Establishment of POP-1 asymmetry in early *C. elegans* embryos

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SUMMARY

In *Caenorhabditis elegans* embryos, the nuclei of sister cells that are born from anterior/posterior divisions show an invariant high/low asymmetry, respectively, in their level of the transcription factor POP-1. Previous studies have shown that POP-1 asymmetry between the daughters of an embryonic cell called EMS results in part from a Wnt-like signal provided by a neighboring cell, called P2. We identify here additional signaling cells that play a role in POP-1 asymmetry for other early embryonic cells. Some of these cells have signaling properties similar to P2, whereas other cells use apparently distinct signaling pathways. Although cell signaling plays a critical role in POP-1 asymmetry during the first few cell divisions, later embryonic cells have an ability to generate POP-1 asymmetry that appears to be independent of prior Wnt signaling.

Key words: *C. elegans*, Frizzled, polarity, POP-1, Wnt

INTRODUCTION

The pattern of cell cleavage and differentiation in *C. elegans* is largely invariant (Sulston et al., 1983; Schnabel et al., 1997). The spindles of most cells are oriented such that mitosis generates an anterior daughter and a posterior daughter, and most of the early anterior/posterior (a/p) cell divisions result in sister cells with different fates. Although these fates are determined through diverse pathways, several studies have suggested that cells throughout the embryo recognize a common a/p coordinate system in choosing their fate (Mello et al., 1992; Hutter and Schnabel, 1994; Mango et al., 1994; Moskowitz et al., 1994; Way et al., 1994). Recent studies have provided considerable evidence for this hypothesis, and have begun to identify molecular components of the a/p system (Lin et al., 1995; Lin et al., 1998; Kaletta et al., 1997; Meneghini et al., 1999; Rocheleau et al., 1997; Rocheleau et al., 1997; Thorpe et al., 1997; Maduro et al., 2002). The transcription factor POP-1 is present in the nuclei of all early embryonic, and several postembryonic, cells. After an embryonic cell divides, its anterior daughter invariably shows a higher level of nuclear POP-1 than its posterior daughter. Thus each a/p pair of sister cells exhibits POP-1 asymmetry with a reproducible high/low polarity. Conditions that result in high POP-1 in the posterior sister cause an anterior transformation in fate, whereas the absence of POP-1 in the anterior sister causes a posterior transformation in fate. POP-1 functions in a variety of cell-type decisions, including mesodermal/endodermal choices and epidermal/neuronal choices. These observations suggest that POP-1 asymmetry provides the a/p coordinate system that collaborates with more widely expressed transcription factors to diversify sister cells.

POP-1 is related to TCF/Pangolin, a transcriptional effector of the canonical Wnt signaling pathway, and POP-1 has been shown to function in canonical Wnt signaling during larval development (Lin et al., 1995; Herman, 2001). However, POP-1 asymmetry in the early embryo is regulated by a non-canonical Wnt pathway, with parallel input from a mitogen-activated protein kinase (MAPK) pathway (for a review, see Korswagen, 2002). Components of these pathways include MOM-2/Wnt, MOM-5/Frizzled, WRM-1/beta-catenin, MOM-4/MAPKKK/TAK1 and LIT-1/Nemo. Sister cells show POP-1 asymmetry because they differ in their nucleo/cytoplasmic distributions of POP-1 (Maduro et al., 2002). Studies with cultured vertebrate cells suggest that WRM-1/beta-catenin can activate LIT-1/Nemo, resulting in phosphorylated POP-1 that accumulates in the cytoplasm (Rocheleau et al., 1999).

How is the a/p polarity system established? The most detailed experimental studies to date have focused on the development of the EMS cell (Goldstein, 1992; Goldstein, 1993). EMS divides into an anterior, mesodermal precursor and a posterior, endodermal precursor. This a/p polarity is induced during the 4-cell stage of embryogenesis by a neighboring cell called P1. For example, removing P1 causes both EMS daughters to have anterior fates (high POP-1) and repositioning P2 on the opposite surface of EMS reverses the polarity of the division.

Relatively little is known about the cellular events that establish a/p polarity in other embryonic cells. At the 2-cell stage, the posterior cell is called P1 (the parent of P2 and EMS) and the anterior cell is called AB (see Fig. 1). Within the AB lineage, POP-1 asymmetry is first detectable after the third division of AB, when there are four a/p sister pairs of AB descendants (Lin et al., 1998); for convenience we refer to this stage as the AB8 stage. POP-1 function is essential for a/p differences in cell fate within each of the four sister pairs of AB8 cells (Lin et al., 1998). Previous studies on how a/p differences are generated in the AB lineage have reached contradictory conclusions. In one set of experiments, AB was...
separated from P₁ and allowed to develop to the AB₁₆ stage (Wittmann et al., 1997). As many as eight of the AB₁₆ cells expressed a transgenic marker that normally is expressed in the eight posterior AB₁₆ cells, suggesting that AB has an inherent a/p polarity. In a different study, videomicroscopy was used to follow AB development after killing P₁ or P₁ descendants (Hutter and Schnabel, 1995). Several AB₈ cells showed posterior to anterior transformations in fate after killing P₁, but not P₁ descendants, suggesting that P₁ induces an a/p polarity in AB that is maintained in a latent form until the AB₈ stage.

