LET-99 opposes Gα/GPR signaling to generate asymmetry for spindle positioning in response to PAR and MES-1/SRC-1 signaling

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Summary

G-protein signaling plays important roles in asymmetric cell division. In *C. elegans* embryos, homologs of receptor-independent G protein activators, GPR-1 and GPR-2 (GPR-1/2), function together with Gα (GOA-1 and GPA-16) to generate asymmetric spindle pole elongation during divisions in the P lineage. Although Gα is uniformly localized at the cell cortex, the cortical localization of GPR-1/2 is asymmetric in dividing P cells. In this report, we show that the asymmetry of GPR-1/2 localization depends on PAR-3 and its downstream intermediate LET-99. Furthermore, in addition to its involvement in spindle elongation, Gα is required for the intrinsically programmed nuclear rotation event that orients the spindle in the one-cell. LET-99 functions antagonistically to the Gα/GPR-1/2 signaling pathway, providing an explanation for how Gα-dependent force is regulated asymmetrically by PAR polarity cues during both nuclear rotation and anaphase spindle elongation. In addition, Gα and LET-99 are required for spindle orientation during the extrinsically polarized division of EMS cells. In this cell, both GPR-1/2 and LET-99 are asymmetrically localized in response to the MES-1/SRC-1 signaling pathway. Their localization patterns at the EMS/P_2 cell boundary are complementary, suggesting that LET-99 and Gα/GPR-1/2 signaling function in opposite ways during this cell division as well. These results provide insight into how polarity cues are transmitted into specific spindle positions in both extrinsic and intrinsic pathways of asymmetric cell division.

Key words: Asymmetric division, Polarity, Spindle orientation, *C. elegans*, Nuclear rotation

Introduction

Asymmetric cell division is a major mechanism through which different cell types are generated during development (Horvitz and Herskowitz, 1992; Ahringer, 2003). In general, asymmetric cell division requires two steps. First, a polarized axis must be established along which cell fate determinants are asymmetrically localized. Second, the mitotic spindle must be oriented on to this polarized axis so that the cell fate determinants are differentially partitioned into the daughter cells. Mechanisms that establish cell polarity and spindle orientation can be intrinsic to the cell, or induced by extrinsic signals (Bowerman and Shelton, 1999). In either case, the coordination between cellular polarity and spindle orientation is essential for a faithful asymmetric cell division. Although a number of proteins have been shown to play roles in asymmetric cell division, the precise molecular mechanism that coordinates spindle orientation with polarity remains to be determined for both intrinsically programmed and extrinsically induced asymmetric divisions.

The early development of *Caenorhabditis elegans* is characterized by asymmetric divisions that produce diverse cell fates (Rose and Kemphues, 1998b; Lyczak, 2002). In the one-cell embryo (P_0), the spindle is oriented on the polarized anterior/posterior (AP) axis. First cleavage is unequal and generates a larger anterior cell called AB and a smaller posterior cell called P_1. P_1 divides unequally with its spindle oriented on the AP axis to produce a larger EMS and a smaller P_2 cell, both of which divide asymmetrically. Divisions of the P lineage are intrinsically programmed (Goldstein et al., 1993; Goldstein, 1995). By contrast, the asymmetric division of EMS along the AP axis absolutely requires contact with its sister P_2 (Goldstein, 1995). However, the AP orientation of spindles in both the P and EMS cells results from a 90° rotation of the nuclear-centrosome complex during prophase, which does not occur in AB.

The intrinsic polarity in P lineage cells is established through the asymmetric distributions of several PAR proteins, which are conserved in many organisms (Ohno, 2001). In the one-cell (P_0), a complex of PAR-3, PAR-6 and PKC-3 (atypical protein kinase-3) are present on the anterior cortex, while the PAR-2 and PAR-1 proteins are present on the posterior cortex. At the two-cell stage, the PAR-3 complex and PAR-2/PAR-1 become asymmetrically localized in anterior and posterior domains again in P_1. The PAR-3 complex is also present uniformly at the cortex of AB. The asymmetric distributions of the PAR proteins result in the polarized distribution of downstream cell fate determinants (Rose and Kemphues, 1998b; Lyczak, 2002). This intrinsic PAR-3/PAR-2 asymmetry is also essential for nuclear rotation and for the asymmetric spindle elongation that results in unequal cleavage in P lineage cells (Tsou et al., 2003; Cheng et al., 1995). The PAR-dependent mechanism causes nuclear rotation to occur centrally in the P_0 and P_1 cells, when the effects of cell shape asymmetry are removed (Tsou et al.,

2002; Tsou et al., 2003). The uniform distribution of PAR-3 in AB is also required to prevent ectopic nuclear rotation directed towards the cell cortex that is caused by the geometry of the cell shape (Tsou et al., 2003). The precise mechanism by which the PARs coordinate polarity with spindle orientation remains to be elucidated, but several key players have been identified.

Heterotrimeric G proteins are required for several aspects of spindle positioning (Zwaal et al., 1996; Gotta and Ahringer, 2001). Using RNA interference experiments, it was shown that asymmetric spindle elongation in the one-cell embryo is dependent on two partially redundant Gα proteins encoded by *goa-1* and *gpa-16* (Gotta and Ahringer, 2001). It was proposed that Gβγ, encoded by *gpb-1* and *gpc-2*, are important in regulating migration of the centrosomes around the nucleus because oblique migration paths were seen in mutant embryos. These observations, coupled with *Gα; Gβ* double mutant analysis led to the interpretation that Gα and Gβγ control distinct microtubule-dependent processes that are required for proper spindle positioning in *C. elegans* embryos (Gotta and Ahringer, 2001). However, depletion of Gβγ also resulted in late nuclear rotation, while *Gα(RNAi)* embryos showed a complete failure of nuclear rotation at the two-cell stage, indicating potential involvement in common processes as well.

