Development 137, 3337-3345 (2010) doi:10.1242/dev.054742
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Extracellular control of PAR protein localization during asymmetric cell division in the *C. elegans* embryo

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SUMMARY

The axis of asymmetric cell division is controlled to determine the future position of differentiated cells during animal development. The asymmetric localization of PAR proteins in the *Drosophila* neuroblast and *C. elegans* embryo are aligned with the axes of the embryo. However, whether extracellular or intracellular signals determine the orientation of the localization of PAR proteins remains controversial. In *C. elegans*, the P0 zygote and germline cells (P1, P2, and P3) undergo a series of asymmetric cell divisions. Interestingly, the axis of the P0 and P1 divisions is opposite to that of the P2 and P3 divisions. PAR-2, a ring-finger protein, and PAR-1, a kinase, relocalize to the anterior side of the P2 and P3 germline precursors at the site of contact with endodermal precursors. Using an in vitro method, we have found that the PAR-2 protein is distributed asymmetrically in the absence of extracellular signals, but the orientation of the protein localization in the P2 and P3 cells is determined by contact with endodermal precursor cells. Our mutant analyses suggest that *mes-1* and *src-1,* which respectively encode a transmembrane protein and a tyrosine kinase, were not required to establish the asymmetric distribution of PAR-2, but were required to determine its orientation at the site of contact with the endodermal precursors. The PAR-2 localization during the asymmetric P2 and P3 divisions is controlled by extracellular signals via MES-1/SRC-1 signaling. Our findings suggest that Src functions as an evolutionarily conserved molecular link that coordinates extrinsic cues with PAR protein localization.

KEY WORDS: PAR proteins, Src tyrosine kinase, Directional control of cell polarity, *C. elegans*

INTRODUCTION

Asymmetric cell division is a fundamental mechanism for generating cell diversity. In animal development, the axis of asymmetric cell division is crucial for determining the future positions of the differentiated progeny. In *Drosophila*, embryonic and larval neuroblasts divide asymmetrically along the apical-basal axis of the epithelial tissues; this results in the daughter neuroblast being positioned on the apical side, and the daughter ganglion mother cell (GMC) on the basal side. The axis of the division remains roughly unchanged through successive rounds of cell division, and the side of GMC delivery is fixed. During the division, the polarity regulator PAR-3 and aPKC are distributed asymmetrically with respect to the apical-basal axis (Januschke and Gonzalez, 2010; Siegrist and Doe, 2006; Zhong and Chia, 2008).

Although these observations suggest that the orientation of the PAR protein localization is determined by extracellular signals, its orientation appears to be determined by different mechanisms in embryonic and larval neuroblasts. In larval neuroblasts, the polarity proteins, including PAR-3, fail to relocalize on the apical side but localize at the cortex adjacent to the centrosome after the centrosome is dislocated, by treatment with a drug that transiently depolymerizes microtubules and in centrosome mutant conditions (Januschke and Gonzalez, 2010). These results indicate that the orientation of the PAR protein localization in *Drosophila* larval neuroblasts is determined by an intrinsic cue. By contrast, in in vitro cultured embryonic neuroblasts obtained from mechanically disrupted *Drosophila* embryos, PAR-3 and aPKC normally localize to the site of epithelial-cell contact only in neuroblasts that maintain contact with multiple epithelial cells, while neuroblasts that contact no or one epithelial cell divide in random orientations with regard to their previous divisions (Siegrist and Doe, 2006). These observations are consistent with the idea that the orientation of the asymmetric PAR protein localization is determined by extracellular signals.

In the *C. elegans* embryo, the P0 zygote and subsequently the P1, P2, and P3 cells in the germline lineage divides asymmetrically to generate a large somatic daughter and a small germline daughter (P1, P2, P3, and P4) (Fig. 1A). During the P0 division, the PAR proteins are distributed asymmetrically in a complementary pattern across the presumptive anterior-posterior axis of the embryo. PAR-3 and another PDZ-domain-containing protein, PAR-6, and PKC-3 (aPKC) are localized anteriorly, while the ring-finger protein PAR-2 and the kinase PAR-1 are localized posteriorly. The localization of the anterior and posterior PAR proteins depends on a mutual exclusion mechanism, as evidenced by the observation that PAR-2 spreads to the anterior side in *par-3* mutants (Cowan and Hyman, 2007). The axis of asymmetric division of the P0 zygote is determined by the location of the sperm components after fertilization (Cowan and Hyman, 2004; Goldstein and Hird, 1996), indicating that sperm provides an important orientation cue.

During asymmetric cell division of the germline cells, the PAR proteins are persistently distributed asymmetrically across the anterior-posterior axis (Boyd et al., 1996; Guo and Kemphues, 1995). Interestingly, the axis of asymmetric cell division of the germline precursor P2 and P3 cells is opposite to that of the P0 and P1 divisions, a phenomenon known as 'polarity reversal' (Schierenberg, 1987). This polarity reversal allows the P2 and P3 cells to generate the germline precursor daughter cells, P3 and P4