To further analyze the cellular basis for a/p polarity in the AB lineage, we analyzed POP-1 levels directly by immunostaining isolated and cultured embryonic cells. Our results indicate that POP-1 asymmetry at the AB₈ stage results from interactions with specific P₁ descendants, rather than with P₁. These interactions are mediated in part by MOM-2/Wnt signaling. Surprisingly, by the AB₁₆ stage embryonic cells have acquired an ability to generate POP-1 asymmetry that appears to be independent of MOM-2/Wnt signaling or prior interactions with other cells, but that requires MOM-5/ Frizzled.

MATERIALS AND METHODS

Strains and alleles
Nematodes were cultured as in Brenner (Brenner, 1974). The wildtype Bristol strain N2 was used. The following mutations were used. LGL, mom-5(or57), dpy-4(or39) and unc-13(e1091); LGV, mom-2(or42) and dpy-11(e1180); LGX, mom-1(or10) and unc-6(n102). The following integrated transgene containing green fluorescent protein (GFP) reporter was used: zds3 (end-1::GFP) (Nance and Priess, 2002).

Cell isolations
Individual embryonic cells were isolated from devitellinized embryos by gently drawing embryos in and out of a drawn-out capillary needle as described previously (Edgar, 1995). Cells were cultured in medium consisting of 5% L-15 (Gibco), 10% fetal calf serum (Gibco), and 4.7% sucrose, adjusted to 320-330 mOsm. To devitellinize embryos, eggs were placed in hypochlorite solution [6% NaOCl, 2.5 N KOH] on an inverted microscope slide for 3.5 minutes, then rinsed three times with 0.25 M HEPIES, pH 7.0 before transfer to culture medium.

Immunostaining and analysis of embryos and cultured blastomeres
Isolated cells were micropipetted directly into a drop of fixative [2% paraformaldehyde, 60 mM PIPES, 25 mM HEPIES [pH 6.8], 10 mM EGTA, 2 mM MgCl₂] on a poly-L-lysine (Sigma)-coated glass slide. After five minutes, excess fixative was removed and slides were placed in –20°C acetone for 5 minutes. Cells were rinsed twice in Tris-Tween [100 mM Tris-HCl (pH 7.5), 200 mM NaCl, 0.1% Tween], then incubated with 10% normal goat serum (Gibco) in Tris-Tween at room temperature for 30 minutes. Cells were then incubated overnight at room temperature with primary antisera. Fixation and immunostaining of intact embryos was as described previously (Lin et al., 1998). The following dilutions of antibodies/antisera were used: anti-POP-1 [1:2500 mouse mABR1L (P4G4) (Lin et al., 1998)]; midbody staining [1:50 rabbit anti-PKL-2 (kindly provided by M. Land)]; P-granules [1:17000 rabbit anti-PGL-1 (Kawasaki et al., 1998)]. Secondary antibodies were conjugated to either Cy-3 (Jackson ImmunoResearch Laboratories) or FITC (Tago). Cells were stained with DAPI (4,6-diamidino-2-phenylindole) at 60 ng/ml for five minutes. POP-1 was scored only when the PKL-2 staining pattern provided an unambiguous indication of sister pairs, thus some results are scored by the number of sister pairs rather than by the number of experiments.

dsRNA-mediated interference (RNAi)
Standard techniques were used to synthesize double-stranded RNA (dsRNA) from T7 promoter-tagged, PCR-amplified genomic DNA for mom-2, mom-5, goa-1, and gpa-16. PCR primers were chosen to span exons and generate fragments between 0.5 and 2 kb in size. L4 or young adult hermaphrodites were soaked overnight with dsRNA.

RESULTS

POP-1 asymmetry in wild-type embryos
The first division of the fertilized egg results in an anterior cell called AB and a posterior cell called P₁ (Fig. 1). Each of the descendants of these cells has a distinct name in C. elegans nomenclature, however it is convenient here to refer to the AB descendants collectively. For example, there are two AB daughters in a 4-cell embryo, so we refer to these daughters as the AB₂ cells and to the developmental stage as the AB₂ stage. The descendants of the P₁ cell are indicated by their standard names such as MS or E. Previous reports have analyzed POP-
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POP-1 asymmetry in all cells up to the 28-cell stage, and POP-1 asymmetry has been observed in E descendants and a few MS descendants in later embryos (Lin et al., 1998; Maduro et al., 2002). To identify sister cells in older embryos and in cell culture experiments, we used a POP-1 antibody in conjunction with an antiserum against the kinase PKL-2. This antiserum stains the midbody, or cell-division remnant, between sister pairs. For each of the sister pairs indicated in D (small arrows), the anterior sister has low POP-1. POP-1 staining is cell cycle-dependent and not detected in prophase or anaphase nuclei (large arrows in B,D) (see also Lin et al., 1995). In all panels anterior is left; in B,D and F ventral is down (arrowhead indicates the P4 cell).