Although canonical heterotrimeric G protein signaling pathways are primarily activated via cell-surface receptors, recent work in *Drosophila* and rat has revealed receptor-independent mechanisms for activation of G-protein signaling (Schaefer et al., 2001; Takesono et al., 1999). In particular, in *Drosophila* neuroblasts the GoLoco domain protein, PINS, is localized asymmetrically and is required for proper spindle orientation. PINS binds to the GDP form of Gα (GDP-Gα) and can cause Gβγ to be released from Gα. *C. elegans* homologs of PINS, called GPR-1 and GPR-2 (GPR-1/2), are required for proper spindle positioning in P lineage cells (Gonczy et al., 2000). This observation suggests that intrinsically activated G-protein signaling may be a conserved pathway for spindle positioning among species. Recent work suggests that Gα and GPR-1/2 act together in the generation of forces needed for anaphase spindle elongation in *C. elegans* (Gonczy et al., 2000; Dechant and Glotzer, 2003; Srinivasan et al., 2003; Colombo et al., 2003; Gotta et al., 2003). In addition, two of three recent studies found that GPR-1/2 are enriched at the posterior pole of the embryo in response to PAR-3 (Colombo et al., 2003; Gotta et al., 2003). These observations, together with previous work (Grill et al., 2001), leads to the model that the asymmetric enrichment of GPR-1/2 results in higher cortical forces at the posterior that cause asymmetric anaphase spindle elongation (Colombo et al., 2003; Gotta et al., 2003).

The LET-99 protein also plays a crucial role in spindle positioning (Rose and Kemphues, 1998a; Tsou et al., 2002). LET-99 is required for nuclear rotation and asymmetric anaphase spindle movements in P_0 and P_1, and LET-99 is enriched in an asymmetrically positioned band in P lineage cells in response to PAR polarity cues (Rose and Kemphues, 1998a; Tsou et al., 2002). Furthermore, the mislocalization of LET-99 correlates with failures in nuclear rotation in *par-3* and *par-2* mutant P_0 and P_1 cells, as well as alterations in anaphase spindle movements in *par-3* embryos (Tsou et al., 2002; Tsou et al., 2003). These observations have led to the model that the cortical LET-99 band is an intermediate that transmits PAR cues into the asymmetric forces needed for nuclear rotation and anaphase spindle movement (Tsou et al., 2002; Tsou et al., 2003). Interestingly, the LET-99 protein contains a DEP domain, which is found in many other molecules involved in G-protein signaling. Thus, LET-99 could provide an asymmetric cue to the G protein signaling pathway.

Asymmetric cell division that occurs in the EMS cell is driven by extrinsic signals from the P_2 cell that both polarizes EMS and orients the spindle (Goldstein, 1995). The conserved Wnt/wingless signaling pathway functions in P_2/EMS signaling, in a partially redundant manner with the MES-1/SRC-1 tyrosine kinase pathway (Schlesinger et al., 1999; Bei et al., 2002). Nuclear rotation in EMS cells is directed toward the posterior cell contact with P_2 (Schlesinger et al., 1999), which is different from the free central nuclear rotation driven by the PAR-dependent mechanism in P_0 and P_1 cells (Tsou et al., 2002; Tsou et al., 2003). In addition, just after rotation in EMS cells, the posterior spindle pole is closely associated with the cortex at the EMS/P_2 boundary (Berkowitz and Strome, 2000), which is not observed for the posterior spindle pole in P_0 and P_1 cells. These differences in nuclear rotation and spindle movements driven by PAR and Wnt/MES-1/SRC-1 signaling suggest that the spatial control of forces that act on the spindle may be different in these cell types. It is not known, however, whether any of the proteins used in positioning spindles in the P lineage also function in EMS.

Whether intrinsically or extrinsically programmed, the coupling between polarity and spindle orientation is essential for asymmetric division. Two characteristics of force generation that must be regulated and coordinated to properly position the spindle are the magnitude of the force and the asymmetry of forces. In this report, we provide evidence for a model that the Gα/GPR-1/2 signaling pathway upregulates the magnitude of force generation in P-lineage cells to drive nuclear and spindle movements, and that LET-99 provides an asymmetric cue and acts antagonistically to Gα/GPR-1/2 signaling. Furthermore, our results indicate that aspects of the Gβ phenotype are due to gain of Gα/GPR-1/2 activity, rather than reflecting a separable role for Gβ in spindle positioning. Finally, we show that Gα/GPR-1/2 signaling and LET-99 are both involved in the asymmetric cell division of EMS cells. Thus, the different polarizing cues used in intrinsic and extrinsically controlled asymmetric divisions use common downstream signaling components.

Materials and methods

Strains and maintenance

C. elegans were cultured using standard conditions (Brenner, 1974). The following strains were used in this study: N2, wild-type Bristol; EU452, *mom-5(zu193) unc-13(e1091) / hT2* I*; + / hT2[bli-4(e937) let(h661)]*; MT2426, *goa-1(n1134)* I; BW1808, *gpa-16(it143) unc-13(e51)* I; KK653, *par-3(it71) unc-32(e189) / qC1* III; RL19, *let-99(or81) unc-22(e66) / DnT1[unc(n754dm) let]* IV; EU660, *let-99(or204ts)* IV; SS149, *mes-1(bn7)* X; and RL41, *gpa-16(it143) unc-13(e51)* I*; unc-22(e66) let-99(or81) / DnT1* IV. Strains were provided by the *C. elegans* Genetics Center (N2, EU452, SS149, MT2426), the Kemphues laboratory (KK653), the Bowermann laboratory (EU660), the Wood laboratory (BW1808), the Mello laboratory (SS149) or constructed in this laboratory. N2 was used for all wild-type controls. Strains were grown at 20°C unless otherwise indicated.

