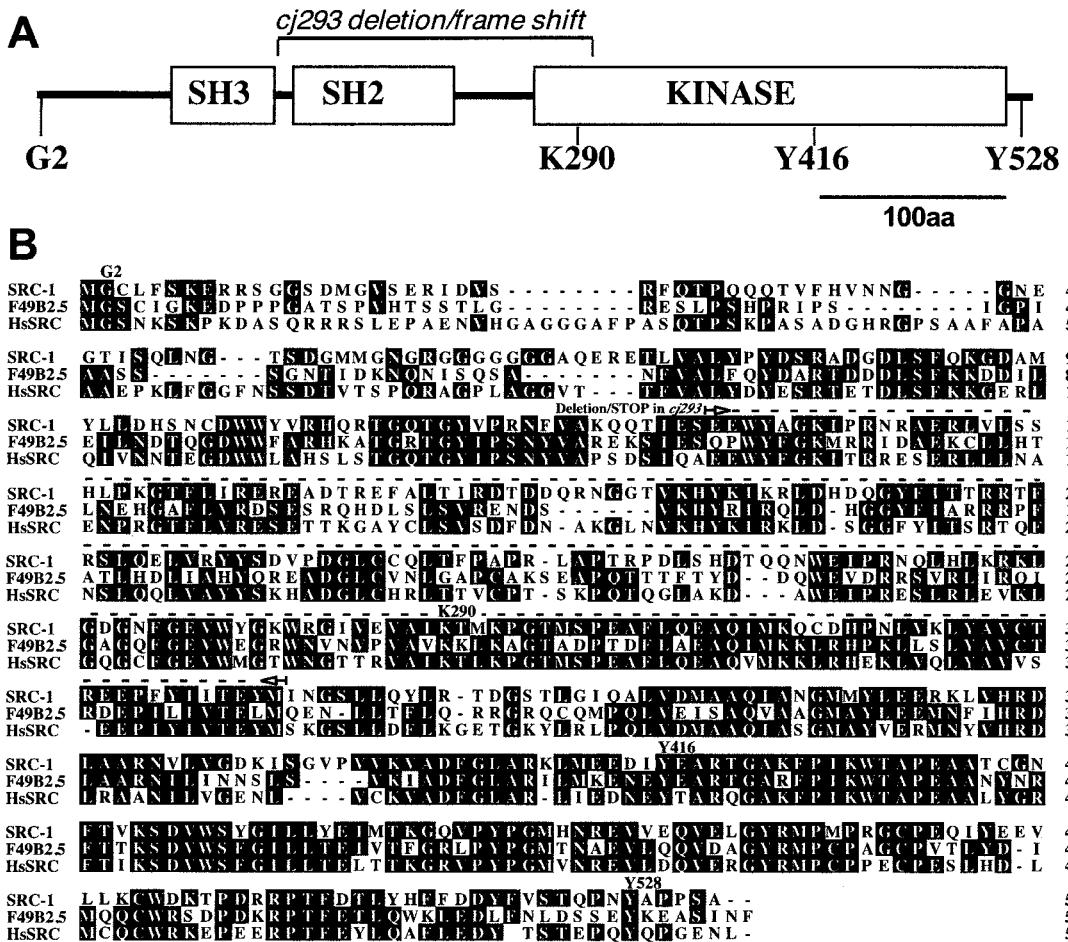


Figure 1. SRC-1 Is a Src Family Kinase

(A) Schematic diagram of protein features and domains conserved in SRC-1. Conserved domains and key residues are indicated, including: the potential myristylation site, glycine 2 (G2), the Src Homology 2 (SH2) and Src homology 3 (SH3) domains, the kinase domain along with the catalytic active-site, lysine 290 (K290), and conserved regulatory tyrosine residues, Y416 and Y528. The region indicated beneath the brackets corresponds to the region deleted in the *cj293* mutant.

(B) Alignment of SRC-1 with a second *C. elegans* Src family kinase (F49B2.5) and human c-Src (HsSRC). The conserved residues indicated in (A) and the position of the *cj293* deletion are indicated above the aligned sequences.

antibody K76, which recognizes the normally germline specific P granules. In wild-type embryos, K76 only stains the P lineage cells, P0, P1, P2, P3, and P4, and at hatching stains only the daughters of P4, Z2 and Z3 (Strome and Wood, 1982). In *src-1* mutant embryos, we found that the P granules were segregated properly to P1 and P2 but were frequently missegregated to both daughters of P2 and P3 as well as to their descendants (Figures 2I–2L; Table 2). Taken together, these findings suggest that SRC-1 is required in the early embryo for proper orientation of the EMS cell division and for proper asymmetry in the P2 and P3 divisions.

We wondered if the morphogenesis defect of *src-1* embryos was caused by the mispositioning of blastomeres due to the early defects in asymmetric divisions, or instead might reflect a second independent role for *src-1* in morphogenesis. We reasoned that the early role for *src-1*(+) in asymmetric division was likely to require maternal *src-1*(+) activity, while the later function in

morphogenesis might be provided either maternally or zygotically. We therefore mated homozygous *src-1*(*cj293*) mothers with wild-type males and analyzed the phenotypes of the resulting heterozygous crossprogeny embryos. We found that these *src-1*(+)/ progeny of *src-1* homozygous mothers were partially rescued for their morphogenesis defects, and approximately 10% (n = 2160) hatched, while 40% (n = 77) underwent extensive body morphogenesis but failed to hatch. The zygotically rescued *src-1*(+)/ progeny exhibited a spectrum of post-embryonic phenotypes including larval lethality and sterility; however, rare fertile adults were also observed. In contrast, the early phenotypes including EMS division orientation defects and the P granule localization defects were not rescued in the *src-1*(+)/ embryos. For example, we found that 16% (n = 18) of EMS blastomeres examined in *src-1*(+)/ embryos divided L/R, and 78% (n = 63) of terminally arrested embryos exhibited mislocalized P granules. These findings indicate that