1 asymmetry in all cells up to the 28-cell stage, and POP-1 asymmetry has been observed in E descendants and a few MS descendants in later embryos (Lin et al., 1998; Maduro et al., 2002). To identify sister cells in older embryos and in cell culture experiments, we used a POP-1 antibody in conjunction with an antiserum against the kinase PKL-2. This antiserum stains the midbody, or cell-division remnant, between sister cells (small arrows in Fig. 2B) (M. Land and C. S. Rubin, unpublished). Using fixed and immunostained embryos at various stages, we identified each of the first 32 descendants of the MS cell, all of the AB8, AB16 and AB32 sister pairs, and a large subset of the AB64 and AB128 sister pairs (Fig. 2A,B and Materials and Methods). For each sister pair, POP-1 was high in the anterior sister and low in the posterior sister, suggesting that POP-1 asymmetry is reiterated with the same polarity after most, if not all, a/p divisions within the embryo.

**P1 descendants are required for POP-1 asymmetry at the AB8 stage**

In normal embryogenesis, POP-1 asymmetry is first evident in AB descendants at the AB8 stage (Lin et al., 1998). To examine POP-1 in cultured cells, we removed the eggshell from 2-cell stage embryos and allowed the devitellinized embryos to develop to the AB8 stage (see Fig. 1 for a comparison of cell positions in normal and devitellinized embryos). POP-1 asymmetry was observed between most sister pairs of AB8 cells in devitellinized but otherwise intact embryos (78%, 32 sister pairs scored) and in embryos in which AB and P1 were separated then immediately recombined (78%, 32 sister pairs). POP-1 asymmetry was not detectable between any sister pairs of AB8 cells when AB was kept separate from P1 (0%, 56 sister pairs scored; Fig. 3A,B) or when AB was allowed to contact P1 only through the first cell cycle (0%, 44 sister pairs). These results indicate that signaling from P1 is not sufficient for POP-1 asymmetry between sister pairs of AB8 cells, and instead suggest that P1 descendants influence POP-1 asymmetry.

**AB cleavage orientation and P2 signaling**

In *C. elegans* and other animals, Wnt proteins are secreted and can signal non-adjacent target cells (Whangbo et al., 2000; Zecca et al., 1996; Neumann and Cohen, 1997). Because P2, a daughter of P1, expresses MOM-2/Wnt and signals EMS, in principle P2 might signal the AB descendants. However, a previous study showed that P2 is not essential for POP-1
asymmetry in the AB^2^ cells (Lin et al., 1998). We have confirmed this result using our modified conditions of cell isolation and culture; most sister pairs of AB^2^ cells show POP-1 asymmetry after P2 is removed from a 4-cell embryo (85%, 48 sister pairs). Moreover, P2 is not sufficient for POP-1 asymmetry; although P2 normally contacts one of the AB^2^ cells, called ABp, the daughters of ABp do not exhibit POP-1 asymmetry.

Why does P2 signaling fail to alter POP-1 levels in the ABp daughters? Because P2 has been shown to interact with ABp, but not EMS, through a separate, Notch-related signaling pathway, we wondered whether this pathway precluded POP-1 asymmetry. However, embryos that were depleted of the receptor GLP-1/Notch by glp-1(RNAi) did not show POP-1 asymmetry at the ABp division, similar to wild-type embryos (0/5). A second possibility is that the transverse divisions of the AB^2^ cells, perpendicular to P2, prevent POP-1 asymmetry. A previous study noted that EMS may fail to respond to induction if it divides perpendicular to, rather than in line with, P2, although this analysis is complicated by the fact that signaling from P2 normally orients the EMS division (Goldstein, 1995). To examine whether division orientation influenced POP-1 asymmetry, we used RNAi to inhibit the functions of the G alpha proteins encoded by goa-1 and gpa-16 (see Materials and Methods); such embryos appear to have random spindle orientations (Gotta and Ahringer, 2001; Zwaal et al., 1996). We observed that a/p, but not transverse, divisions of EMS resulted in POP-1 asymmetry (6/6 and 0/5, respectively; Fig. 4A). Similarly, a/p, but not transverse, divisions of the AB^2^ cells resulted in POP-1 asymmetry (4/4 and 0/4, respectively; Fig. 4A). When the functions of goa-1 and gpa-16 were inhibited in mom-2(or42) mutant embryos, POP-1 asymmetry was not detectable after either an a/p, or transverse, division of the AB^2^ daughters (0/12 and 0/6, respectively). As a second method for altering the AB^2^ division axis, a laser microbeam was used to fuse the AB^2^ daughters together immediately after their birth. As a fused AB^2^ cell enters mitosis, it often develops two largely separate spindles that orient a/p. After the tetrapolar division, the posterior daughters usually showed lower levels of nuclear POP-1 than the anterior daughters (16/18 sister pairs; Fig. 4C).