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Accepted 3 August 2010

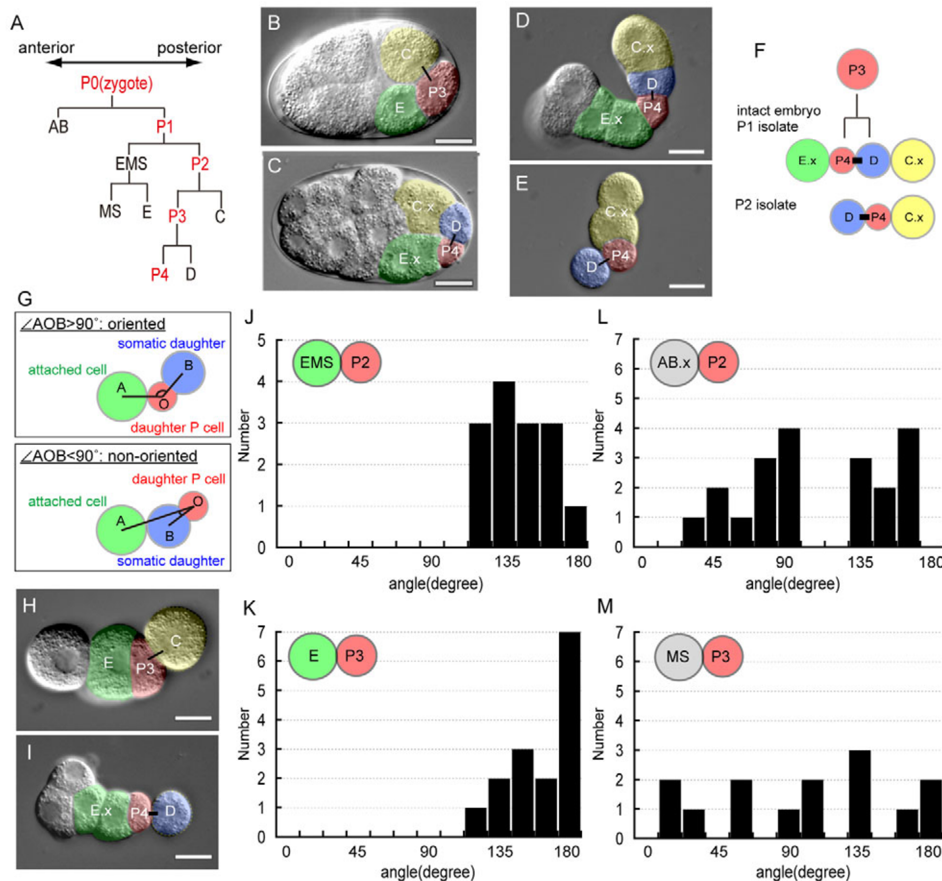

Fig. 1. Extracellular control of the asymmetric cell division of germline precursors P2 and P3. (**A**) Cell lineage in early embryonic *C. elegans* development. The left-right position of the cells reflects the anterior-posterior position in the embryo. The P0 zygote and germline cells (P1, P2, P3 and P4) are indicated in red. (**B**,**C**) DIC images of germline and somatic daughters born after the P2 and P3 divisions. Germline daughters (P3 and P4, red) were in contact with an endodermal precursor (E or E.x, green), whereas the somatic daughters (C cell, yellow in B; D cell, blue in C) were distal to the endodermal precursors in intact embryos. Anterior is leftwards and ventral is downwards. (**D**,**E**) DIC images of the progeny of an isolated P1 (D) and P2 cell (E) cultured in vitro. In the isolated P1 cell cultures, the germline daughter (P4, red) was in contact with the endodermal precursor (E.x, green), whereas one somatic daughter (D, blue) was in contact with the somatic C daughter cell (C.x, yellow). In P2 isolates, the P4 cell was in contact with the C daughter cell, and the somatic daughter D was not. 'x' indicates a daughter cell (e.g. 'E.x' indicates a daughter of the E cell). (**F**) Summary of the position of the P4 cell born after the P3 division in intact embryos, P1 isolates and P2 isolates. (**G**) Diagram showing the measured angle of the P2 and P3 divisions. (**H**,**I**) DIC images of germline daughters (P3 or P4, red), somatic daughters (C cell, yellow; D cell, blue) born after the P2 and P3 division, and attached endodermal precursors (E or E.x, green). (**J-M**) Histograms of the angle of P2 and P3 division when P2 was attached to EMS (J) or AB.x (L), or when P3 was attached to E (K) or MS (M). Scale bars: 10 μm.

in contact with or adjacent to endodermal precursors (E and E.x), while the somatic daughters of P2 and P3 (C and D) are distal to the endodermal precursors (Fig. 1B,C). Consequently, the primordial germ cell, P4, makes physical contact with the endodermal precursors that will generate the intestine, a source of yolk proteins in *C. elegans* (Kimble and Sharrock, 1983). During the P2 and P3 divisions, the PAR-2 and PAR-1 proteins are relocalized to the anterior side, at the site of contact with endodermal precursors (Boyd et al., 1996; Guo and Kemphues, 1995). Although these data suggest that the localization of the PAR protein is under extracellular control, previous results suggested that the polarity reversal is induced by an intrinsic mechanism (Goldstein, 1995b; Schierenberg, 1987).

mes-1 and *src-1*, which, respectively, encode a transmembrane protein and a tyrosine kinase, are involved in the control of spindle orientation in asymmetric divisions of the P2 cell and mesendoderm EMS cell, which are neighboring cells in the four-cell stage of *C. elegans* embryos (Bei et al., 2002; Berkowitz and Strome, 2000). MES-1 protein localizes specifically to the P2-EMS boundary, a site of SRC-1-dependent tyrosine phosphorylation (Bei et al., 2002). MES-1/SRC-1 signaling cooperatively functions with Wnt signaling in the EMS division (Bei et al., 2002). However, it remains unknown whether the MES-1/SRC-1 singling controls the PAR protein localization in the P2 division during the polarity reversal.

In this study, we re-examined whether the orientation of PAR protein localization during polarity reversal is determined by extracellular signals from the endodermal precursors, by isolating cells and placing them in direct contact with endodermal precursors in a different orientation from their original contact site in vitro. We found that the PAR-2 and PAR-6 were localized asymmetrically and in a complementary pattern during the P2 and P3 divisions in the absence of extracellular signals, and the PAR-2 protein was always located at the contact site with endodermal precursors, even when the contact site was randomized. Cell recombination assays revealed that the P2 cell was responsive to the extracellular signals only at the initial phase of the cell cycle. Mutant and mosaic

analyses suggest that MES-1 functions both in sending extracellular signals from the endodermal precursors, and in receiving these signals in the germline precursors, whereas SRC-1 transduces the signal(s) that control PAR-2 relocalization during polarity reversal. Therefore, the PAR protein localization is oriented by extracellular signals via MES-1/SRC-1 signaling. This is the first experimental demonstration, altering cell positions to directly test a role of cell signaling on directional control of PAR protein localization during asymmetric cell division.

MATERIALS AND METHODS

C. elegans strains and culture conditions

SS149: *mes-1(bn7)* (Berkowitz and Strome, 2000) was maintained at 15°C, and cultured at 25°C to observe its phenotype. Homozygous *src-1(cj293)* embryos were isolated from OV10: *src-1(cj293)*/hT2 animals (Itoh et al., 2005). In addition, the following strains were used: HS1996, *gfp::tbg-1(ddIs6)*; AZ235, *gfp::beta-tubulin(ruIs48)*; JH1512, *gfp::par-6*; KK866, *gfp::par-2*; and TH120, *gfp::par-6* and *gfp::par-2* (Schonegg et al., 2007).

In vitro cell manipulation

Embryonic cells were obtained and cultured as described previously (Edgar, 1995), except for the media (Shelton and Bowerman, 1996) and the modified Chitinase-Chymotrypsin solution: Yatalase (5 U of Chitinase activity) (Takara Bio, Japan), 10 U each of Chitinase from *Trichoderma viride* and *Streptomyces griseus*, and 10 mg of alpha-chymotrypsin from bovine pancreas (Sigma, USA) were dissolved in 1 ml of the egg buffer (Edgar, 1995) containing 25 mM HEPES (pH 6.0).