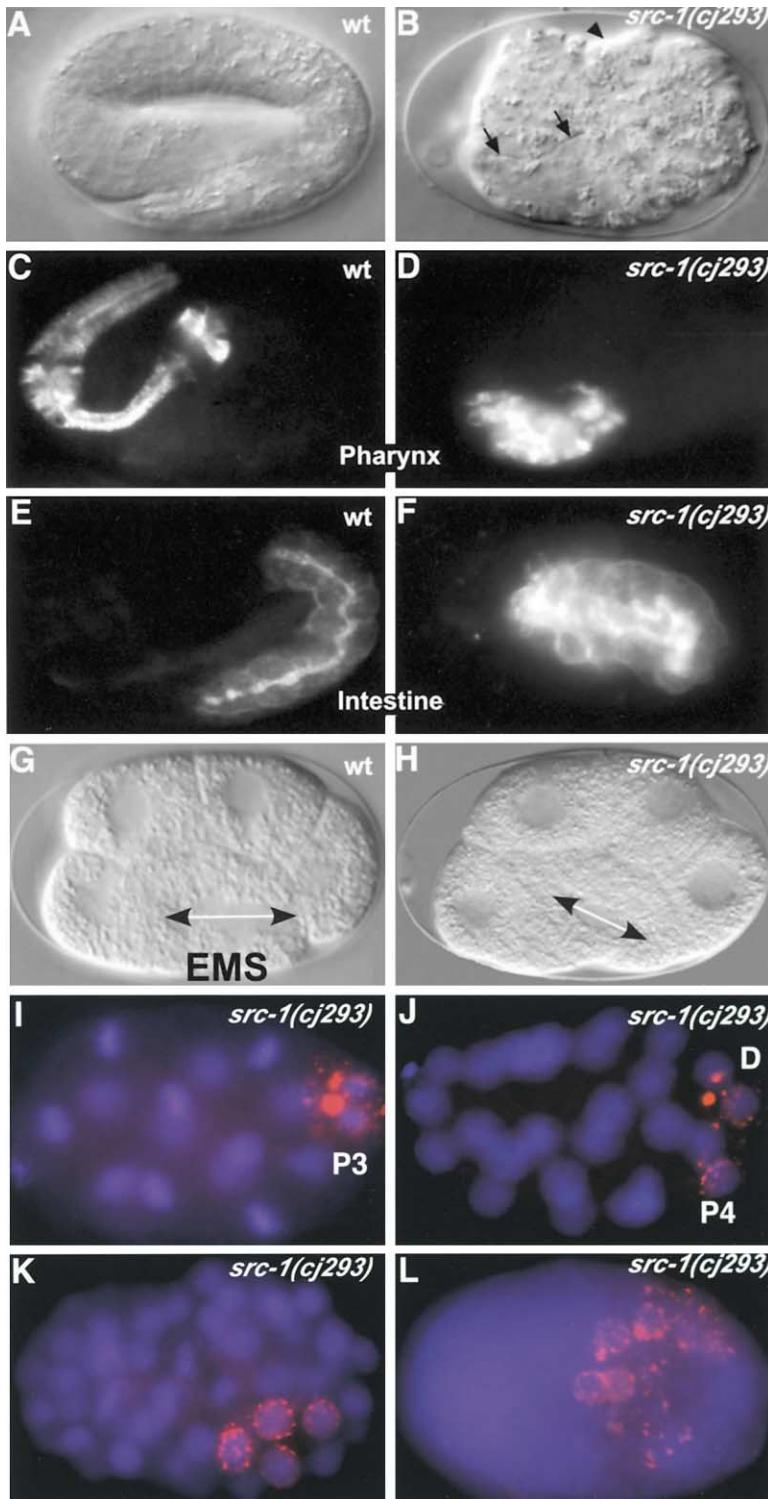


Figure 2. *src-1* Is Required for Morphogenesis and Cell Polarity

(A-F) *src-1* is required for morphogenesis.

(A-B) Light micrographs showing morphogenesis in (A) wild-type and (B) *src-1(cj293)* embryos (anterior is to the left and dorsal is up in all micrographs shown). The wild-type embryo (A) has elongated and is ready to hatch. The *src-1* embryo (B) contains a wild-type number of well-differentiated cells, but has an amorphous appearance with hypodermal cells located on the dorsal side (arrowhead) and normally internal organs including the pharynx (arrows) located outside on the ventral surface of the embryo.

(C-G) Immunofluorescence micrographs showing pharyngeal differentiation (C and D) and intestinal differentiation (E and F) in wild-type and *src-1* embryos. In *src-1* embryos (D and F), both the pharynx and intestine are correctly specified but are disorganized, fail to elongate, and fail to define clear basal-apical polarity.

(G-J) *scr-1* is required for proper cell division polarity.

(G and H) Light micrographs showing a wild-type EMS cell division (G) and a skewed *src-1* mutant EMS cell division (H).

(I-L) Immunofluorescence images showing progressively later staged *src-1* early embryos stained with the P granule monoclonal antibody K76 (red) and nuclear stain DAPI (blue). The embryo (I) is shown with the germline cell P3 in telophase, P granules are distributed uniformly on both sides of the dividing cell. A slightly older 28-cell stage embryo (J) is shown after the P granules have been partitioned into both descendants of P3 (named P4 and D). After one more division (K) and in terminally arrested embryos (L), P granules are observed in 4 and 16 descendants (respectively) of P3.

SRC-1 functions in two distinct developmental events. First, maternally provided SRC-1 functions in the early embryo to control proper specification of EMS and P lineage division axes; second, later in embryogenesis, zygotic and maternal products function together in body morphogenesis.

src-1 and the Receptor Tyrosine Kinase-Related Gene *mes-1* Function Together to Direct Phosphotyrosine Accumulation between EMS and P2

The germline phenotypes observed in *src-1* mutant embryos appeared similar to phenotypes associated with

Table 1. Genetics of P2/EMS Signaling

Embryo type	% Embryos with L-R EMS daughters (n)	% Embryos lacking intestine (n)
<i>src-1(cj293)</i>	15 (59)	0 (n > 500)
<i>src-1(RNAi)</i>	10 (42)	0 (n > 500)
<i>mes-1(bn74)</i>	0 (28)	0 (n > 500)
<i>mes-1(RNAi)</i>	0 (17)	0 (n > 500)
<i>src-1(RNAi);mes-1(bn74)</i>	13 (52)	0 (356)
<i>mom-1(or10)</i>	0 (12)	48 (208)
<i>mom-1(or10),mes-1(RNAi)</i>	100 (11)	100 (97)
<i>mom-1(or10);src-1(RNAi)</i>	100 (9)	100 (104)
<i>mom-2(ne141)</i>	0 (21)	66 (212)
<i>mom-2(ne141);mes-1(bn74)</i>	90 (31)	100 (534)
<i>pop-1(RNAi)</i>	0 (12)	0 (336)
<i>mom-2(ne141);mes-1(bn74);pop-1(RNAi)</i>	91 (22)	0 (389)
<i>mom-2(ne141);src-1(RNAi)</i>	94 (17)	99 (461)
<i>followed by pop-1(RNAi)</i>	n.d.	0 (49)
<i>mom-3(zu21)</i>	0 (14)	56 (182)
<i>mom-3(zu21); mes-1(bn74)</i>	100 (17)	100 (374)
<i>mom-3(zu21);src-1(RNAi)</i>	100 (9)	99 (103)
<i>mom-5(zu193)</i>	0 (11)	4 (137)
<i>mom-5(RNAi)</i>	0 (12)	3 (184)
<i>mom-5(RNAi);mom-1(or10)</i>	0 (12)	18 (81)
<i>mom-5(zu193);mes-1(bn74)</i>	100 (19)	100 (489)
<i>mom-5(zu193);mes-1(bn74);pop-1(RNAi)</i>	100 (16)	0 (197)
<i>mom-5(zu193);src-1(RNAi)</i>	97 (30)	100 (247)
<i>followed by pop-1(RNAi)</i>	nd	0 (79)
<i>dsh-2(RNAi);mig-5(RNAi)</i>	0 (27)	4 (348)
<i>dsh-2(RNAi);mig-5(RNAi);mom-2(ne141)</i>	0 (9)	7 (415)
<i>dsh-2(RNAi);mig-5(RNAi);mom-5(zu193)</i>	0 (11)	8 (351)
<i>dsh-2(RNAi);mig-5(RNAi);mes-1(bn74)</i>	97 (27)	99 (353)
<i>dsh-2(RNAi);mig-5(RNAi);src-1(RNAi)</i>	94 (17)	100 (573)
<i>dsh-2(RNAi);mig-5(RNAi);src-1(cj293)</i>	100 (12)	100 (254)
<i>gsk-3(RNAi)</i>	0 (18)	11(491),E to MS fate 56(22)*
<i>gsk-3(RNAi);mes-1(bn74)</i>	97 (35)	11(255),E to MS fate 100(15)*
<i>gsk-3(RNAi);src-1(RNAi)</i>	95 (22)	11(228),E to MS fate 100(12)*
<i>mom-4(ne19)</i>	0 (15)	46 (384)
<i>mom-4(ne19);dsh-2(RNAi);mig-5(RNAi)</i>	0 (14)	100 (357)
<i>mom-4(ne19);mes-1(bn74)</i>	0 (12)	57 (702)
<i>mom-4(ne19);src-1(RNAi)</i>	12 (34)	98 (273)
<i>apr-1(RNAi)</i>	0 (17)	31 (396)
<i>apr-1(RNAi);dsh-2(RNAi);mig-5(RNAi)</i>	0 (10)	100 (228)
<i>apr-1(RNAi);mes-1(bn74)</i>	0 (19)	33 (218)
<i>apr-1(RNAi);src-1(cj293)</i>	19 (26)	99 (215)
<i>lit-1(RNAi)</i>	0 (18)	100 (237)
<i>lit-1(RNAi);mes-1(bn74)</i>	0 (19)	100 (389)
<i>lit-1(RNAi);src-1(cj293)</i>	16 (19)	100 (187)
<i>wrm-1(RNAi)</i>	0 (15)	100 (372)
<i>wrm-1(RNAi);mes-1(bn74)</i>	0 (24)	100 (447)
<i>wrm-1(RNAi);src-1(cj293)</i>	17 (24)	100 (235)
<i>pop-1(zu189)</i>	0 (14)	0 (n > 500)
<i>pop-1(RNAi);mes-1(bn74)</i>	0 (11)	0 (247)
<i>pop-1(zu189),src-1(RNAi)</i>	9 (22)	0 (364)