We used devitellinized embryos to further examine interactions between P2 and the AB^2^ cells. In devitellinized embryos, as in normal embryos, the division axes of the AB^2^ cells are oriented transversely with respect to the position of EMS (asterisks in Fig. 1). We found that the AB^2^ divisions remained approximately transverse after P2 was removed (32/32 embryos), or after a graft P2 was placed on various surfaces of one or both AB^2^ cells (22/22 embryos). We conclude that the presence or absence of P2 does not significantly alter the transverse division axis of an AB^2^ cell. When a graft P2 was positioned perpendicular to the predicted AB^2^ division axis (short red arrow in Fig. 1), POP-1 asymmetry was not observed between either pair of AB^2^ daughters (0/8 embryos). In the next set of experiments the graft P2 was placed in line with the predicted division axis of one of the AB^2^ cells (long red arrow in Fig. 1); we refer to this experimental paradigm as the AB^2^ polarization assay. In each of these experiments, the AB^2^ cell in contact with the graft P2 divided into daughters with POP-1 asymmetry (6/6; Fig. 4E,F).

The daughter distal to P2 had high POP-1 and the proximal daughter had low POP-1, similar to the high/low POP-1 polarity that P2 normally induces in the distal/proximal daughters of EMS. The AB^2^ cell that was not in contact with the graft P2 divided into daughters lacking POP-1 asymmetry (6/6). In a reciprocal experiment, a pair of AB^2^ cells was isolated and grafted onto the P2 cell of an intact, devitellinized host embryo (position indicated by blue arrow in Fig. 1). In this configuration, the P2 cell of the host embryo is in contact simultaneously with the host EMS and one of the graft AB^2^ cells, analogous to the pattern of cell contacts that P2 makes in normal 4-cell embryos. In each case in which the graft AB^2^ cell divided in line with P2, the daughters of that AB^2^ cell showed high/low POP-1 polarity with respect to P2 (5/5). Taken together, these results suggest that ABp in normal embryogenesis has the potential to respond to signaling from P2, and that interactions between P2 and EMS do not preclude P2 from simultaneously signaling ABp. Instead, the transverse
division of ABp appears to prevent it from responding to P2, such that POP-1 remains high in both ABp daughters.

**P1 descendants induce high/low POP-1 polarity**

The AB2 polarization assay described above (long red arrow in Fig. 1) was used to examine the signaling properties of several cells in the early embryo. We found that graft AB2, AB4, AB8, P1 and EMS cells could not induce POP-1 asymmetry in this assay (Table 1). In contrast, the P1 descendants E, C and P3 appeared equivalent to P2 in their ability to induce POP-1 asymmetry with high/low polarity (Table 1). The P1 descendant MS usually induced POP-1 asymmetry, however the asymmetry was markedly less than that observed with the other P1 descendants (data not shown).

Previous experiments by others have demonstrated that a signal from P2 aligns the EMS division axis and induces the proximal EMS daughter (E) to lengthen its cell cycle and to undergo endodermal differentiation (Goldstein, 1992; Goldstein, 1993). To further compare the signaling properties of MS, E and C with P2, each of these cells was grafted onto an isolated EMS cell. Similar to P2, both E and C were able to align the EMS division axis and to induce the proximal EMS daughter to lengthen its cell cycle and to undergo endodermal differentiation (6/7, 8/8 and 8/8 experiments, respectively; Fig. 5E,F). MS was unable to align the EMS spindle and did not induce endodermal-specific differentiation (0/9 experiments). We conclude that E, C and P2, and to a lesser extent MS, can induce POP-1 polarity, and that the signaling properties of E and C appear identical to those reported previously for P2.

MS, E and C are born during the AB4 stage and make extensive cell contacts with AB descendants (see below). Thus interactions at the AB4 stage might generate POP-1 asymmetry at the AB8 stage. We found that the daughters of an isolated AB4 cell did not show POP-1 asymmetry (Table 1). However, when an MS, E or C cell was grafted onto an isolated AB4 cell, the AB4 daughters showed high/low POP-1 polarity in almost all cases (Fig. 5G,H; Table 1). Surprisingly, in each experiment the spindle of the AB4 cell appeared to align with the signaling cell, in contrast to the spindles of AB2 cells. Grafting an AB4 cell onto a second AB4 cell never resulted in POP-1 asymmetry (Table 1), and these cells showed only infrequent, and probably random, alignment of their spindles (7/38 had aligned spindles). Thus, MS, E and C have the ability to align the AB4 spindle with the AB4 daughter.