Table 1. Nuclear, centrosome and spindle positions in one-cell embryos*

Genotype	Position of pronuclear meeting†	Position of pronuclei at NEBD‡	Spindle midpoint at late anaphase§	*n*¶
Wild type	67.1±3.6	50.1±0.9	60.2±2.1	10
goa-1(RNAi);gpa-16(RNAi)	66.0±1.5	49.3±1.1	49.3±1.2	10
gpr-1/2(RNAi)	65.1±2.3	49.8±1.0	52.9±2.7	13
gpb-1(RNAi)	68.1±3.2	61.6±2.8	63.2±1.1	10
let-99(or81)	65.6±2.1	62.2±2.3	60.4±1.5	10
goa-1(n1134);gpa-16(RNAi);gpb-1(RNAi)	66.3±2.7	50.2±1.2	50.6±0.6	10
goa-1(RNAi);gpa-16(RNAi);let-99(or81)	65.7±1.8	49.9±0.6	49.9±1.0	10
gpr-1/2(RNAi);gpb-1(RNAi)	65.4±2.1	50.3±0.9	52.8±2.4	12
gpr-1/2(RNAi;let-99(or81)	65.5±2.0	50.1±0.6	49.6±1.1	10

*Positions are expressed as percentage of egg length (mean±s.d.) with anterior equal to 0%.
†Position of midpoint between the pronuclei at meeting.
‡Position of the midpoint between the pronuclei just after nuclear envelope breakdown (NEBD).
§Position of the midpoint between the spindle poles at late anaphase when the spindle is at its most posterior point, before the onset of cytokinesis.
¶*n*=number of embryos.

RNA interference

Antisense and sense RNAs were transcribed in vitro from linearized full-length cDNA templates (Ambion MEGAscript). Double-stranded RNAs (dsRNA) were annealed as described by Fire et al. (Fire et al., 1998). Young adult worms were soaked in dsRNA solution (1.5 mg/ml) for 8-10 hours at room temperature. The progeny of soaked worms were analyzed between 16 and 32 hours post-soaking.

Microscopy and analysis of living embryos

Embryos were mounted to avoid flattening the embryo, and examined under DIC optics using time-lapse video microscopy (Rose and Kemphues, 1998a). Nuclear and spindle positions were measured from video images as described in Table 1. Hyperactive centrosome movements were quantified by measuring the angular velocity of the nuclear-centrosome complex, which was then converted to a linear velocity using the radius of the complex. Spherical cells were generated as described previously (Tsou et al., 2002). All filming was at room temperature of 23-25°C.

Antibodies and immunolocalization

A full-length *gpr-2* cDNA was cloned into the pGEX protein purification vector (Amersham Bioscience), expressed in bacteria, purified using Gluthione S-Transferase resin and injected into a rabbit (Covance). Antisera were affinity-column purified using the GST:GPR-2 fusion protein. Rabbit antibodies against LET-99 were prepared as described previously (Tsou et al., 2002). The same material and procedure were used to obtain polyclonal anti-LET-99 antibodies from rat.

For in situ immunolocalization, worms were cut in egg buffer on poly-lysine coated slides, freeze-fractured and fixed with methanol (Miller and Shakes, 1995). For staining of *let-99*ts embryos, embryos were temperature shifted as described in the text, and those undergoing a normal P1 division were fixed during prophase of the EMS cell cycle. Antibody incubation was carried out at 4°C overnight for both anti-LET-99 and anti-GPR-2 (1:50 in PBS) and at room temperature for 1-2 hours with FITC-conjugated goat anti-rabbit or Rhodamine-conjugated goat anti-rat (1:200 in PBS). Primary and secondary antibodies were pre-absorbed with acetone powders of GST-expressing bacteria and wild-type worms respectively. Embryos were staged by DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) staining of the nuclei. Images were obtained on a Leica Confocal. Single-section confocal images taken at a mid-embryo focal plane were analyzed using IP Images software (Scanalytic). To quantify levels of GPR-1/2 staining, the line tool was used to mark the entire cortex, and the average pixel value of the marked region was measured. The unit of relative intensity in Fig. 4 is expressed as a ratio of cortical staining to cytoplasmic staining as in (Tsou et al., 2002).

Results

Loss of Gα and Gβ function produces opposite phenotypes: less active versus hyperactive nuclear and spindle pole movements

In *C. elegans* embryos, Gα (GOA-1 and GPA-16) and Gβ (GPB-1) have been proposed to function in two distinct process, asymmetric spindle positioning and centrosome migration patterns (Gotta and Ahringer, 2001). However, depletion of either also results in spindle orientation defects at second cleavage. To further explore the relationship between Gα, Gβ and spindle movements, we re-examined the phenotypes of *Gα* and *Gβ* mutant embryos. We focused in particular on two phenotypes: the overall movement of nuclei and spindles, and whether the movements were polarized. The speed at which spindle poles move has been taken as evidence of the net forces from the cortex acting on the centrosomes (Grill et al., 2001; Colombo et al., 2003), which assumes that viscosity is not altered in mutant backgrounds. Similarly, in this study we use the overall speed of nuclear and spindle movements as an indicator of the relative magnitude of the net forces acting on the centrosomes. We use the polarity of movements as an indicator of whether the net forces are asymmetric.