nd = not determined.

*In approximately 90% of *gsk-3(RNAi)* embryos, the C blastomere makes intestine (Y.B. unpublished results; Schlesinger et al., 1999). A similar frequency of intestinal differentiation was observed in the C lineage of double mutants *gsk-3;src-1* and *gsk-3;mes-1*.

loss-of-function alleles of a previously described gene, *mes-1*. Like *src-1* mutants, mutations in *mes-1* lead to defects in the asymmetric divisions that give rise to the germline blastomere P4. In *mes-1* mutants, P2 and P3 exhibit a partial loss of polarity and the P granules are mislocalized to both descendants of these divisions (Strome et al., 1995). Also as observed in *src-1* mutants, we found that 63% (n = 27) of *mes-1* mutant embryos exhibited an initially skewed alignment of the EMS spindle. However, whereas 15% of *src-1* mutant embryos

exhibited a fully L/R EMS division axis, we did not observe L/R EMS divisions in the *mes-1* mutant embryos examined (Table 1). Furthermore, unlike *src-1* mutants, the majority (>85%) of *mes-1* mutants properly execute morphogenesis and develop into sterile adults (Strome et al., 1995).

Since both SRC-1 and MES-1 are related to protein tyrosine kinases, we asked if phosphotyrosine, pTyr, staining in the early embryo was correlated with their activities. We found that the pTyr-specific monoclonal

Table 2. *src-1* Is Required for P-Granule Localization

Stage (# of Cells)	Cells with P-Granules	% of Cells with P-Granule (n)
2-3	P1	100 (n>50)
4-7	P2	100 (n>500)
8-15	P3	79 (24)
	P3+C	21 (24)
16-55	P4	21 (51)
	P4+D	69 (51)
	P4+D+C	10 (51)
terminal	Z2+Z3	20 (113)
	4-6 cells	30 (113)
	>10 cells	49 (113)

Embryos were scored at 25°C.

antibody, pY99, stained cell-cell contact sites both in early embryo and throughout development (Figure 3A and data not shown). Interestingly, at the four-cell stage in wild-type embryos, the junctional pTyr staining between P2 and EMS was enhanced relative to other contact sites (Figures 3A, 3C, 3E, and 3F). This enhanced pTyr staining overlapped with MES-1 protein localization throughout the stages when MES-1 protein is detectable, including at the P2/EMS contact site (Figures 3B-3D) as well as the P3/E and P4/Ep contact sites (data not shown).

We next asked if this enhanced pTyr staining required *mes-1*(+) and *src-1*(+) activities. We found that throughout the four-cell stage *mes-1* mutants exhibited normal levels of pTyr staining at other cell junctions but exhibited a reduced level of staining at the P2/EMS junction (compare Figures 3E and 3F to Figures 3G and 3H). Likewise, pTyr staining was reduced at the P3/E and P4/Ep contact sites (data not shown). In contrast, *src-1* mutants exhibited greatly reduced, uniformly low levels of pTyr staining at all cell contact sites, both throughout the four-cell stage (Figures 11 and 1J) and until at least the forty-four-cell stage in development (data not shown). Thus, at the four-cell stage, *mes-1* is required for enhanced pTyr staining at the P2/EMS contact site while *src-1* is required for most of the visible pTyr staining.

The wild-type MES-1 protein is not predicted to encode an active kinase domain (Berkowitz and Strome, 2000), therefore these findings suggest that MES-1 localized at the P2/EMS contact site activates SRC-1 to direct tyrosine phosphorylation of junctional proteins. Consistent with this idea, we found that MES-1 protein localization did not depend on SRC-1 activity (data not shown). Furthermore, we found that double mutants between *mes-1*(*bn74*) and *src-1*(*cj293*) did not exhibit enhanced or novel phenotypes and instead appeared identical to *src-1* single mutants (Table 1). These findings are consistent with the idea that MES-1 functions upstream of SRC-1 in a cell contact-mediated signaling process that controls P lineage asymmetries after the four-cell stage and may also contribute to the specification of the EMS division axis.

src-1 and *mes-1* Interact Genetically with Wnt Signaling Components

The defects in the EMS division orientation of many *src-1* and *mes-1* mutant embryos resemble a partially

penetrant phenotype observed in mutants of several Wnt-pathway related genes required for the P2/EMS cell-cell interaction (Schlesinger et al., 1999). We therefore decided to ask if *src-1* and *mes-1* interact genetically with previously described genes implicated in P2/EMS signaling. To do this, we constructed double mutants between *mes-1* and *src-1* and each of the known components of the P2/EMS signaling pathway (Table 1). We found that several of these double mutants exhibited strong synergistic interactions consistent with a complete loss of P2/EMS signaling. This synergy included an endoderm to mesoderm transformation in the E cell lineage (as assayed by laser ablation; see Experimental Procedures) and a fully L/R division axis in EMS (Table 1). The L/R division in EMS appeared to result from a failure of the nascent mitotic apparatus to rotate onto the A/P axis prior to division. As in wild-type embryos, we observed that the newly duplicated centrosomes in EMS migrated properly around the nuclear envelope ending up positioned correctly on the L/R axis, orthogonal to the previous A/P division axis. In wild-type embryos, astral microtubules at one pole appear to capture a cortical site and rotate the spindle complex onto the A/P axis. This rotation was not observed in the double mutants resulting in a uniform L/R division axis (Table 1 and data not shown).