**Table 1. Identification of signaling cells**

<table>
<thead>
<tr>
<th>Candidate</th>
<th>Assay*</th>
<th>Daughters with POP-1 asymmetry/n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>AB2</td>
<td>0/3</td>
</tr>
<tr>
<td>AB4</td>
<td>AB2</td>
<td>0/7</td>
</tr>
<tr>
<td>AB8</td>
<td>AB2</td>
<td>0/13</td>
</tr>
<tr>
<td>P1</td>
<td>AB2</td>
<td>0/5</td>
</tr>
<tr>
<td>EMS</td>
<td>AB2</td>
<td>0/11</td>
</tr>
<tr>
<td>P2</td>
<td>AB2</td>
<td>6/6</td>
</tr>
<tr>
<td>P3</td>
<td>AB2</td>
<td>4/4</td>
</tr>
<tr>
<td>MS</td>
<td>AB2</td>
<td>5/6 (weak)</td>
</tr>
<tr>
<td>E</td>
<td>AB2</td>
<td>3/3</td>
</tr>
<tr>
<td>C</td>
<td>AB2</td>
<td>8/8</td>
</tr>
<tr>
<td>C</td>
<td>AB2</td>
<td>0/12</td>
</tr>
<tr>
<td>MS</td>
<td>AB2</td>
<td>3/6 (weak)</td>
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<tr>
<td>wild type</td>
<td>None</td>
<td>0/10</td>
</tr>
<tr>
<td>AB4</td>
<td>AB4</td>
<td>0/38</td>
</tr>
<tr>
<td>MS</td>
<td>AB4</td>
<td>9/9</td>
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<tr>
<td>E</td>
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<td>6/7</td>
</tr>
<tr>
<td>C</td>
<td>AB4</td>
<td>12/13</td>
</tr>
<tr>
<td>C</td>
<td>AB4</td>
<td>0/6</td>
</tr>
<tr>
<td>MS</td>
<td>AB4</td>
<td>15/15</td>
</tr>
</tbody>
</table>

*Assays using AB2 cells were on devitellinized wild-type embryos (AB2 polarization assay). All other assays were on individual wild-type cells as listed.

**Fig. 5.** Signaling by P1 descendants. (A-D) Nomarski photomicrographs showing the division of ABpr in (A) wild-type and (B) mom-2(or42) embryos, and the division of ABpl in (C) wild-type and (D) mom-2(or42) embryos; the asterisk indicates a division into the focal plane. (E) GFP expression of an endoderm-specific transgene after combining isolated EMS and C cells; DAPI staining of the same cells is shown in F. At the time of fixation the EMS daughter distal to C had divided twice, whereas the proximal EMS daughter divided only once. (G) POP-1 expression after the division of combined AB4 and MS cells; DAPI staining is shown in H. (I) POP-1 expression after the division of the AB4 cells in a devitellinized embryo. Prior to cell division, a graft E cell was placed on one of the AB4 cells. Note that AB4 daughter proximal to E has low POP-1. J shows superimposed DAPI and PKL-2 images with arrows indicating midbodies.
spindles and to induce high/low POP-1 polarity in the AB4 daughters. The ability of AB descendants to respond to signaling persists until at least the AB16 stage; isolated AB16 cells that were combined with C divided into daughters with high/low POP-1 polarity relative to C (Table 1).

Videomicroscopy was used to examine the cell contacts and spindle orientations of the four AB4 cells in live embryos (n=12); these cells are named ABal, ABar, ABpl and ABpr. ABal contacts only MS, and its spindle normally orients toward MS. ABar and ABpl contact MS, E and C simultaneously, however their spindles orient toward E and often move slightly toward E before cell division (Fig. 5A,C). Finally, ABar contacts C and MS, and its spindle is oriented approximately between C and MS. The observation that three of the AB4 cells contact multiple signaling cells simultaneously suggests that the AB4 cells distinguish, or integrate, the various signals. We used devitellinized 8-cell embryos to test whether an AB4 cell could distinguish between signals from MS and E. In devitellinized embryos, the spindles of the AB4 cells usually orient toward MS. ABar and ABpl contact MS, E and C simultaneously, however their spindles orient toward E and often move slightly toward E before cell division (Fig. 5A,C).