To examine the extrinsic control of asymmetric P3 division, the E and MS cells were identified by the timing of their sister cells' (E.x and MS.x) division. E and MS were used for experiments only when the cell divisions of the E.x and MS.x were separated by more than 17 minutes.

The alteration of the contact site of P2 cells to various cells in vitro was confirmed by the centrosome position in P2. The centrosome position in P2 was displaced about 45° and 90° (Fig. 3M, 0 min; Fig. 3N, 0 min, respectively), and 0°, 135° or 180° (data not shown).

In vitro mosaic analysis

The fluorescence of GFP-fused TBG-1 (HS1996) was used to distinguish the genetic backgrounds of cells under a fluorescence-equipped dissecting microscope (MZFLIII with Fluo Combi, Leica, Switzerland). In this study, defects in the orientation of EMS cell division reported previously (Bei et al., 2002) were reproduced in our chimeric P2-EMS pairs.

RNA interference

Double-stranded RNAs synthesized from full-length cDNAs contained in *yk496g2 (mes-1)* or *yk117f2 (src-1)* was microinjected into the gonads of young adult animals (Fire et al., 1998). The embryos were observed after an overnight incubation.

Fluorescence microscopy

A DeltaVision sectioning microscope (Applied Biosystems, USA) was used to capture the fluorescent images.

RESULTS

Endodermal precursors orient the asymmetric cell division of germline precursors in *C. elegans* embryos

When P1, P2, and P3 cells were isolated just after their birth and cultured as single cells in vitro, they divided asymmetrically, producing different-sized daughter cells, and the daughter cells of the isolated P1, P2, and P3 cells divided in a similar pattern to their in vivo counterparts (data not shown). Therefore, P1, P2, and P3 divide asymmetrically without cell-cell contact, and the progeny of the cultured P1, P2, and P3 cells were identifiable by their size and time of division, as described in previous studies (Goldstein, 1995b; Lee and Goldstein, 2003; Schierenberg, 1987).

When we observed the position of the progeny of the P1 and P2 isolates, we found that the P4 cell in the P1 isolates was generated in contact with the endodermal precursor (E.x), and a somatic daughter, D, which was in contact with the C daughter cell (C.x) (3/3 cases) (Fig. 1D), as observed in intact embryos (20/20 cases). However, we were surprised to find that the P4 cell in the P2 isolates was frequently generated in an inverted position; the P4 cell, but not D was in contact with C.x (28/30 cases, Fig. 1E,F). Which position the P4 cell was generated in correlated with the presence or absence of the endodermal precursor E (Fig. 1F), suggesting that the axis of the asymmetric cell division of P3 is determined by extracellular signals from endodermal precursors. In the absence of an E cell, the C daughter cell may have a marginal ability to affect the axis of the P3 division.

Next, to reveal whether contact with endodermal precursors is sufficient to orient the asymmetric cell division of P3 as well as P2, we examined the axis of their asymmetric cell divisions by experimentally altering the site of contact between the germline and endodermal precursors. We did this by isolating a P2 or P3 cell just after their birth and re-attaching it to the endodermal precursor at various orientations (see Movie 1 in supplementary material). The alteration of the contact site was confirmed by examining the position of the centrosome in the P2 cells, because it is originally on the side distal to the contact site between P2 and EMS in intact embryos (see Materials and methods). The axis of the asymmetric P2 and P3 divisions was defined as 'oriented' if the angle formed by the three cells was greater than 90°. In this situation, the daughter P cell (P3 or P4) was generated in contact with or proximal to the attached cell (Fig. 1G). If the angle was less than 90°, the P2 or P3 division was defined as 'non-oriented' ($\angle$AOB; Fig. 1G).

We found that when the isolated P2 or P3 cell was attached to an endodermal precursor cell, the daughter P cell born after the P2 or P3 division was generated in contact with the endodermal precursor (Fig. 1H,I), and the axis of the P2 and P3 divisions was always 'oriented' (14/14 P2-EMS pairs, and 15/15 P3-E pairs; Fig. 1J,K). By contrast, when the P2 or P3 cell was attached to a control cell (AB.x or MS), the angles of the P2 and P3 divisions were highly variable, indicating that the axis was not specified (Fig. 1L,M). The difference in the effect between contact with endodermal precursors or with control cells was statistically significant ($P<0.005$ by Fisher's exact test). These results indicated that the axis of asymmetric P2 and P3 divisions is specifically oriented by contact with the endodermal precursors. Collectively, these findings show that the germline precursor cells P2 and P3 have two properties: they establish their cell polarity without cell-cell contact and determine their axis of the division in response to extracellular signals.

To examine the time window of the cells' ability to send or receive the extracellular signals, we cultured the signal-sending EMS or signal-receiving P2 cells in isolation for various periods of time, and then attached the cultured cells to a newly born signal-receiving P2 or signal-sending EMS cell isolated from another embryo (Fig. 2A). When the EMS cell was cultured in isolation, its effect on the axis of asymmetric P2 division was statistically significant until at least 8 minutes after the EMS birth (Table 1). By contrast, when the P2 cell was cultured in isolation, the effect of EMS on the axis of asymmetric P2 division was statistically significant until only 2 minutes after the P2 birth (Table 1). These results suggest that P2 responds to extracellular signals during the initial period of the P2 cell cycle (which is approximately 17 minutes long), whereas EMS sends signals during and beyond the time when P2 can respond to them.

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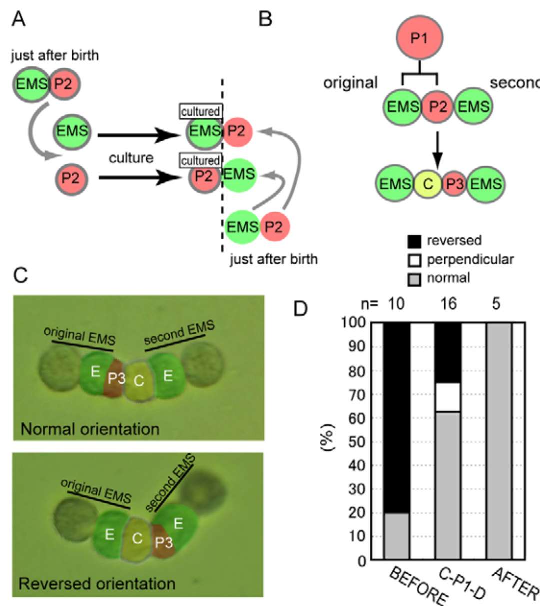