Synergy was observed between *mes-1* or *src-1* mutants and each of the following previously described mutants: *mom-1* (Porcupine), *mom-2* (Wnt/Wg), *mom-5* (Frizzled), *sgg-1* (GSK-3), and *mom-3* (uncloned). In addition, we observed identical synergies in the phenotypes of embryos produced by *mes-1* or *src-1* homozygotes after injection with a mixture of two double-stranded RNAs targeting the *C. elegans* Disheveled homologs *dsh-2* (C27A2.6) and *mig-5* (T05C12.6). RNAi targeting these Disheveled homologs individually did not induce visible defects in P2/EMS signaling (Table 1 and data not shown).

SRC-1 Signaling and Wnt Signaling Appear to Act in Parallel during P2/EMS Signaling

Previous studies have shown that double mutants between Wnt pathway components fail to exhibit enhanced defects in P2/EMS signaling (Rocheleau et al., 1997; Thorpe et al., 1997). For example, in double mutants between *mom-2/Wnt* and its presumptive receptor, *mom-5/Frizzled*, greater than 90% of embryos produced by homozygous mothers correctly specified intestine from the posterior daughter of EMS and 100% of the EMS blastomeres lineage produced A/P daughters. Thus, the less penetrant maternal-effect gutless phenotype of *mom-5* mutants partially suppresses the more penetrant gutless phenotype of *mom-2* mutants (Rocheleau et al., 1997). This finding suggests that signaling via MOM-5 can interfere with gut specification when the MOM-2 ligand is absent. The *mom-1* gene appears to be the only *C. elegans* homolog of *Porcupine*, a gene required for the proper secretion of Wnt/Wg in *Drosophila* (Kadowaki et al., 1996). Presumptive null alleles of *mom-1* result in egg-laying defects as well as maternal-effect embryonic lethality, phenotypes consistent with defects in both embryonic and postembryonic Wnt signaling (Rocheleau et al., 1997; Thorpe et al.,

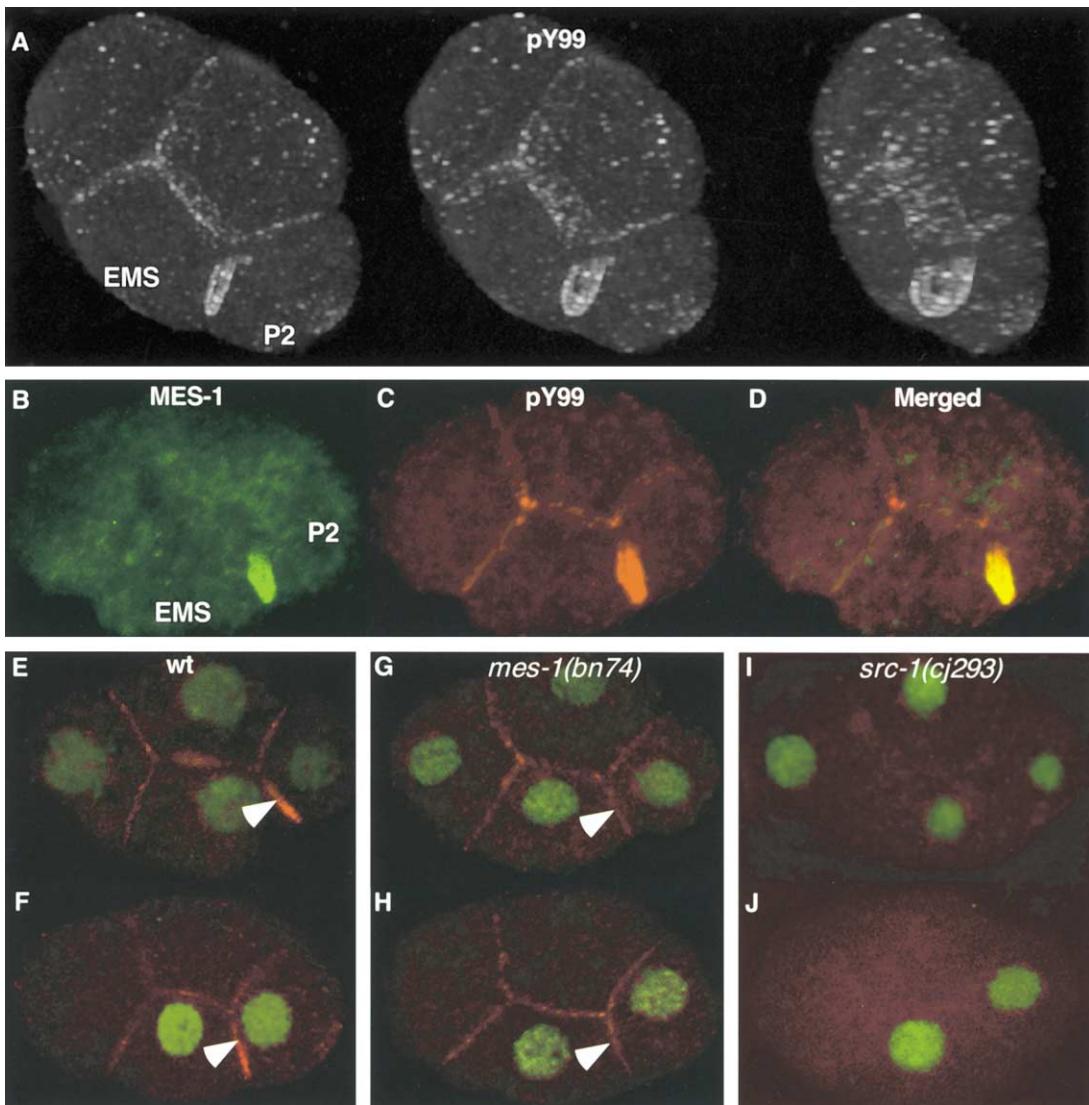


Figure 3. MES-1 and SRC-1 Are Required for Asymmetric Phosphotyrosine Staining in Four-Cell Stage Embryos
 (A) Three-dimensional reconstruction of phosphotyrosine junctional staining in a wild-type embryo. Each successive image is rotated by 5° to reveal the phosphotyrosine signal that lies along the plane of each cell contact.
 (B–D) A single embryo is shown stained with MES-1 specific antibody (B) and with pY99 antibody (C). The merged image (D) reveals extensive overlap (yellow) at the junction between EMS and P2.
 (E–J) Genetic analysis of phosphotyrosine localization during the four-cell stage in wild-type embryos (E and F), *mes-1* mutant embryos (G and H), and *src-1* mutant embryos (I and J). Confocal microscopy was used to image phosphotyrosine, pY99, staining (red), and interphase nuclei (green). Nuclei were stained using an antibody that recognizes an epitope absent in mitotic cells (anterior and dorsal two cells in [F], [H], and [J]).