We wanted to determine whether POP-1 asymmetry in sister pairs of AB4 cells required signaling between AB descendants. For these experiments we separated AB from P1, then separated each of the successive descendants of AB immediately after each cell division. The sequentially isolated cell was allowed to divide one additional time before staining the resulting sister pair for POP-1. As expected, POP-1 asymmetry in sister pairs of AB4 cells was not observed between sequentially isolated sister pairs of AB16 and AB32 cells (0/11 and 0/10, respectively). In contrast, POP-1 asymmetry was usually present between sequentially isolated sister pairs of AB16 and AB32 cells (18/35 and 29/29, respectively; Fig. 3E,F).
Table 3. Development of sequentially isolated AB8 cells

<table>
<thead>
<tr>
<th>Embryo type</th>
<th>Wild type</th>
<th>mom-2(or42)</th>
<th>mom-2(RNAi); mom-1(or10)</th>
<th>mom-5(or52)</th>
<th>mom-4(or39)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daughters with POP-1 asymmetry [%/(n)]</td>
<td>51 (35)</td>
<td>56 (27)</td>
<td>58 (12)</td>
<td>0 (28)</td>
<td>0 (20)</td>
</tr>
<tr>
<td>Granddaughters with POP-1 asymmetry [%/(n)]</td>
<td>100 (32)</td>
<td>99 (75)</td>
<td>100 (30)</td>
<td>0 (13)</td>
<td>0 (19)</td>
</tr>
<tr>
<td>Granddaughters aligned linearly [%/(n)]</td>
<td>94 (32)</td>
<td>92 (75)</td>
<td>87 (30)</td>
<td>0 (13)</td>
<td>84 (19)</td>
</tr>
<tr>
<td>Low/high-high/low POP-1 [%/(n)]</td>
<td>94 (32)</td>
<td>91 (75)</td>
<td>87 (30)</td>
<td>0 (13)</td>
<td>0 (19)</td>
</tr>
</tbody>
</table>

n, number of isolations examined.

*POP-1 asymmetry* indicates that a sister cell pair within an isolation had a difference in POP-1 levels comparable with wild-type differences.

### DISCUSSION

MOM-5/Frizzled, but not MOM-2/Wnt, is essential for isolated AB descendants to develop POP-1 asymmetry

Intact mom-2(or42) mutant embryos show a variable reduction or loss of POP-1 asymmetry between the EMS daughters and between sister pairs of AB8 cells (Table 2). However, mom-2(or42) mutants and mom-2(RNAi) embryos analyzed after the AB8 stage showed strong POP-1 asymmetry between most sister pairs of AB or EMS descendants (Fig. 2C,D; Table 2 and data not shown). POP-1 polarity was abnormal in these mutants, for example, many transverse sister pairs showed POP-1 asymmetry, and some a/p sister pairs had low/high polarity. We sequentially isolated AB8 cells from mom-2(or42) embryos and from mom-1(or10); mom-2(RNAi) double mutant embryos, then allowed these cells to divide once or twice in culture before immunostaining for POP-1. The mom-1 gene encodes the only C. elegans protein related to Prc, and should thus be required for secretion of MOM-2 and other Wnt family members (Kadowaki et al., 1996; Rocheleau et al., 1997). We found that the daughters and granddaughters of the sequentially isolated AB8 cells usually showed robust POP-1 asymmetry (Table 3). Thus, MOM-2/Wnt is not essential for POP-1 asymmetry after the AB8 stage, but has a role in POP-1 polarity.

We found that neither the daughters nor granddaughters of a sequentially isolated AB8 cell from a mom-5(or57) mutant embryo showed POP-1 asymmetry (Table 3). Similarly, an AB cell that was isolated from a mom-5(or37) mutant and allowed to divide to the AB16 or AB32 stages did not show POP-1 asymmetry between any sister pairs (0/8 and 0/4 experiments, respectively). These results suggest that MOM-5/Frizzled has a role in POP-1 asymmetry that is independent of MOM-2/Wnt signaling. Because sister pairs of AB16 or AB32 cells usually show POP-1 asymmetry in intact embryos that have been depleted of MOM-5/Frizzled by RNAi or by mutation, we examined intact embryos that were depleted of both MOM-2/Wnt and MOM-5/Frizzled. These latter embryos lacked POP-1 asymmetry in both AB and EMS descendants at the AB16 and AB32 stages (Fig. 2E,F; Table 2).

Interactions resulting in low/high POP-1 polarity

A wild-type, sequentially isolated AB16 cell can divide into a pair of sister cells with POP-1 asymmetry. However, when two adjacent AB16 cells were allowed to divide, their spindles almost invariably aligned to generate a line of two sister pairs with low/high-high/low POP-1 polarity (Fig. 3G). This was the case irrespective of whether two sequentially isolated AB16 cells were combined and allowed to divide once (19/21 experiments), or a sequentially isolated AB8 cell was allowed to divide twice (30/32). Identical results were obtained for the sequentially isolated daughters of the EMS cell (5/5). These results suggest that each parental cell aligned the spindle of the other, with a reciprocal induction of low/high POP-1 polarity. Thus, these cells induce low/high POP-1 in the distal/proximal daughters of the responding cell, in contrast to the high/low POP-1 induced by MOM-2/Wnt signaling.