In addition to the phenotypes described previously (Gotta and Ahringer, 2001; Zwaal et al., 1996), we observed that *gpb-1(RNAi)* embryos (also referred to as *Gβ* mutants) exhibit hyperactive nuclear and spindle movements (rocking) in all cells from early prophase to metaphase. Instead of the centering and smooth rotational movement of the nucleus seen in wild-type embryos, the nuclear-centrosome complex in *gpb-1(RNAi)* embryos rocked vigorously and did not center completely (Fig. 1A, Table 1). However, the centrosomes aligned along the AP axis by metaphase (Fig. 1A). The speed of nuclear rocking during prophase (0.55±0.09 μm/second, *n*=8) was six times faster than the speed of the nuclear rotation seen in wild-type embryos (0.09 μm/second) (Tsou, 2002). During anaphase in *gpb-1(RNAi)* embryos, the oscillations of the spindle poles were asymmetric and resemble those seen in wild type (Fig. 1A), suggesting that asymmetric forces are present (Grill et al., 2001). At the two-cell stage in *gpb-1(RNAi)* embryos, the nuclei exhibited rocking during prophase (not shown), and P1 nuclear rotation often occurred late, during nuclear envelope breakdown (Gotta and Ahringer,

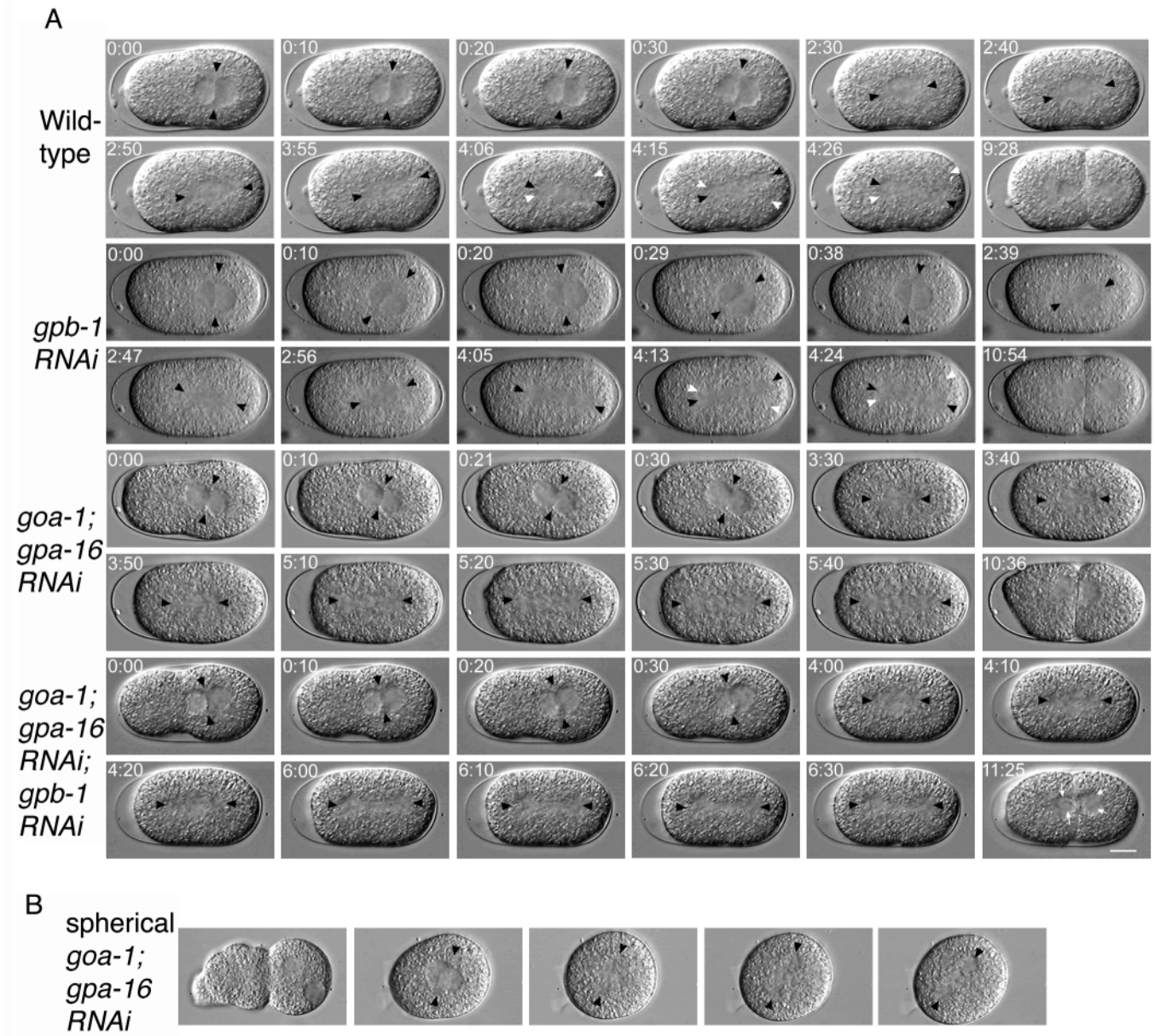

Fig. 1. *Gα* and *Gβ* mutants display opposite phenotypes and the *Gβ* phenotypes are due to gain of Gα activity. (A) DIC images of live one-cell embryos recorded by time-lapse video microscopy in various genetic backgrounds (as indicated) undergoing the first division. Black arrowheads indicate the current position of centrosomes in each image. White arrowheads indicate the position of the centrosome in the previous image during anaphase spindle pole oscillations. White arrows indicate multiple nuclei. Relative time points are indicated (minutes:seconds). Note the rapid changes in centrosome position in *gpb-1(RNAi)* embryos (rocking), and the lack of anaphase spindle pole oscillations in *Gα* mutant embryos. (B) Spherical *Gα(RNAi)* embryos. Anterior is towards the left in this and all subsequent figures. Scale bar: 10 μm.