1997). Double mutants between *mom-1* and *mom-2* do not exhibit enhanced defects in gut specification (Thorpe et al., 1997). Similarly, we found that *mom-5(RNAi)* in the *mom-1(or10)* mutant background failed to enhance P2/EMS signaling defects and instead partially suppressed the gutless defect of *mom-1(or10)*, resulting in 18% rather than 48% gutless embryos (Table 1). Likewise, we found that *dsh-2(RNAi); mig-5(RNAi)* failed to significantly enhance defects in P2/EMS signaling in doubles with *mom-5(zu193)* (Table 1). Furthermore, like *mom-5* mutants, *dsh-2(RNAi); mig-5(RNAi)* partially suppressed the *mom-2* gutless phenotype (Table 1). These findings suggest that MOM-5 and DSH-2;MIG-5 function

together downstream of MOM-1 and MOM-2, and that in the absence of MOM-2 activity, the activities of MOM-5 and DSH-2;MIG-5 can interfere with a parallel mechanism for gut induction.

Taken together, the findings described here suggest that Wnt signaling and Src signaling function in parallel during P2/EMS signaling. In support of this model, all of the existing embryonic lethal mutants in Wnt pathway components, including presumptive null alleles at various steps in the pathway, exhibit only a partial loss of P2/EMS signaling (Rochelleau et al., 1997). Also, double mutant combinations between mutants that affect distinct steps in the Wnt pathway do not exhibit enhanced

P2/EMS signaling defects and can instead result in reduced penetrance of P2/EMS signaling defects (Rocheleau et al., 1997; Table 1). These findings are in contrast to expectations for a combination of weak alleles wherein double mutants are expected to exhibit the phenotype consistent with the stronger allele or may show an enhanced phenotype. Finally, the finding that double mutant combinations between Wnt pathway mutants and *src-1* or *mes-1* cause a nearly total loss of P2/EMS signaling (Table 1) suggests, first of all, that the Wnt components do indeed function together within a pathway and second, that this pathway functions in parallel to *src-1*, *mes-1* signaling.

SRC-1 Signaling and Wnt Signaling Converge to Regulate POP-1

Endoderm induction is correlated with a downregulation of the POP-1 protein in the E blastomere (Rocheleau et al., 1997; Thorpe et al., 1997). Therefore, we examined whether inhibition of *pop-1* could restore endoderm specification in double mutants. Consistent with this idea, we found that inhibiting *pop-1* by RNAi resulted in restored differentiation of the endoderm but did not restore the A/P division orientation of the EMS cell (Table 1). Finally, we examined POP-1 levels in *mes-1(bn74)*; *mom-2(ne141)* and *src-1(RNAi)*; *mom-2(ne141)* double mutants and found that POP-1 protein which is normally restricted to MS as a result of P2/EMS signaling was present at equal, high levels in both daughters of EMS (data not shown). These findings indicate that MES-1/SRC-1 signaling collaborates with Wnt-signaling factors to downregulate POP-1 and to specify the E cell fate.

Several genes previously implicated in P2/EMS signaling behaved as though they function in parallel with both Wnt and Src signaling or lie below the convergence in signaling. These included *mom-4* (TAK1) and *apr-1* (APC), as well as *wrm-1* (β -catenin) and *lit-1* (Nemo/NLK; Table 1). Consistent with previous studies, none of these genes appeared to have a role in controlling the division orientation of EMS (Rocheleau et al., 1997; Thorpe et al., 1997; Schlesinger et al., 1999), and mutants in these genes failed to exhibit synergy for EMS division defects in doubles with either Wnt or Src components (Table 1 and data not shown). For example, just as *mom-4* and *apr-1* mutants enhanced endoderm defects observed in canonical Wnt components (Rocheleau et al., 1997), we found that these mutants also exhibited enhanced endoderm defects in doubles with *src-1* mutants (Table 1). This finding suggests that *mom-4* and *apr-1* function in parallel with both pathways or function downstream of the convergence between Wnt and Src signaling. *mom-4* and *apr-1* exhibited little or no genetic interaction with *mes-1* (Table 1), supporting the idea that *src-1* is more critical than *mes-1* for P2/EMS signaling (see Discussion).

The finding that *wrm-1* and *lit-1* do not exhibit synergy for the control of EMS division orientation (Table 1) is consistent with previous data that place WRM-1 and LIT-1 as direct regulators of POP-1 (Ishitani et al., 1999; Meneghini et al., 1999; Rocheleau et al., 1999). Taken together, these findings are consistent with a model in which MES-1 and SRC-1 function in parallel with certain upstream Wnt signaling factors and converge with this

parallel pathway to downregulate POP-1 and to specify the A/P division axis of EMS. Finally, consistent with this model, we found that MES-1 protein localization and phosphotyrosine accumulation were not affected in the Mom mutants examined including *mom-2*, *mom-3*, *mom-4*, *mom-5*, and *wrm-1* (Berkowitz and Strome, 2000 and data not shown).

Autonomy and Nonautonomy in P2/EMS Signaling

Previous studies on P2/EMS signaling described methods that permit isolated blastomeres and chimeric embryos to be cultured in vitro (Goldstein, 1992). We used these techniques to examine the genetic requirements for EMS spindle orientation in four-cell stage embryos. As shown previously (Goldstein, 1995b), isolated wild-type EMS cells placed back in contact with P2 (but not two EMS cells placed together), correctly orient their mitotic spindles in accordance with the P2 contact site (Figures 4A and 4B). We next examined chimeric embryos assembled using a combination of wild-type and *src-1* mutant blastomeres. We found that chimeric embryos lacking *src-1* function in P2 exhibited a wild-type orientation of the EMS division axis (Figure 4C), while embryos lacking *src-1* function in EMS always failed to do so (Figure 4D). An identical set of experiments were performed on *mes-1* mutant embryos. Interestingly, we found that *mes-1(+)* activity was required in both EMS and P2 to orient the mitotic spindle of EMS (Figures 4E and 4F). Thus, SRC-1 functions cell autonomously in EMS to control the EMS division axis, while MES-1 functions in both cells.

We next wanted to examine the genetic requirements for MES-1 protein localization. To do this, we first removed P2 from a wild-type four-cell embryo and then immediately placed it back in a new orientation. We found that when P2 was placed back in such a way that it made contact with EMS, MES-1 protein localization was restored at the newly established P2/EMS contact site (arrow in Figure 4G). MES-1 protein localization was never observed at other P2 cell contacts. For example, when placed away from EMS in contact only with the anterior blastomeres, no MES-1 protein was observed at P2 cell contact sites (Figure 4H). As described previously (Berkowitz and Strome, 2000), a remnant of the previous P2/EMS junctional staining was almost always retained in the P2 cell (see arrowheads in Figures 4G, 4H, and 4K). Consistent with the idea that MES-1 is required in both cells, the junctional MES-1 staining was not restored if MES-1 was absent in either P2 or EMS (Figures 4I and 4J). As expected from previous staining of intact *src-1* mutant embryos, *src-1(+)* activity was not required in either P2 or EMS for MES-1 protein localization (data not shown). Furthermore, as in intact embryos, we found that intense pTyr staining at junctions was correlated with MES-1 protein localization at those junctions (data not shown). Thus, just as proper control of EMS division orientation requires MES-1 in both cells, MES-1 protein localization at the P2/EMS contact site also requires MES-1 protein in both cells.