We observed two additional examples in which low/high POP-1 polarity appeared to be induced in the distal/proximal daughters of a responding cell. First, experiments combining an AB4 cell with an MS, E or C cell, or combining an AB16 cell with a C cell, resulted in a high/low-high/low pattern of POP-1 polarity (Fig. 5G,H). This pattern is consistent with the hypothesis that the AB4 cell induced low/high POP-1 polarity in the MS daughters, whereas MS simultaneously induced high/low POP-1 polarity in the AB4 daughters. The second example involved POP-1 polarity in C and P3, the daughters of the P2 cell (see legend to Fig. 6 for details). In wild-type or mom-2(or42) mutant embryos, C is born proximal to ABp and has high POP-1, whereas P3 is distal and has low POP-1 (Fig. 6A, and see Fig. 1). In a devitellinized embryo, in which P2 does not contact ABp, we found that POP-1 polarity was reversed in the P2 daughters (Fig. 6C). We observed the same polarity reversal when isolated EMS and P2 cells were combined and allowed to divide (Fig. 6E). However, the normal pattern of POP-1 polarity was restored after ABp and ABa cells were grafted onto the P2 cell of a devitellinized wild-type host embryo (position of blue arrow in Fig. 1). This was the case irrespective of whether the graft ABp and ABa cells originated from wild-type embryos (10/10), or from mom-2(or42) mutants (5/5; Fig. 6G). Thus, AB2 cells can induce low/high POP-1 polarity in the P2 daughters.

We examined whether MOM-2/Wnt, MOM-1/Prc, MOM-5/Frizzled or MOM-4/MAPKKK are required for the polarized division of adjacent AB16 cells (the granddaughters of a sequentially isolated AB8 cell; Table 3). The mom-2 and mom-1 mutant cells usually had aligned spindles and low/high-high/low POP-1 polarity. In contrast, the cells from mom-5 or mom-4 mutants did not show POP-1 asymmetry. The mom-4 mutant cells usually aligned their spindles, however the mom-5 mutant cells did not (Table 3). We conclude that MOM-5/Frizzled and MOM-4/MAPKKK, but not MOM-2/Wnt, play a role in low/high POP-1 polarity.

The a/p sister cells in early C. elegans embryos have an
the AB8 cells after P1 is removed, so all cells should adopt signaling if the AB2 spindle and P2 are aligned, either by transformations at the AB8 stage; POP-1 remains high in all of the AB2 spindle. In normal development the perpendicular, anterior fates (Hutter and Schnabel, 1995). Our results also previous study that killing P1 caused posterior to anterior fate isolated AB. These results are consistent with those from a and AB16 cells. Previous studies reached contradictory daugther with POP-1 asymmetry, as do the subsequent AB8 cells. We have shown that an AB2 cell can respond to signaling if the AB2 spindle and P2 are aligned, either by altering the position of P2 or by altering the orientation of the AB2 spindle. In normal development the perpendicular, left/right division of ABp produces daughters with very similar fates, and the parallel differentiation of these left/right daughters is a major source of bilateral symmetry in C. elegans (Sulston et al., 1983). If the ABp spindle were to align with P2, the a/p division would result in daughters with different levels of POP-1 and presumably different fates.

At the next round of cell division, each AB4 cell divides into daughters with POP-1 asymmetry, as do the subsequent AB8 and AB16 cells. Previous studies reached contradictory conclusions about whether asymmetry in the AB lineage was determined intrinsically or through cell interactions (see Introduction). Our present study provides a resolution of this paradox. We observed POP-1 asymmetry in sister pairs of AB16 cells, but not AB8 cells, which were derived from an isolated AB. These results are consistent with those from a previous study that killing P1 caused posterior to anterior fate transformations at the AB8 stage; POP-1 remains high in all of the AB8 cells after P1 is removed, so all cells should adopt anterior fates (Hutter and Schnabel, 1995). Our results also support the study of Wittmann et al., which suggested AB descendants had an intrinsic asymmetry independent of P1; their study used a transgenic marker that is expressed at the AB16 stage (Wittmann et al., 1997). Thus, cell interactions are essential for POP-1 asymmetry at, but not after, the AB8 stage. Reported examples of AB8 cells that correctly adopted posterior fates after killing or removing P1 may represent cells in transition between the two modes of generating POP-1 asymmetry (Gendreau et al., 1994; Hutter and Schnabel, 1995).

**POP-1 asymmetry at the AB8 stage**

Although we propose that cell interactions determine POP-1 asymmetry in the early AB lineage, our results argue against a previous model that the primary interaction is between AB and P1 (latent polarity model), rather than between AB descendants and P1 descendants (Hutter and Schnabel, 1995). We have shown that exposing AB to P1 is not sufficient to generate POP-1 asymmetry at the AB8 stage, and that P1 does not provide signaling activity in our assays. In the previous study, laser irradiation of the P1 descendants did not prevent a/p polarity at the AB8 stage, suggesting that the P1 descendants were not essential for polarization (Hutter and Schnabel, 1995). However, laser-irradiation does not effectively prevent P2 from signaling EMS, nor does it prevent P2 from signaling ABp through a separate, Notch-related signaling pathway (Mello et al., 1994). All of the known genes that are involved in POP-1 asymmetry in the early embryo are expressed maternally, as are the components of the Notch pathway. Thus, it may be difficult to eliminate translation of maternally provided mRNAs by laser-irradiating the early embryonic cells.