Fig. 2. Asymmetric cell division of the germline precursor P2 cell is highly cell-autonomous and responsive to extracellular signals in *C. elegans*. (**A**) Experimental procedure for examining the time window for P2 or EMS to send or receive signals. P2 and EMS were isolated just after birth, cultured in isolation for various times and then attached to a newly born P2 or EMS cell isolated from another embryo. The results are shown in Table 1. (**B**) Schematic procedure for examining whether the axis of the asymmetric P2 division is oriented to a second EMS. A second EMS was placed against a P2 cell on the opposite side of the original EMS in a P2-EMS pair generated from a P1 isolate. The axis was defined as having a 'normal' orientation if the daughter P3 cell was generated on the original EMS side, and a 'reversed' orientation if it was generated on the second EMS side. The germline P1, P2 and P3 cells are red, the somatic daughter C born after the P2 division is yellow, and the endodermal precursors (EMS) are green. (**C**) Dissecting microscopy images of the germline daughter P3 (red) and somatic daughter C (yellow) after the P2 division in the presence of a second EMS. In the upper panel, the axis of the P2 division was in the normal orientation; in the lower panel, the axis was reversed. (**D**) Summary of the axis of P2 division in the presence of a second EMS. The normal, reversed or perpendicular orientation is shown by a gray, black or white bar, respectively. 'C-P1-D' (completion of P1 division) was defined as the period 4-6 minutes after the mother P1 cell initiated cleavage. 'BEFORE' and 'AFTER' were the periods 3 minutes before and after 'C-P1-D', respectively.

When P2 was sandwiched between the original and a second endodermal precursor EMS cell (Fig. 2B and see Movie 2 in the supplementary material), the axis of the asymmetric cell division of P2 was oriented to the contact site with one or the other of the signal-sending EMSs, but in no case did P2 fail to divide asymmetrically (Fig. 2C,D). The position of daughter P3 correlated with the timing of the attachment of the extra EMS cell (Fig. 2D). That is, if the second EMS cell was attached after the completion of the P1 division, the daughter P3 was often generated on the side of the original EMS (Fig. 2D; AFTER; 5/5 cases), as occurs in the absence of a second EMS. By contrast, if the second EMS was attached to the P2 cell before the completion of the P1 division (C-P1-D), the daughter P3 was frequently generated on the side of the second EMS (Fig. 2D; BEFORE; 8/10 cases, $P<0.001$, compared with the 'AFTER' experiment, by Fisher's exact test). Thus, once the axis of the asymmetric P2 division was determined, during the initial phase of the P2 cell cycle, the signal coming from the other side was ignored, indicating that the asymmetric P2 division is a highly cell-autonomous process.

The orientation of the PAR-2 localization is controlled by extracellular signals

In intact embryos, PAR-2 was distributed asymmetrically, to the site of contact with endodermal precursors, and to the apical cortex of the P2 and P3 cells (Fig. 3A,B; arrowheads and arrowheads with asterisk), whereas PAR-6 was distributed in a complementary pattern to PAR-2 (Fig. 3C,D). To examine how the asymmetric localization of the PAR proteins in P2 and P3 develops, we isolated P2 or P3 cells just after their birth and cultured them as single cells. In the isolated P2 cells, the cortical PAR-2 and PAR-6 proteins were distributed asymmetrically, at the presumptive P3 and C cell sides, respectively [Fig. 3G,H; in P2 cells expressing GFP-fused PAR-2 (8/8 cases) or GFP-fused PAR-6 (8/8 cases)]. The same was true for the P3 isolates, although the signal level of the GFP-fused PAR-6 protein was very low [P3 cells expressing GFP-fused PAR-2 (9/9 cases) or GFP-fused PAR-6 (2/2 cases)]. These results indicate that the asymmetric localization of PAR proteins in the germline precursors P2 and P3 develops without extracellular signals.

We next examined how the asymmetric PAR-2 localization is controlled by extracellular signals. When a P2 or P3 cell was attached to an endodermal precursor at various orientations in vitro, the PAR-2 protein in the P2 or P3 cell was located asymmetrically to the site of contact, before cell division (5/5 P2-EMS pairs, Fig. 3I; 10 minutes, and 9/10 P3-E pairs, Fig. 3K; 21 minutes). In addition, in P2-EMS pairs, some PAR-2 protein spread over the non-contact area of the P2 cell (Fig. 3I; 10 minutes, arrowheads with asterisk). This distribution pattern was reminiscent of the PAR-2 localization at the apical cortex in intact embryos. By contrast, when P2 or P3 was attached to a control cell (AB.x or MS), the PAR-2 became distributed asymmetrically, but its localization was not oriented to the area of cell-cell contact [Fig. 3J; 10 minutes, 0/3 P2-AB.x pairs ($P<0.05$ by Fisher's exact test), and Fig. 3L; 14 min, 0/4 P3-MS pairs ($P<0.005$)]. These results indicated that the asymmetric PAR-2 localization is specifically oriented to the contact site by extracellular signals from the endodermal precursor.

During the P2 and P3 divisions in intact embryos, the mitotic spindle was oriented along the proximal-distal axis relative to the germline-endodermal precursor contact site, by centrosome migration. Just after chromosome duplication in the P2 cell, one of the centrosomes migrated to the site of the endodermal-precursor contact, from the position where the centrosome was duplicated before the initiation of cell division (n=5) (Fig. 3E and see Fig. S1A in the supplementary material; 0-8 minutes). By contrast, in the P3 cell, one centrosome migrated away from the position where the centrosome duplicated, whereas the other remained at the site of contact (n=5) (Fig. 3F and see Fig. S1B in the supplementary material; 0 minutes-19 minutes).

When the site of contact of P2 or P3 with the endodermal precursor was altered in vitro, the centrosome of the presumptive germline daughter P cell was always located adjacent to the region of cell contact before the initiation of mitotic spindle elongation (9/9 P2-EMS pairs and 7/7 P3-EMS pairs in Fig. 3M,O). By contrast, when a control cell (AB.x or MS) was attached, the centrosome frequently failed to locate to the region of cell contact [3/8 P2-AB.x pairs ($P<0.01$ by Fisher's exact test) and 1/4 P3-MS pairs ($P<0.05$) in Fig. 3N,P]. These results indicated that the centrosome position is controlled by extracellular signals from the endodermal precursors, which specify the alignment of the mitotic spindle in P2 and P3,