We noticed that although junctional staining was only observed at the P2/EMS contact site, all blastomeres including the AB blastomeres contain abundant MES-1 protein. This nonjunctional MES-1 protein often appeared to localize as small punctae, perhaps indicating

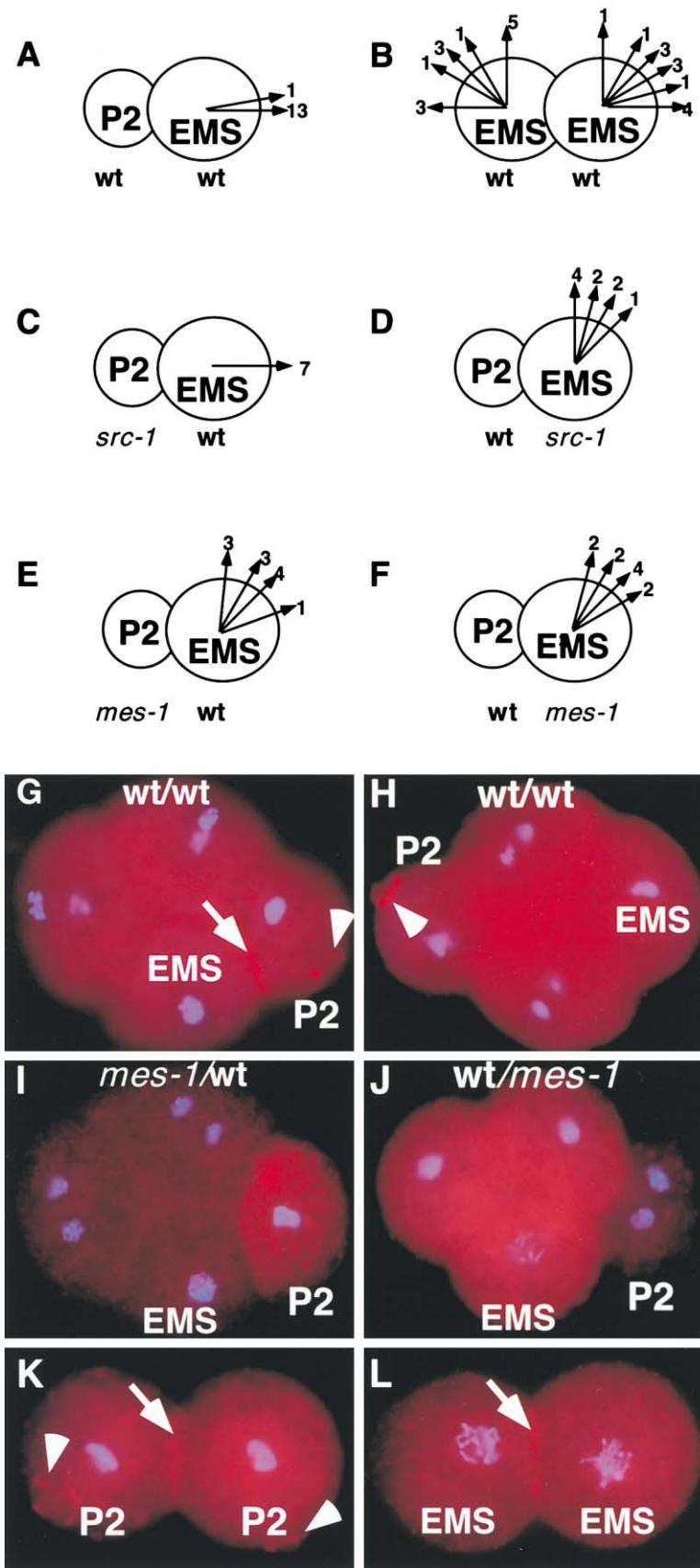


Figure 4. Autonomy and Nonautonomy in SRC-1/MES-1 Signaling

(A–F) Diagrams representing experiments in which early blastomeres from one or more embryos were first isolated and then placed in contact with each other as shown. The arrows indicate the direction of the EMS cell division and the number in front of the arrow indicates the number of experiments in which that orientation of division was observed. The origins of each blastomere, *wt* (wild-type), *mes-1*, or *src-1* are indicated beneath each diagram. The division axis of the P2 cell was not determined.

(G–L) P2/EMS, EMS/EMS, or P2/P2 contacts can direct MES-1 protein localization. Immunofluorescence micrographs showing MES-1 protein localization (red) and nuclei stained with DAPI (blue). Embryos were assembled from wild-type or mutant blastomeres as indicated.

(G and H) Wild-type chimeras assembled by first removing P2 and then placing it back in contact either in its correct, posterior position, next to EMS (G) or at the anterior, away from EMS (H).

(I and J) Similar experiments in which either EMS (I) or P2 (J) was derived from a *mes-1* mutant embryo.

(K and L) Chimeras assembled by placing two wild-type P2 cells (K) or two wild-type EMS cells (L) in contact with each other. The arrows in (G), (K), and (L) indicate restored MES-1 staining. A remnant of cortical MES-1 associated with the previous P2/EMS contact is indicated with an arrowhead (G, H, and K).

the existence of MES-1-containing vesicles in the cytoplasm (compare *mes-1*(-) and *mes-1*(+) blastomeres in Figures 4I and 4J). This punctate appearance was often especially intense near the former EMS contact site within P2 or EMS cells (Figures 4G, 4H, and 4K, and data not shown). These findings suggest that there is some property unique to the P2/EMS junction that permits MES-1 to relocate from the cytoplasm to the cell-cell junction. In order to ask if this ability to recruit MES-1 to the junction required factors unique to P2 or EMS, we placed wild-type P2 in contact with wild-type P2 (Figure 4K) and wild-type EMS in contact with wild-type EMS (Figure 4L). We found that in both types of experiment the junctional MES-1 staining was restored (Figures 4K and 4L). Thus, P2/EMS, EMS/EMS, and P2/P2 cell contacts can all induce cortical localization of MES-1 protein, while cell contacts with or between other blastomeres fail to do so. Taken together, these studies indicate that P2 and EMS undergo a dynamic interaction that involves signaling in both directions.

Discussion

Convergence between SRC and Wnt Signaling

The present study provides evidence for a genetic *in vivo* link between the Wnt and Src pathways during P2/EMS signaling. We have shown that mutations in six different genes that are implicated in a canonical Wnt-like signaling pathway, including *mom-1* (Porcupine), *mom-2* (Wnt/Wg), *mom-3* (uncloned), and *mom-5* (Frizzled), as well as two Disheveled homologs *dsh-2* and *mig-5*, all exhibit identical synergistic interactions with a *C. elegans* Src family tyrosine kinase, *src-1*. Furthermore, we have shown that SRC-1 functions together with a second, novel protein, MES-1, related to receptor type tyrosine kinases. Together, SRC-1 and MES-1 are required for the enhanced accumulation of phosphotyrosine at the junction between EMS and P2 and function along with Wnt signaling components to specify endoderm and the A/P division orientation of the EMS cell.