The AB4 cells contact one or more of the P1 descendants MS, E, C and P3. We have shown that each of these P1 descendants can induce high/low POP-1 polarity in AB descendants, similar to the ability of P2 to induce high/low POP-1 polarity in the daughters of EMS. Indeed, at least two of the P1 descendants, E and C, appear to be fully equivalent to P2 in their ability to polarize EMS. C and P2 require MOM-2/Wnt for signaling, however the MS cell appears to provide a different high/low signal. We have shown that MOM-2/Wnt can induce high/low POP-1 polarity in the daughters of EMS, and that P1 does not provide signaling activity in our assays. In the previous study, laser irradiation of the P1 descendants did not prevent a/p polarity at the AB8 stage, suggesting that the P1 descendants were not essential for polarization (Hutter and Schnabel, 1995). However, laser-irradiation does not effectively prevent P2 from signaling EMS, nor does it prevent P2 from signaling ABp through a separate, Notch-related signaling pathway (Mello et al., 1994). All of the known genes that are involved in POP-1 asymmetry in the early embryo are expressed maternally, as are the components of the Notch pathway. Thus, it may be difficult to eliminate translation of maternally provided mRNAs by laser-irradiating the early embryonic cells.

The AB4 spindles, in contrast to the AB2 spindles, align with
the signaling cells P2, MS, E or C in cell culture experiments. This difference might result from the synthesis of new regulators in the AB4 cells, or from the degradation of inhibitors present in the AB2 cells. In normal development each AB4 division results in an anterior/posterior pair of daughters. However, all of these divisions are oblique, and some are nearly transverse, with respect to the a/p axis of the egg (see Fig. 5A,C). The alignment of the AB4 spindles with posterior-localized signaling cells such as E provides a partial explanation for the a/p pattern of high/low POP-1 polarity at the AB8 stage. In addition, the ability of MS to align the AB8 spindle may be crucial for the normal pattern of Notch-mediated interactions. MS can interact with the ABal spindles. This conclusion is based on experiments in which cells were sequentially isolated in culture, but is not present in the AB2 cells. In normal development each AB2 cell can divide with POP-1 asymmetry, contact with signaling cells P2, MS, E or C in cell culture experiments. Therefore MOM-5/Frizzled may be a component of the signaling pathway that generates low/high POP-1 polarity independent of MOM-2/Wnt. Drosophila Frizzled is an essential component of the planar cell polarity pathway, however the role of Wnt proteins has not been determined (Lawrence et al., 2002). It will be of interest to determine whether other genes involved in Drosophila planar cell polarity have functions in low/high signaling in C. elegans. MOM-4/MAPKKK and proteins such as LIT-1/Nemo and WRM-1/Beta-catenin are essential for POP-1 asymmetry in AB descendants, and thus appear to be core components of the asymmetry-generating machinery (this study) (Kaletta et al., 1997; Lin et al., 1998; Meneghini et al., 1999; Rocheleau et al., 1997; Rocheleau et al., 1999).

The observation that POP-1 asymmetry is present in intact mom-5 mutant embryos, but not in mom-2;mom-5 double mutant embryos, suggests that MOM-2/Wnt signaling can induce POP-1 asymmetry independent of MOM-5/Frizzled. These results support the view from previous genetic studies that MOM-2/Wnt and MOM-5/Frizzled have overlapping, but distinct roles in the early embryo (Rocheleau et al., 1997; Schlesinger et al., 1999). A survey of gene expression patterns in C. elegans embryos has detected mRNAs corresponding to at least two additional Frizzled-related proteins that are candidate receptors for MOM-2/Wnt (Y. Kohara, personal communication; http://nematode.lab.nig.ac.jp/). However, our present study indicates that these Frizzleds cannot be functionally redundant with MOM-5 for the POP-1 asymmetry shown by isolated, cultured cells.

When two adjacent AB8 cells divide in culture they generate a line of two sister pairs with low/high-high/low POP-1 polarity. In an intact, normal embryo, similarly oriented divisions of adjacent AB8 cells would be expected to produce sister pairs with high/low-high/low POP-1 polarity. Thus, the behavior of the isolated cells does not reproduce the normal pattern of POP-1 polarity. Among the cell culture experiments described here, the only condition that resulted in high/low-high/low POP-1 polarity involved combining a low/high signaling cell (AB4) with a high/low signaling cell such as MS, E or C. Thus, it is possible that the normal pattern of POP-1 involves two distinct signaling pathways. We have shown that isolated AB descendants remain responsive to MOM-2/Wnt signaling until at least the AB16 stage, however we do not yet know whether Wnt signals persist in normal embryos at the AB16 and later stages.

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