2001; Zwaal et al., 1996). These results together suggest that the net forces acting on nuclei and spindles in *Gβ* mutant cells are hyperactive but still act asymmetrically. We therefore propose that GPB-1 is required for controlling the magnitude of the net forces acting on centrosomes, but is not required for generating asymmetric forces in early *C. elegans* embryos.

In contrast to the *gpb-1(RNAi)* phenotype described above, *goa-1(RNAi); gpa-16(RNAi)* embryos [also referred to as *Gα(RNAi)* or *Gα* mutant embryos] exhibited no nuclear rocking movements from prophase to metaphase. Anaphase spindle elongation was symmetric with neither spindle pole undergoing transverse oscillations (Fig. 1A). At the two-cell stage, *Gα* mutant embryos had nuclei mispositioned close to the cell contact region (Fig. 1A), often had multiple nuclei in each cell, and failed to exhibit nuclear rotation as previously reported (Gotta and Ahringer, 2001).

Although nuclear rotation was observed in some one-cell *Gα* mutants embryos (6/18 embryos), in many embryos the two centrosomes were prematurely positioned on the AP axis before pronuclear meeting (12/18 embryos). To further examine nuclear rotation in *Gα* mutants, we examined embryos in which the eggshell was removed by chitinase

digestion. Our recent studies showed that although wild-type embryos have a PAR polarity-dependent mechanism for nuclear rotation that is cell-shape independent (Hyman and White, 1987; Tsou et al., 2003), ectopic rotation in certain polarity and spindle orientation mutants can be driven by the oval shape of the embryo (Tsou et al., 2002; Tsou et al., 2003). Thus, removal of the eggshell to produce a spherical embryo is essential to determine if the intrinsic polarity-dependent mechanism of nuclear rotation remains functional. In spherical *Gα(RNAi)* embryos in which the centrosomes were normally positioned at pronuclear meeting, nuclear rotation failed to occur (*n*=3; Fig. 1B). This result indicates that Gα not only is required for asymmetric anaphase spindle positioning as described previously, but is also essential for intrinsically controlled nuclear rotation in the one-cell embryo.

Overall, the less active nuclear and spindle movements of *Gα* mutant embryos suggest that the net forces acting on nuclei and spindles are much smaller than in wild-type and *Gβ* mutant cells. It is not clear whether Gα is directly required for the asymmetry of forces, or if there is simply insufficient force to respond to asymmetric cues in the absence of Gα. Taken together, these results suggest that Gα and Gβ depletion cause opposite effects in the one-cell embryo: depletion of Gβ causes hyperactive but polarized nuclear and spindle movements, while depletion of Gα causes less active and non-polarized nuclear and spindle movements.

The hyperactive spindle movements of *gpb-1(RNAi)* embryos are due to excess Gα activity

The opposite phenotypes of Gα and Gβ mutant embryos described above can be explained by three hypotheses: (1) Gα and Gβγ have distinct downstream effectors and function independently to affect nuclear and spindle movements; (2) Gβγ is the major regulator while the less-active nuclear and spindle movements seen in Gα mutant embryos are due to gain of Gβγ activity; and (3) Gα is the major regulator while the hyperactive nuclear and spindle movements seen in the Gβγ mutant embryos are gain of Gα phenotypes. Simultaneous depletion of both Gα and Gβ should distinguish among these possibilities. Previous triple RNAi analyses were interpreted as support for the first hypothesis, however those analyses did not examine all of the phenotypes reported here. Therefore, we re-examined *Gα; Gβ* double mutants using RNA interference of *gpa-16* and *gpb-1* function in a *goa-1* mutant background.

The phenotype of *goa-1(n1134); gpa-16(RNAi); gpb-1(RNAi*) embryos (*n*=13) was indistinguishable from that of *Gα* single mutant embryos. Significantly, the centration defects and hyperactive nuclear movements during prophase and metaphase that are indicative of Gβ depletion were completely suppressed (Fig. 1A, Table 1). During anaphase, neither spindle pole exhibited oscillations and division was symmetric. In addition, these embryos showed the nuclear mispositioning defect and multiple nuclei seen in *Gα* embryos (Fig. 1A). Control RNAi experiments done in parallel using RNAi of *gpb-1* in wild-type worms produced the characteristic *gpb-1* phenotype, and RNAi of *gpb-1* in *goa-1(n1134)* worms produced a phenotype intermediate between that of *goa-1* and *gpb-1* mutant embryos (not shown). These results indicate that RNAi of *gpb-1* was effective and therefore we conclude that Gα loss of function is epistatic to Gβ loss of function. The absence of the nuclear rocking and centration defects in the *Gα;Gβ* double mutants is consistent with the hypothesis that the hyperactive movements of nuclei in *gpb-1(RNAi)* embryos are due to excess Gα activity, rather than the loss of a *gpb-1* specific function.