Our findings indicate that the activities of the Wnt and Src pathways independently target a common set of downstream factors that control the developmental outcomes of P2/EMS signaling. The alleles analyzed include presumptive null alleles in both Wnt signaling and Src signaling components, and more importantly, synergy occurs only between components of distinct pathways and not between components of the same pathway. For example, double mutants between *mom-2* (Wnt) and its presumptive receptor *mom-5* (Frizzled) fail to exhibit synergy and exhibit wild-type P2/EMS signaling in greater than 90% of the embryos analyzed, a frequency similar to that observed in *mom-5* single mutants (Rochefeuille et al., 1997). In contrast, double mutants between either *mom-2* or *mom-5* and *src-1* exhibit striking synergy and lack endoderm in nearly 100% of the embryos analyzed. The lack of synergy between mutants that affect different steps within the same pathway suggests that the alleles used strongly inhibit signaling through the pathway. Thus it is not plausible, for example, that *src-1* mutants enhance P2/EMS signaling defects by reducing signaling through the Wnt pathway. Rather, these findings indicate that the Wnt and Src

pathways are redundant and converge to regulate a common set of downstream factors that control cell fate as well as the orientation of cell division (see model, Figure 5).

MES-1/SRC-1 Signaling

Our findings suggest that the P2 and EMS cells signal in both directions via MES-1 and SRC-1. In the EMS cell, MES-1/SRC-1 signaling functions in parallel with Wnt signaling, as discussed above, while in P2 it functions to control germline asymmetries including the localization of the P granules. MES-1 is predicted to be a single-pass transmembrane protein with a 471 amino acid extracellular domain and a 500 amino acid cytoplasmic domain with similarity to receptor-type protein tyrosine kinases (Berkowitz and Strome, 2000). MES-1 may therefore function directly as both ligand and receptor in a homotypic interaction between P2 and EMS. Although all early blastomeres contain MES-1, the junctional localization of MES-1 is observed only at the P2/EMS contact site. The localization of MES-1 protein to the cell junction requires MES-1 protein in both EMS and P2, but can also occur when two EMS or two P2 cells are placed in contact. The P2/EMS interaction that leads to MES-1 protein localization does not require SRC-1 or any of the Wnt signaling factors examined in this study. Thus, MES-1 protein and other as yet unidentified factors unique to P2 and EMS are required for MES-1 to become localized to the cell junction.

Interestingly, despite the fact that both EMS and P2 can direct MES-1 protein localization to the cortex, cortical remnants containing MES-1 protein are often found on P2 but rarely on EMS. Thus, MES-1 is either more firmly anchored at the cortex of P2, or P2 differs from EMS in having a slower recycling process for removing cortical MES-1. These findings suggest a possible explanation for previous observations that P lineage cells, including P2, can retain their polarity when isolated (Goldstein, 1995b; Schierenberg, 1988). The retention of cortical MES-1 in P2 may reflect a greater potential for P lineage cells (in general) to retain localized cortical factors. Retention of localized cortical factors from one cell division to the next could in turn serve to provide an intrinsic source of polarity in P lineage cells.

Signaling downstream of MES-1 requires SRC-1 activity in the EMS cell. However, it is clear that SRC-1 has additional activities that do not depend on MES-1 activity. SRC-1 is required for phosphotyrosine staining that outlines each cell-cell junction in the early embryo until at least the forty-four-cell stage, and *src-1* mutants arrest embryogenesis without completing body morphogenesis. Furthermore, SRC-1 directs basal levels of tyrosine phosphorylation at the P2/EMS junction even in the absence of MES-1. Perhaps consistent with this finding, SRC-1 is more critical than MES-1 in controlling EMS division orientation and endoderm induction (see Table 1). For example, *src-1* mutants exhibited L/R EMS divisions in 15% of embryos examined, while *mes-1* single mutants were never observed to undergo L/R EMS divisions. Similarly, *src-1* mutants but not *mes-1* mutants enhanced endoderm defects of *mom-4* and *apr-1*. These findings suggest that SRC-1 signals from the P2/EMS cell junction even when MES-1 is absent. Alternatively,

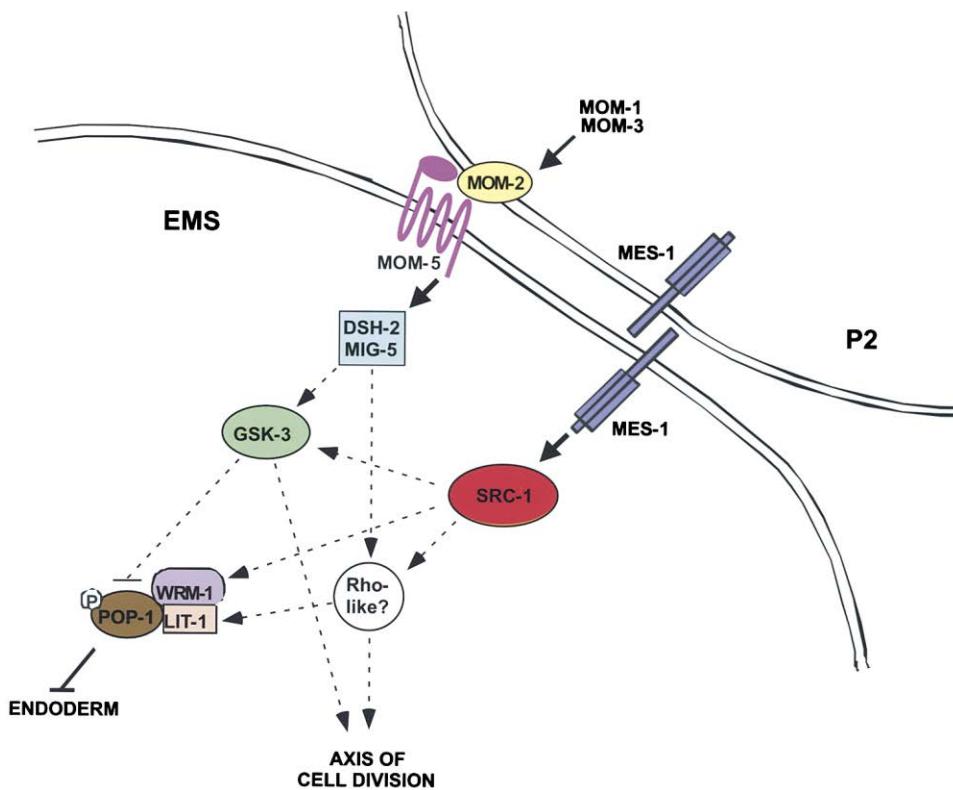


Figure 5. Model for Induction of Endoderm and Cell Division Axis in EMS

it is possible that Wnt signaling or other signaling pathways contribute to SRC-1 activation.