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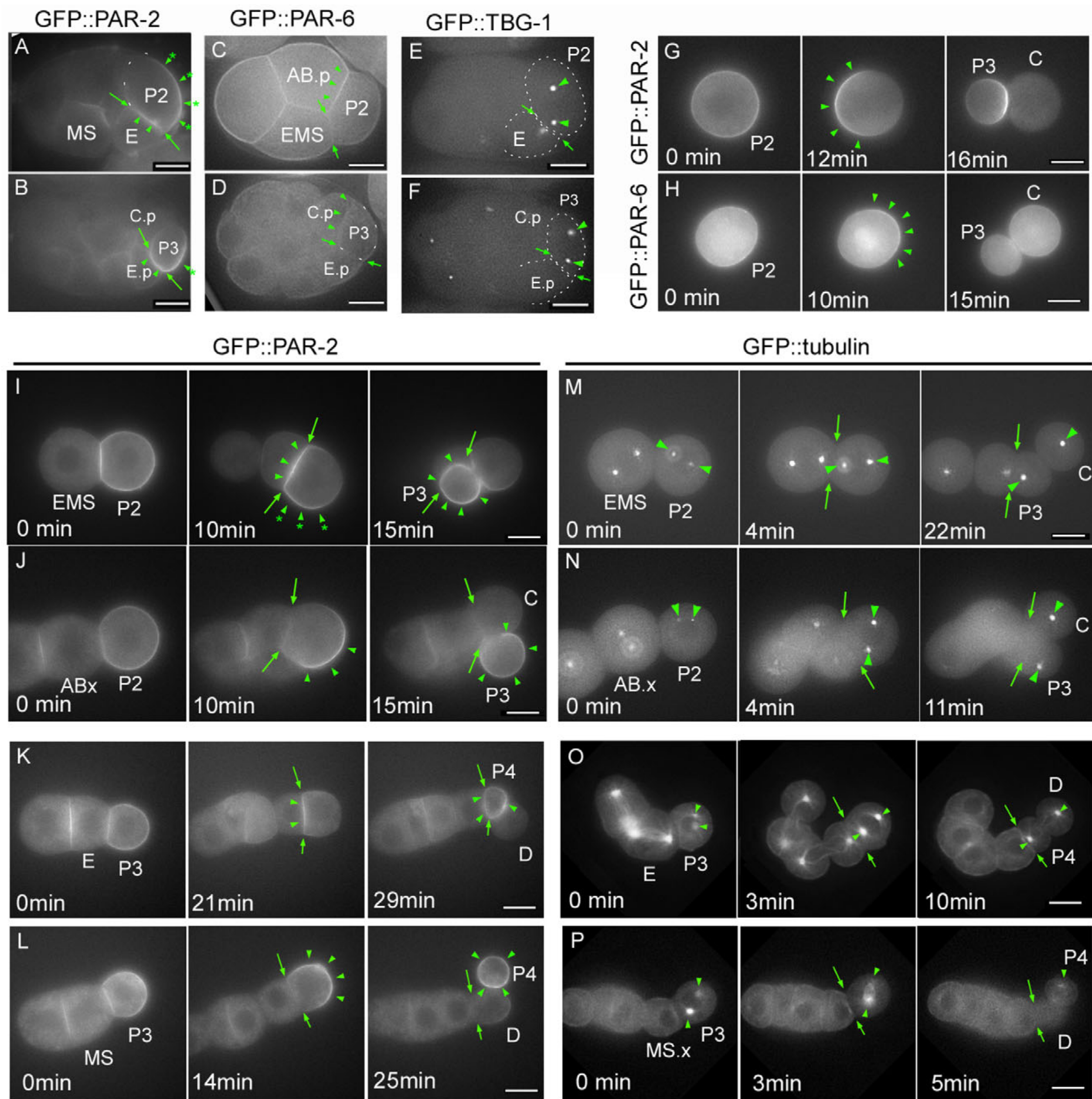

Fig. 3. PAR-2 localization and spindle orientation are guided by extracellular signals. (**A-F**) Fluorescence microscopy images of GFP-fused PAR-2 (A,B), GFP-fused PAR-6 (C,D) and GFP-fused TBG-1 (γ-tubulin) (E,F) in the P2 or P3 cell in intact embryos. The paired arrows indicate the cell boundary between the P cell (P2 or P3) and the endodermal precursor (EMS, E or E.p). Throughout this figure, the arrowheads indicate the PAR protein or centrosome positions. The arrowheads with an asterisk indicate PAR protein at the apical cortex or non-cell-contact area. Cell boundaries without the cortical GFP signal are marked by broken white lines (A,D-F). Anterior is leftwards and ventral is downwards. (**G**,**H**) Series of fluorescence microscopy images of GFP-fused PAR-2 (G) or GFP-fused PAR-6 (H) in P2 isolates. (**I-P**) Series of fluorescence microscopy images of GFP-fused PAR-2 (I-L) or GFP-fused tubulin [TBG-1 (γ-tubulin) (M,N) or β-tubulin (O,P)] in the P2 or P3 cell attached to various cells in vitro. Minute 0 is the time at which observation started. Scale bars: 10 μm.

relative to the germline-endodermal contact site. Taken together, our data indicate that extracellular signals from the endodermal precursors orient the asymmetric cell division of the germline precursors by regulating the localization of PAR-2 and the spindle orientation.

During the P2 and P3 division in intact embryos, the centrosome positioned at the center of the contact area was subsequently shifted to the edge of the contact area (arrowheads with asterisk; see Fig. S1A in the supplementary material; 9.5-12.5 minutes and see Fig. S1B in the supplementary material; 22.5-25 minutes), so that the spindle orientation was tilted to the ventral-dorsal axis of the embryo. The axis tilting of the P2 and P3 division appears to be important for the proper positioning of the P2 daughters (P3 and C) in embryos, such that P3 is located on the ventral side of the C cell (Fig. 1B). The shift in the centrosome position and the axis tilting were also seen in P2-EMS pairs in vitro (Fig. 3M, Fig. 1J). Therefore, the axis tilting is at least partially caused by a cell intrinsic mechanism, rather than by an extrinsic mechanism, such as physical constraint from the eggshell.

Table 1. Time windows for EMS to P2 signaling to orient the asymmetric P2 division

Cell	Culture time (minutes)	Oriented	Non-oriented	Total	P value
EMS	0-2	21	0	21	4.77×10^{-7}**
	3-5	8	0	8	0.0039*
	6-8	7	0	7	0.0078*
P2	0-2	21	0	21	4.77×10^{-7}**
	3-5	5	3	8	0.36
	6-8	2	4	6	0.89

See Fig. 2A for the experimental procedure. The asymmetric P2 division was defined as 'oriented' if the angle of the P2 division (∠ AOB as shown in Fig. 1G) was 90°-180°, and as 'non-oriented' if the angle was 0°-90°. One-sided binomial tests were performed under the condition that the hypothesized probability of oriented divisions is 0.5, because the P2 division would fail to orient in half of the cases in the absence of signaling. ***P*<0.0033 and **P*<0.017 were defined as statistically significant based on the Bonferroni correction.