Our results are in contrast to previous studies in which *Gα;Gβ* triple RNAi embryos were generated that exhibited intermediate or additive phenotypes compared with *Gα* and *Gβ* single RNAi mutants (Gotta and Ahringer, 2001; Srinivasan et al., 2003). Our use of a strong loss-of-function mutant and only double RNAi may have resulted in a stronger phenotype because the efficacy of RNAi drops for some genes when carried out using three or more RNAs (Gonczy et al., 2000). In addition, Gα and Gβγ naturally sequester each other, and reducing one subunit will release and increase the free form of the other subunit. Thus, if Gα double RNAi does not completely deplete the protein, any remaining Gα will probably be sequestered by excess Gβγ, resulting in a strong Gα phenotype. This Gα could then be released and become active when Gβ is removed by RNAi, resulting in a 'synthetic' intermediate or weaker phenotype.

RNA interference of the receptor independent G-protein regulators GPR-1/2 produces a similar phenotype to that of *Gα(RNAi)* and suppresses the *Gβ(RNAi)* phenotype

Recent work has shown that two *C. elegans* homologs of receptor independent activators of G protein signaling, called GPR-1 and GPR-2 (GPR-1/2), are involved in spindle positioning. *gpr-1/2(RNAi)* embryos have a phenotype very similar to that of *Gα (RNAi)* (Gonczy et al., 2000; Srinivasan et al., 2003; Colombo et al., 2003; Gotta et al., 2003), and thus GPR-1/2 is thought to be required positively for Gα signaling. Based on studies of GPR homologs in other systems (Schaefer et al., 2001), loss of GPR should result in more inactive GαGβγ trimeric complexes and thus cause a loss of function for both Gα and Gβ. The strong similarity in phenotype between *Gα(RNAi)* and *gpr-1/2(RNAi)* embryos is thus consistent with our interpretation that the Gβ phenotype is due to excess Gα, rather than to a loss of Gβ specific effector function. Furthermore, if this hypothesis is correct, then *gpr-1/2* should mimic Gα in all double mutant combinations.

To provide a baseline for double mutant analysis, we first examined the *gpr-1/2(RNAi)* phenotype using RNA interference of *gpr-2*. Because *gpr-2* is 96% identical to *gpr-1* at the nucleotide level, RNAi is expected to inhibit the function of both genes. Antibody staining with anti-GPR-2 antibodies showed that RNA interference did deplete GPR-1/2 protein (see below). In *gpr-1/2(RNAi)* one-cell embryos, less active nuclear and spindle movements like those seen in Gα mutants were observed (Fig. 2). During prophase, no rocking of the nucleus was observed (*n*=13). During anaphase, no oscillations of the spindle poles were observed in any embryos, but in many embryos (62%) the spindle still elongated asymmetrically toward the posterior pole. This asymmetric spindle elongation in *gpr-1/2(RNAi)* embryos was reduced compared with wild type, but did result in slightly unequal cleavage (compare Fig. 2 with Fig. 1, Table 1). Nuclear mispositioning after cell division was also observed in *gpr-1/2(RNAi)* cells (Fig. 2), as in Gα mutants.

If GPR-1/2 functions together with Gα, then loss of GPR-1/2 should be able to suppress the hyperactive nuclear and

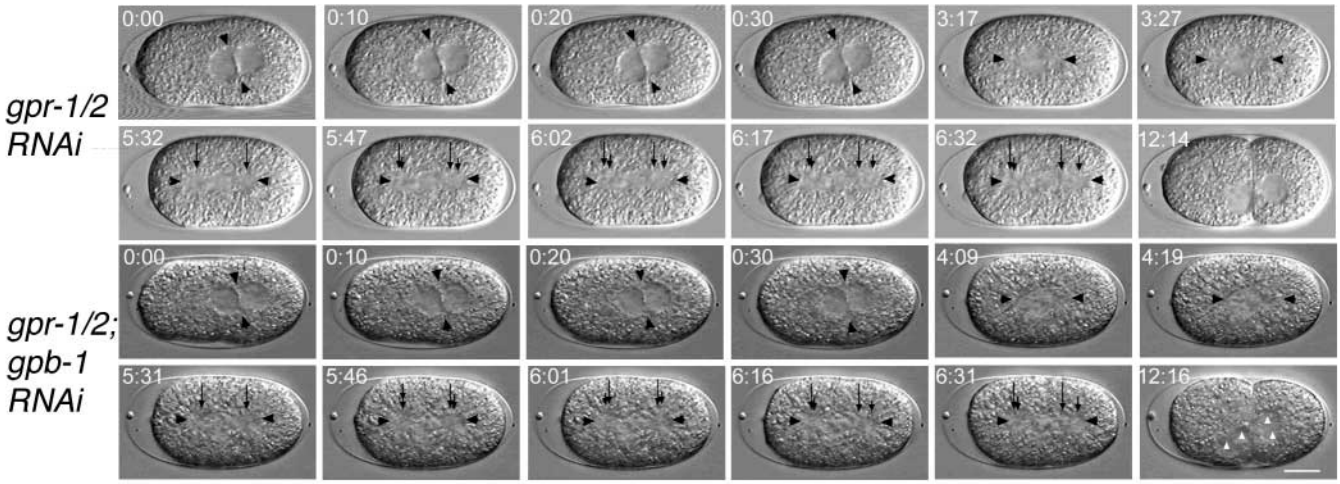

Fig. 2. *gpr-1/2(RNAi)* embryos have similar phenotypes to those seen in *Gα* mutants. DIC images of live *gpr-1/2(RNAi)* and *gpr-1/2(RNAi); gpb-1(RNAi)* one-cell embryos recorded by time-lapse video microscopy undergoing the first division. Black arrowheads mark the current position of centrosomes. Short arrows mark the current position of the spindle poles on the AP axis during spindle elongation, and long arrows indicate the original position of the spindle poles before spindle elongation onset. White arrowheads indicate multiple nuclei. Note the lack of hyperactive nuclear rocking and lack of anaphase spindle pole oscillations (compare with Fig. 1). Relative time points are indicated (minutes:seconds). Scale bar: 10 μm.