A Network of Signaling Downstream of Cell Contacts

The Wnt and Src signaling pathways converge to control diverse cellular and genetic targets (Figure 5). For example, both pathways control EMS division orientation and both contribute to asymmetries in the level of the TCF/LEF-related protein POP-1. POP-1 is downregulated in response to phosphorylation by a protein kinase complex that consists of WRM-1 (β -catenin) and LIT-1 (Nemo/NLK) (Ishitani et al., 1999; Meneghini et al., 1999; Rocheleau et al., 1999). Thus, Wnt and Src signaling could downregulate POP-1 by activating WRM-1 or LIT-1. The GSK-3 protein functions positively along with MOM-2 (Wnt/Wg) in P2/EMS signaling and may negatively regulate POP-1. Although we have indicated potential activation of GSK-3 by both pathways (Figure 5), the genetics of GSK-3 suggests that it has multiple functions and may function in a constitutive rather than activated manner (Figure 5; Schlesinger et al., 1999; and our unpublished data).

The literature on Src and Wnt signaling suggests numerous potential convergence points for these signaling pathways (dashed lines in Figure 5). These include potential convergence directly on WRM-1 (β -catenin) (Hinck et al., 1994; Papkoff, 1997), on GSK-3 (Hughes et al., 1993; Kim et al., 1999; reviewed in Peifer and Polakis, 2000; Wang et al., 1994), or on G proteins of the Rho family (Arthur et al., 2000; Billuart et al., 2001;

Crespo et al., 1997; Habas et al., 2001; Winter et al., 2001). We have shown that two *C. elegans* Disheveled-related genes, *dsh-2* and *mig-5*, contribute to P2/EMS signaling and exhibit a phenotype very similar to that of *mom-5* (Frizzled). In other systems, Disheveled is thought to act downstream of Frizzled and behaves like a branchpoint in signaling via β -catenin/Armadillo and via Rho and MAP kinase (Boutros et al., 1998; Strutt et al., 1997). In *C. elegans*, P2/EMS signaling was previously shown to involve both canonical Wnt-like signaling via WRM-1 (β -catenin) and via proteins similar to MAP-kinase (LIT-1) and MAP kinase kinase kinase (MOM-4) (Ishitani et al., 1999; Meneghini et al., 1999; Rocheleau et al., 1999; Shin et al., 1999). Perhaps P2/EMS signaling via both Wnt and Src pathways activates an as yet unidentified Rho-like G protein that in turn directs spindle orientation through its effects on the actin cytoskeleton and activates endoderm induction via a MAP kinase-like signaling cascade that targets LIT-1 (Figure 5).

Recent work on dorsal closure in *Drosophila* has identified a possible convergence between Src and Wnt signaling at the level of regulation of the Jun N-terminal kinase (JNK). Dorsal closure is the process in which epithelial sheets spread over and enclose the dorsal region of the *Drosophila* embryo during morphogenesis. JNK signaling is essential for dorsal closure and mutants lacking JNK exhibit a dorsal-open phenotype and also exhibit loss of expression of a TGF- β homolog *decapentaplegic* (*dpp*) in the epithelial cells that lead the closure process (Hou et al., 1997; Riesgo-Escovar and Hafen,

1997). Recent genetic studies have implicated both Src-like kinases (Tateno et al., 2000) and Wnt signaling components (McEwen et al., 2000) in the dorsal closure process and in regulating the expression of *dpp*. Mutations in Src and Wnt signaling factors produce dorsal closure phenotypes similar to JNK mutants and activation of JNK signaling can partially suppress defects caused by these mutants. These findings suggest that Wnt and Src may converge to regulate JNK activity and dorsal closure in *Drosophila* and thus provide evidence from another system for interactions between these pathways in a developmental process.

Our studies in *C. elegans* indicate that Wnt and SRC signaling function redundantly in a relatively simple developmental interaction between two early embryonic cells. The genetic tools developed here should facilitate the identification of additional components of both pathways. In the future, it will be interesting to learn whether convergence between these two important signaling pathways occurs on a single key intermediate, or instead as seems more likely, occurs on multiple levels to coordinate control both cell fate and cell division axis.

Experimental Procedures

Strains and Alleles

The Bristol strain N2 was used as the standard wild-type strain. Marker mutations, deficiencies, and balancer chromosomes used are listed by chromosome as follows: *LG1*: *unc-13(e51)*, *mom-4(ne19)*, *mom-5(zu193)*, *pop-1(zu189)*, *src-1(cj293)*, *szT1(l; X)*; *LGII*: *unc-4(e120)*, *mom-3(zu21)*, *mnc1*; *LGIV*: *him-3(e1147)*; *LGV*: *dpy-11(e224)*, *mom-2(ne141)*; *X*: *mes-1(bn74)*, *unc-6(n102)*, *mom-1(or10)*. *C. elegans* culture and genetics were as described in Brenner (1974).

Isolation of the *src-1* Gene and Deletion Allele

The *src-1* gene was identified by screening a mixed stage *C. elegans* cDNA library with a *v-src* probe. The *src-1* cDNA sequence differs from the GeneFinder predictions for Y92H12A.1 and is detailed in the GenBank accession number listed in this paper. To identify a mutation in *src-1*, approximately 30,000 haploid genomes were screened by PCR according to protocols of Zwaal et al. (1993).

Microinjection and Molecular Biology

RNAi was performed as described in Fire et al. (1998) and Rocheleau et al. (1997). *src-1* dsRNA was prepared from a full-length *src-1* cDNA. yk365b3, yk46b11, and yk55h11 were used to prepare dsRNA for *mes-1*, *mig-5*, and *dsh-2*, respectively.

Microscopy

Light and immunofluorescence microscopy and laser microsurgery were described previously (Rocheleau et al., 1997). Tissue differentiation of *src-1* embryos was analyzed using morphological criteria at the light microscope and with tissue-specific antibodies. Antibodies used include: monoclonal antibodies 9.2.1 and J126 to visualize pharyngeal muscle and intestinal valve cells, respectively. The P4 cell fate was determined by isolating the P4 blastomere using laser microsurgery to kill other blastomeres. POP-1 staining was done as described (Lin et al., 1995). pY99 and MES-1 staining were done using the POP-1 immunostaining protocol with one modification; after freeze cracking and methanol fixation, the slides were incubated for 5 min in ice-cold acetone and then air-dried. pY99 antibody was obtained from Santa Cruz Biotechnology and used at 1:300 dilution.

Blastomere Isolation

Blastomeres were isolated as described by Edgar and Wood (1993) and Goldstein (1995b) using predrawn 35 μ m glass capillaries (FHC Incorporated, Bowdoinham, ME). Briefly, after chitinase treatment, embryos were transferred into SGM medium with 35% calf serum.

The blastomeres were then separated by mouth pipetting them through the glass capillary. For the EMS cleavage axis experiments, P1 was isolated from two-cell stage embryo. EMS and P2 were then separated right after P1 divided. Blastomeres were immediately put in contact with each other and EMS division axis was examined under light microscope. Reconstituted blastomeres were cultured for 5 min or more, fixed with 4% paraformaldehyde (5 min), and washed with Tris-Tween (5 min) followed by antibody incubation.

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