MES-1/SRC-1 signaling is required to orient the asymmetric cell division of the germline precursor P2 cell

To search for candidate molecules that mediate the extracellular signals that control the orientation of the asymmetric P2 division, we first investigated the involvement of Wnt signaling. In the intact embryos of *mom-2/wnt* (48/48) or *mom-5/frizzled* (23/23) mutants, and in P2-EMS pairs generated from isolated P1 cells of the *mom-2/wnt* (7/7) or *mom-5/frizzled* (13/13) mutants, the daughter P3 cell was generated normally, in contact with endodermal precursors, suggesting that *mom-2* and *mom-5* are not required for the asymmetric cell division of P2. Although in *mom-2* and *mom-5* mutant embryos, the endoderm precursor E cell often aberrantly transform into their sister MS-like cell, the MES-1 protein still localizes normally to the P2-EMS boundary (Bei et al., 2002; Berkowitz and Strome, 2000).

Next, we tested whether MES-1/SRC-1 signaling was involved in the asymmetric division of the P2 cell. In loss-of-function *mes-1* or *src-1* embryos, we found that the difference in cell size between the P2 daughters was diminished (Table 2), consistent with previous reports (Bei et al., 2002; Strome et al., 1995). In addition, P2 divided abnormally, along the anterior-posterior axis of the embryos, so that the small daughter P3 was not in contact with the endodermal precursor, but instead protruded on the posterior side (Fig. 4A,B; Table 2). Thus, loss-of-function *mes-1* and *src-1* embryos exhibited defects in the asymmetry and the axis of the P2 division.

To examine how MES-1/SRC-1 signaling is involved in the asymmetric P2 division, we observed PAR-2 localization before the division. In wild-type embryos, PAR-2 was distributed asymmetrically to the site of endodermal-precursor contact and the apical cortex (Fig. 4C; 0-11 minutes). In the loss-of-function *mes-1* and *src-1* embryos, PAR-2 was distributed asymmetrically at the apical cortex, but frequently failed to accumulate at the site of the endodermal-precursor contact (paired arrows and arrowheads with asterisks in Fig. 4D, 12 minutes; Fig. 4E, 8 minutes; Table 3, Phenotype I). These results indicated that *mes-1* and *src-1* are required not for establishing the asymmetric distribution of PAR-2, but for orienting the localization of PAR-2 to the endodermal-precursor contact site.

During the P2 division, the relationship between the PAR-2 localization and spindle orientation was abnormal in some loss-of-function *mes-1* and *src-1* embryos. In wild-type embryos, the PAR-2 at the apical cortex became restricted to the ventral side (Fig. 4C; 11-14 minutes), and the PAR-2 at both the apical cortex and the endodermal-precursor contact site was segregated into the P3 cell (Fig. 4C; 14-16 minutes). In some loss-of-function *mes-1* and *src-1* embryos, in the absence of PAR-2 protein at the contact site, the apical PAR-2 was segregated normally into the smaller daughter, which was abnormally positioned on the posterior side (Fig. 4D, 12-17 minutes; Table 3, Phenotype II). In these cases, the segregation of the apical PAR-2 was coupled with an abnormal axis of division. This suggested that the asymmetry of the P2 division was normal, but the axis of its division was improperly specified in these *mes-1* and *src-1* embryos.

In other loss-of-function *mes-1* and *src-1* embryos, the segregation of the apical PAR-2 and the axis of division axis were uncoupled, so that the apical PAR-2 protein was mis-segregated into both or either daughter cell (Fig. 4E, 18-21 minutes; Table 3, Phenotype III). Although this observation suggests that the uncoupling causes the defects in the asymmetry of the P2 division, this uncoupling phenotype may not be directly owing to the loss of MES-1/SRC-1 signaling. When P2 cells were isolated from intact *mes-1(bn7)* embryos, PAR-2 localization and the axis of division were coupled normally, and PAR-2 segregated into the smaller daughter [Fig. 4F; 7/7 cases *P*<0.001, compared with intact *mes-1(bn7)* embryos by Fisher's exact test]. These results suggested that, in the absence of MES-1/SRC-1 signaling, unknown extrinsic signals (e.g. signals that direct apical-basal polarity of embryos) or the effects of cell shape on spindle orientation may have caused the uncoupling. Thus, the observed defects in the asymmetry of the P2 division may be secondary defects in embryos lacking *mes-1* or *src-1* function. Taking these data together, we confirmed that *mes-1* and *src-1* are required to direct the PAR-2 protein localization and spindle orientation to the contact site, but not to establish the asymmetric localization of the PAR-2 protein.

To reveal whether the MES-1 and SRC-1 activities are required in the signal-sending EMS cell or the signal-receiving P2 cell, we performed in vitro mosaic analyses, in which both the P2 and EMS

Table 2. Summary of defects in the axis of asymmetric P2 division and size asymmetry between P2 daughter cells (C and P3)

Genotype	Size asymmetry defect (%)*	Axis defect[†]	*n*
N2 (wild type)	0	0	21
mes-1(bn7)	30.0	0	50
mes-1(RNAi)	42.9	25.0	14
mes-1(bn74)	33.3	63.6	21
src-1(cj293)	24.0	73.1	25
src-1(RNAi)	42.9	92.9	33

*The size asymmetry was defined as defective when the ratio of the area of C to P3 in the DIC image was less than 1.3.
[†]The axis of division was defined as defective when the daughter P3 cell was not generated in contact with the endodermal precursor.