spindle movements in *Gβ* mutants, just as loss of Gα did. In *gpr-1/2(RNAi); gpb-1(RNAi)* embryos, hyperactive movements were completely suppressed during prophase and metaphase, and centration and nuclear rotation resembled that seen in *gpr-1/2* or *Gα* single mutants (Fig. 2, Table 1). Similarly, *gpr-1/2(RNAi); gpb-1(RNAi)* embryos showed no spindle pole oscillations during anaphase and spindle pole elongation was reduced and only slightly asymmetric, as in *gpr-1/2(RNAi)* embryos (Fig. 2, Table 1). The fact that *gpr-1/2(RNAi); gpb-1(RNAi)* embryos resemble *Gα; Gβ* double mutant embryos further supports the hypothesis that Gα and GPR-1/2 act together to regulate forces that affect spindle positioning, and that the phenotypes seen in Gβ mutants are due to gain of Gα/GPR-1/2 activity.

Asymmetric localization of GPR-1/2 at the cortex depends on PAR-3 and LET-99

GPR-1/2 were recently shown to be asymmetrically localized at the cell cortex in response to PAR proteins (Colombo et al., 2003; Gotta et al., 2003). Because LET-99 is also asymmetrically localized in a PAR-dependent manner, we sought to determine the relationship between PAR-3, LET-99 and GPR-1/2 localization. We first confirmed the asymmetric localization of GPR using affinity-purified antibodies against a full-length GPR-2 fusion protein, which are also expected to recognize the 97% identical GPR-1 protein. In early wild-type embryos, GPR-1/2 were localized both on the asters and the cell cortex (Fig. 3A-I). These staining patterns were absent in *gpr-1/2(RNAi)* embryos, suggesting that they are specific (Fig. 3J); by contrast, nuclear staining observed in all cells was present in *gpr-1/2(RNAi)* embryos, suggesting that this staining is not GPR-specific (Fig. 3J). The cortical localization of GPR-1/2 changed with the cell cycle. In one-cell embryos, GPR-1/2 were uniformly present at a low level on the cortex from early prophase to metaphase. The level of cortical localization of GPR-1/2 increased and became weakly enriched at the posterior pole of most embryos during anaphase (78%, *n*=42; Fig. 3C). In some embryos with enriched posterior staining, GPR-1/2 also appeared to be slightly enriched at the anterior pole of the embryo, compared with lateral regions (Fig. 3D and Fig. 4B). GPR-1/2 asymmetry became more pronounced during cytokinesis and interphase of the two-cell stage. In the P1 blastomere during interphase (Fig. 3E,F), GPR-1/2 were highly enriched around the posterior pole of the cell (100%, *n*=53), and were present at low levels uniformly around the cortex of AB. As the cell cycle progressed, GPR-1/2 asymmetry in P_1 disappeared; GPR-1/2 were uniformly localized around the cortex through out prophase, metaphase and early anaphase (Fig. 3G,H). During late anaphase and telophase, GPR-1/2 were once again enriched at the posterior part of the P_1 cell (Fig. 3I). These results indicate that GPR-1/2 is asymmetrically localized in the P lineage.

If Gα and GPR-1/2 function together as a complex, the localization of GPR-1/2 should depend on Gα but not Gβ. Cortical localization of GPR-1/2 was no longer observed in *Gα* mutant embryos at any stage of the cell cycle (*n*=21, Fig. 3K-O). In particular, at interphase in two-cell and four-cell embryos when GPR-1/2 enrichment is most evident in wild type (Fig. 3E,F,I), GPR-1/2 staining at the cortex and cell contact regions was undetectable in *Gα(RNAi)* embryos (Fig. 3M-O). By contrast, in *Gβ* mutant embryos, GPR-1/2 were still localized at the cortex in all cells (Fig. 3P-T). Posterior enrichment of GPR-1/2 were observed during late anaphase through the next interphase in P cells (Fig. 3S,T), although in some embryos the posterior domain in the P_1 cell appeared larger than in wild type. These observations indicate that Gβ is not required for cortical localization and posterior enrichment of GPR-1/2, which is consistent with our observations that Gβ is not required for polarized spindle movements. Interestingly, the staining intensity of cortical GPR-1/2 from prophase to metaphase appeared higher in *Gβ* mutant embryos than that in wild-type cells (Fig. 3P) and quantification of average fluorescence intensity was consistent with this observation (Fig. 4P). At later stages, when anaphase