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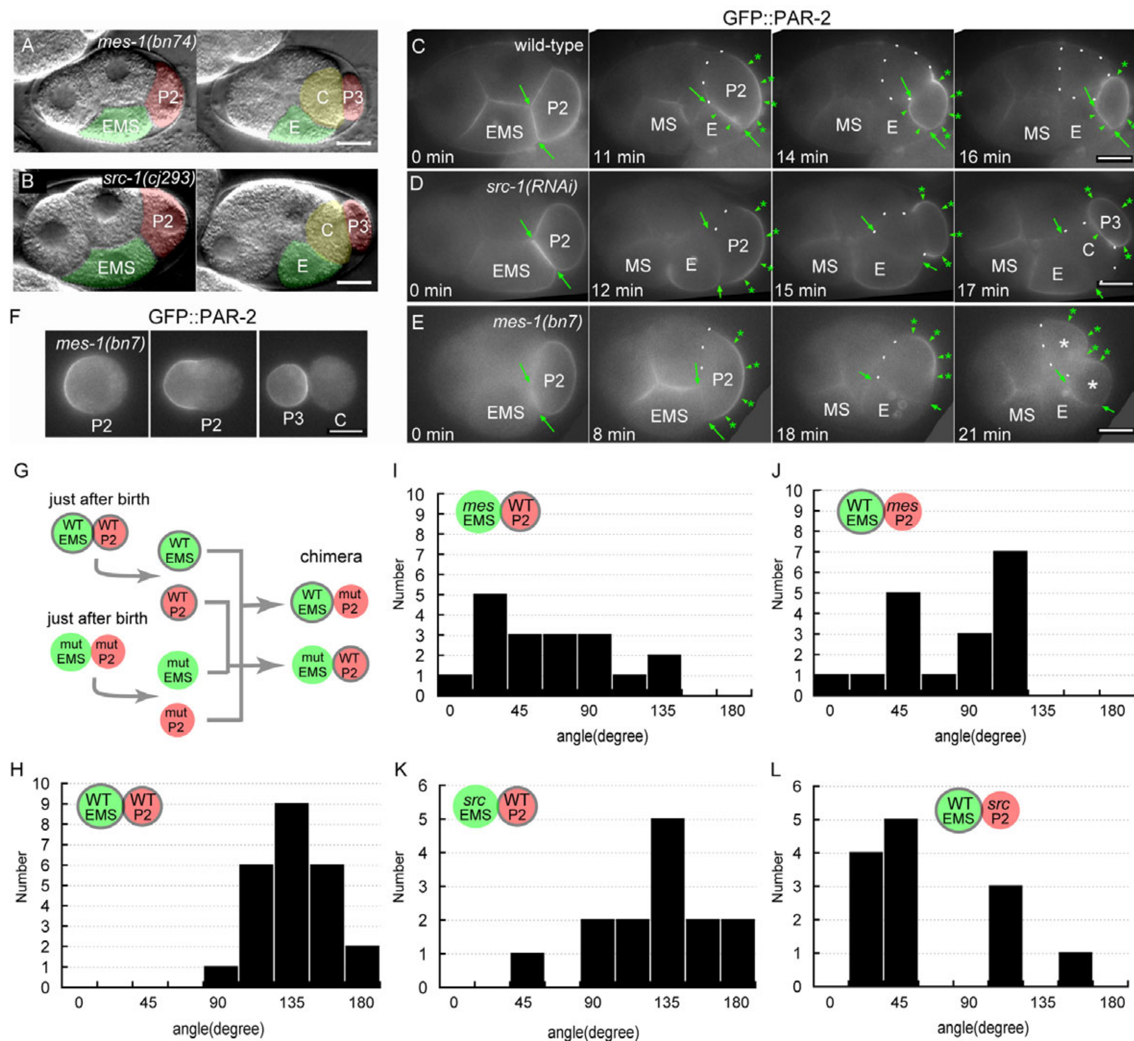

Fig. 4. MES-1/SRC-1 signaling is required to orient the asymmetric cell division of the germline precursor P2 cell. (**A**,**B**) DIC images of the P2 division in *mes-1* or *src-1* mutants. Germline precursor P2 and its germline daughter P3 (red) and somatic daughter C (yellow). Endodermal precursors (EMS and E) are green. (**C-E**) Fluorescence microscopy images of GFP-fused PAR-2 in wild-type (C), *src-1(RNAi)* (D) and *mes-1(bn7)* (E) embryos. Paired arrows indicate the cell boundary between the P2 cell and endodermal precursor (EMS and E). Green arrowheads indicate PAR-2 protein at the contact site with endodermal precursors, and green arrowheads with an asterisk indicate PAR-2 protein localized to the apical cortex. Cell boundaries without PAR-2 signals are marked by broken white lines. Minute 0 is the P2 birth time (at the completion of the mother P1 division). Anterior is leftwards and ventral is downwards. (**F**) Fluorescence microscopy images of GFP-fused PAR-2 protein in a P2 cell isolated from a *mes-1(bn7)* embryo. (**G**) Schematic diagram for making chimeric P2-EMS pairs. The P2 and EMS were isolated, and one was replaced with a cell isolated from a mutant embryo, producing a chimeric P2-EMS pair. (**H-L**) Histogram of the axis of P2 division in a chimeric P2-EMS pair. (H) A wild-type cell was replaced with a wild-type cell from another embryo. (I-L) A wild-type cell was replaced with a mutant cell, resulting in pairs with a wild-type P2 and *mes-1* mutant EMS (I), *mes-1* mutant P2 and wild-type EMS (J), wild-type P2 and *src-1* mutant EMS (K), or *src-1* mutant P2 and wild-type EMS (L). The angle of the P2 division was estimated at 22.5° intervals on a dissecting microscope. Scale bars 10 μm.

cell were isolated, and one was replaced with the equivalent cell isolated from a mutant embryo, producing a chimeric P2-EMS pair (Fig. 4G). When the P2 or EMS cell was replaced with another wild-type counterpart, the asymmetric P2 division was oriented normally to the site of contact with the EMS cell, only when the difference in the birth time between P2 and EMS was within 2 minutes (Fig. 4H, Table 1; 0-2 minutes).

On the other hand, when either the wild-type P2 or EMS was replaced with a *mes-1* mutant cell, the axis of the asymmetric P2 division failed to be oriented with respect to the contact site (Fig. 4I,J; $P<0.001$ compared with Fig. 4H by the Mann-Whitney test), indicating that *mes-1* is required in both the signal-sending EMS cell and the signal-receiving P2 cell. In chimeras of a *src-1* mutant P2 and wild-type EMS, the P2 division failed to be oriented (Fig. 4L; $P<0.001$), but was almost normal in chimeras of a wild-type P2 and a *src-1* mutant EMS (Fig. 4K; $P>0.2$), indicating that *src-1* is required only in the signal-receiving P2 cell. Because MES-1 is a transmembrane protein, MES-1 may function to send and receive the signals as a ligand and receptor, whereas SRC-1 may act as an intracellular transducer of the extracellular signals that determine the axis of the asymmetric P2 division.

A similar signaling process was proposed for the asymmetric division of the *C. elegans* EMS (Bei et al., 2002). Therefore, the MES-1/SRC-1 signaling regulates Wnt- and PAR-regulated asymmetric cell divisions in adjacent blastomeres in early *C. elegans* embryos.

Table 3. Summary of defects in PAR-2 localization in *mes-1* and *src-1* loss-of-function embryos

Genotype	Phenotype I Before P2 division (%)	Phenotype II During P2 division (%)	Phenotype III During P2 division (%)	*n*
mes-1(bn7)	92.9	7.1	50	14
mes-1(RNAi)	71.4	42.9	14.3	7
src-1(cj293)	77.8	77.8	0	9
src-1(RNAi)	80.0	40.0	60.0	5

Phenotype I: loss of PAR-2 localization at the site of endodermal-precursor contact before the P2 division, as shown in Fig. 4D and 4E.
Phenotype II: the axis of the asymmetric P2 division was defective, as shown in Fig. 4D.
Phenotype III: the axis of the P2 division was uncoupled with the PAR-2 localization, as shown in Fig. 4E.

DISCUSSION

In this study, we showed that endodermal precursors specify the orientation of PAR-2 localization and mitotic spindle in the asymmetric division of *C. elegans* germline precursors P2 and P3. We also show that the P2 cell develops its cell polarity without cell-cell contact and adopts a specific orientation for their divisions in response to extracellular signals. Thus, its contact with endodermal precursors provides an orientation cue for its asymmetric cell division. These dual properties of cell polarization without cell-cell contact and non-autonomous determination of the axis of polarization are in clear contrast with the mechanisms of cell polarization in the *C. elegans* EMS and mammalian epithelial cells, which require extracellular signals to become polarized (Goldstein, 1995a; Martin-Belmonte and Rodriguez-Fraticelli, 2009). On the other hand, similar to *C. elegans* germline precursors, mammalian neurons develop polarity in vitro without extracellular cues, whereas their contact with extracellular substrate molecules can influence which neurite becomes an axon (Menager et al., 2004). In addition, in budding yeast, the polarity of asymmetric cell division is oriented by a cue from the remnant of the previous cell division, called the bud scar. In mutants in which signals from the bud scar are disrupted, the cells become polarized in random orientations (Wedlich-Soldner and Li, 2003). Thus, in budding yeast, the mechanisms for cell polarization and for determining the axis of polarization are separable. Although our results do not exclude the possibility that the cell division remnant plays a role in cell polarization, as it does in budding yeast, the mechanisms regulating cell polarization in the *C. elegans* germline precursors may be similar to that in budding yeast, as well as mammalian neurons.

Src may function as an evolutionarily conserved molecular link between extrinsic cues and PAR proteins in asymmetric cell division

Src has been suggested to be involved in the directional control of PAR protein localization in mammalian T lymphocytes. T lymphocytes are activated in response to an antigen presented on the surface of an antigen-presenting cell (APC), by forming an adhesive contact called an immunological synapse (IS) (Huppa and Davis, 2003). In response to the IS formation, polarity proteins, including PAR proteins, are located to the site of contact with the APC: PAR-3 localizes to the IS and PAR-1b accumulates at the cytoplasmic region near the synapse (Lin et al., 2009; Ludford-Menting et al., 2005). Experiments using the Src family of tyrosine kinases (SFK) inhibitor PP2 showed that SFK activity is required for the PAR-1b accumulation near the synapse, suggesting that SFK functions upstream of the PAR-1b redistribution (Lin et al., 2009). A recent study strongly suggests that the IS-induced polarity leads to asymmetric cell division (Chang et al., 2007). These reports and our study collectively suggest that Src may function as an evolutionarily conserved molecular link between extrinsic cues and PAR protein distribution in asymmetric cell division.

Possible molecular mechanisms for oriented PAR-2 distribution

How PAR-2 is directed to the site of endodermal-precursor contact in *C. elegans* embryos remains an unanswered question. PAR-2 associates dynamically with the cell cortex in the P0 zygote (Cheeks et al., 2004). Therefore, its intracellular movement might be under the control of SRC-1 in the P2 cell. One possibility is that the direct phosphorylation of PAR-2 by SRC-1 targets PAR-2 for capture by the protein-transport system. However, a mutant PAR-2 with a substitution at the tyrosine residue (Y390A) localized normally during P2 division (data not shown). Therefore, PAR-2 may not be a direct substrate of SRC-1 kinase, although it may contain another as yet unidentified phosphorylation site.

A second possibility is that SRC-1 controls the intracellular movement of the PAR-2 protein by an indirect mechanism, such as through cytoskeletal regulation. Mammalian Src regulates actin filament formation near the cell membrane and controls endosomal motility via actin filament regulation near the endosomal membrane (Randazzo, 2003; Young and Copeland, 2010). Therefore, SRC-1 may regulate the transport of PAR-2 protein along the cell cortex via cortical actin filaments, or between the cytoplasm and cortex via endocytosis or vesicle transport in the *C. elegans* germline precursors.

Novel implications for the polarity reversal in the germline lineage during *C. elegans* development

The asymmetric division of P2 has previously been shown to be insensitive to contact with an endodermal precursor (Goldstein, 1995b; Schierenberg, 1987), which is inconsistent with our present findings. In a study by Schierenberg, P2 was separated from the EMS cell by squeezing the EMS out of a hole in the eggshell created by a laser beam, and the axis of asymmetric P2 division was observed in the eggshell (Schierenberg, 1987). That method may have taken longer to remove the EMS from the eggshell than our method. Furthermore, in Schierenberg's method, a small region of the removed EMS appears to remain at the original contact site (Schierenberg, 1987). Our method improved both the timing and the thoroughness of the EMS cell removal. In this study, we showed that the time window in which the P2 cell is competent to receive extracellular signals is very narrow. Therefore, the temporal resolution of the experimental method is crucial to uncovering the extrinsic control of asymmetric P2 division.

The other previous report, by Goldstein, used the same method as in the present study to manipulate the cells in vitro; however, that study used a different definition to determine the angle of asymmetric P2 division. In that report, the angle of the P2 division was measured, without distinguishing between the daughter P3 and C cells (Goldstein, 1995b). In the present study, the angle formed by the P2 daughters and attached cell was measured using the small daughter P3 cell as the triangular vertex. Thus, this definition was crucial to our discovery of the extrinsic control of the orientation

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of division. Taken together, our detailed characterizations using improved methods led us to discover the extrinsic mechanism for determining the axis of asymmetric cell division in the germline precursor P2 cell.

Acknowledgements
We thank Einhard Schierenberg for valuable discussion, Yuji Kohara for cDNA and the *Caenorhabditis* Genetics Center, funded by the NIH National Center for Research Resources, for *C. elegans* strains. This work was supported by the Special Postdoctoral Researcher Program in RIKEN to Y.A.; by a grant from the Japanese Ministry of Education, Culture, Sports, Science and Technology to Y.A. and H.S.; by a grant from the Japan Society for the Promotion of Science to H.S.; and by NIH R01GM083071 to B.G. Deposited in PMC for release after 12 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.054742/-/DC1

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