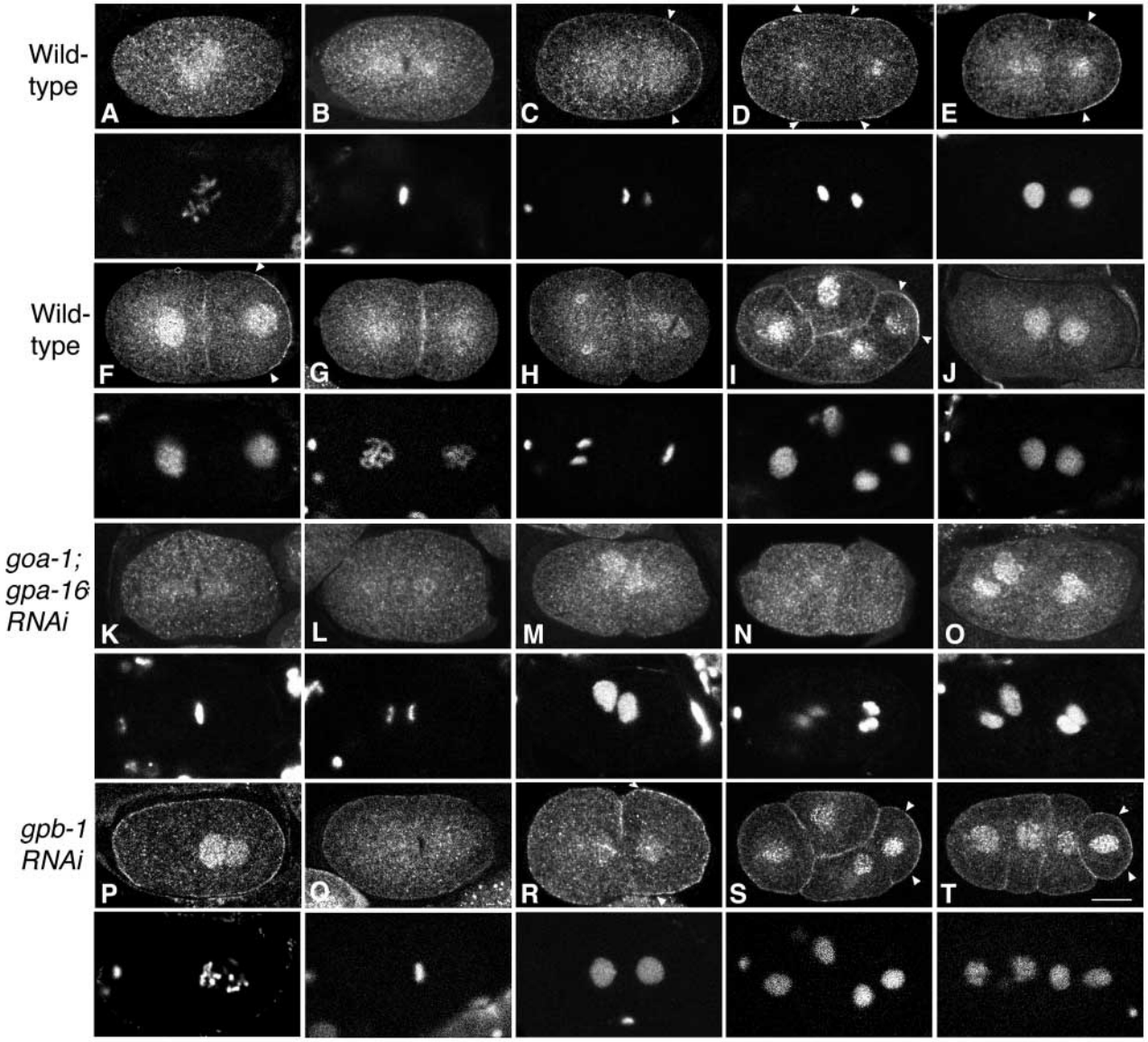

Fig. 3. Localization of GPR-1/2. Confocal sections of wild-type (A-I), *gpr-1/2(RNAi)* (J), *Gα(RNAi)* (K-O) and *gpb-1(RNAi)* (P-T) embryos stained with anti GPR-1/2 antibodies (top panels for each series) and DAPI to visualize DNA (bottom). (A,P) One-cell prophase embryos. (B,K,Q) One-cell metaphase embryos. (C,D,L) One-cell anaphase embryos. (E) One-cell telophase embryo. (F,J,M,R) Two-cell interphase embryos. (G) Two-cell prophase embryo. (H) Two-cell embryo where P1 is at metaphase. (I,N,O,S,T) Four-cell interphase embryos. White arrowheads indicate the boundaries of the domains enriched GPR-1/2. Scale bar: 10 μm.

spindle pole oscillations occur in both *Gβ* mutant and wild-type embryos, the cortical GPR-1/2 staining levels in Gβ mutants resembled that in wild-type embryos (Fig. 3I,S). These findings are consistent with the idea that in *Gβ(RNAi)* embryos, the loss of Gβ results in more Gα and GPR-1/2 at the cortex in prophase, resulting in excess Gα/GPR-1/2 activity. In contrast to the effects on cortical localization, GPR-1/2 can still localize to the microtubule asters in either *Gα* or *Gβ* mutant embryos (Fig. 3K-T), indicating that different mechanisms are used to localize GPR-1/2 to asters.

Recent studies showed that asymmetric enrichment of GPR-1/2 in the P lineage depends on the PAR-3 polarity protein, and it was observed that GPR-1/2 levels are high in both the AB and P1 cells just after division in *par-3* embryos (Colombo et al., 2003; Gotta et al., 2003). Our observations confirm this result, but we also note that the higher levels appear late in the first cell cycle. In prophase and metaphase of one-cell *par-3* embryos, cortical GPR-1/2 staining appeared similar to wild type (compare Fig. 4E with 4A). During late anaphase and telophase in one-cell *par-3* embryos, the posterior enrichment of GPR-1/2 was not observed (*n*=11; Fig. 4F) and instead the entire cortex showed higher staining intensity for GPR. During interphase of the next cell cycle, strong GPR1/2 staining was observed in both daughter cells (*n*=13; Fig. 4G), instead of being restricted to the posterior pole of P1 (Fig. 4C). In some embryos GPR-1/2 levels appeared higher at the poles of both AB and P_1 (Fig. 4G). In four-cell embryos, during interphase, many cells showed a cap of GRP-2 enrichment at

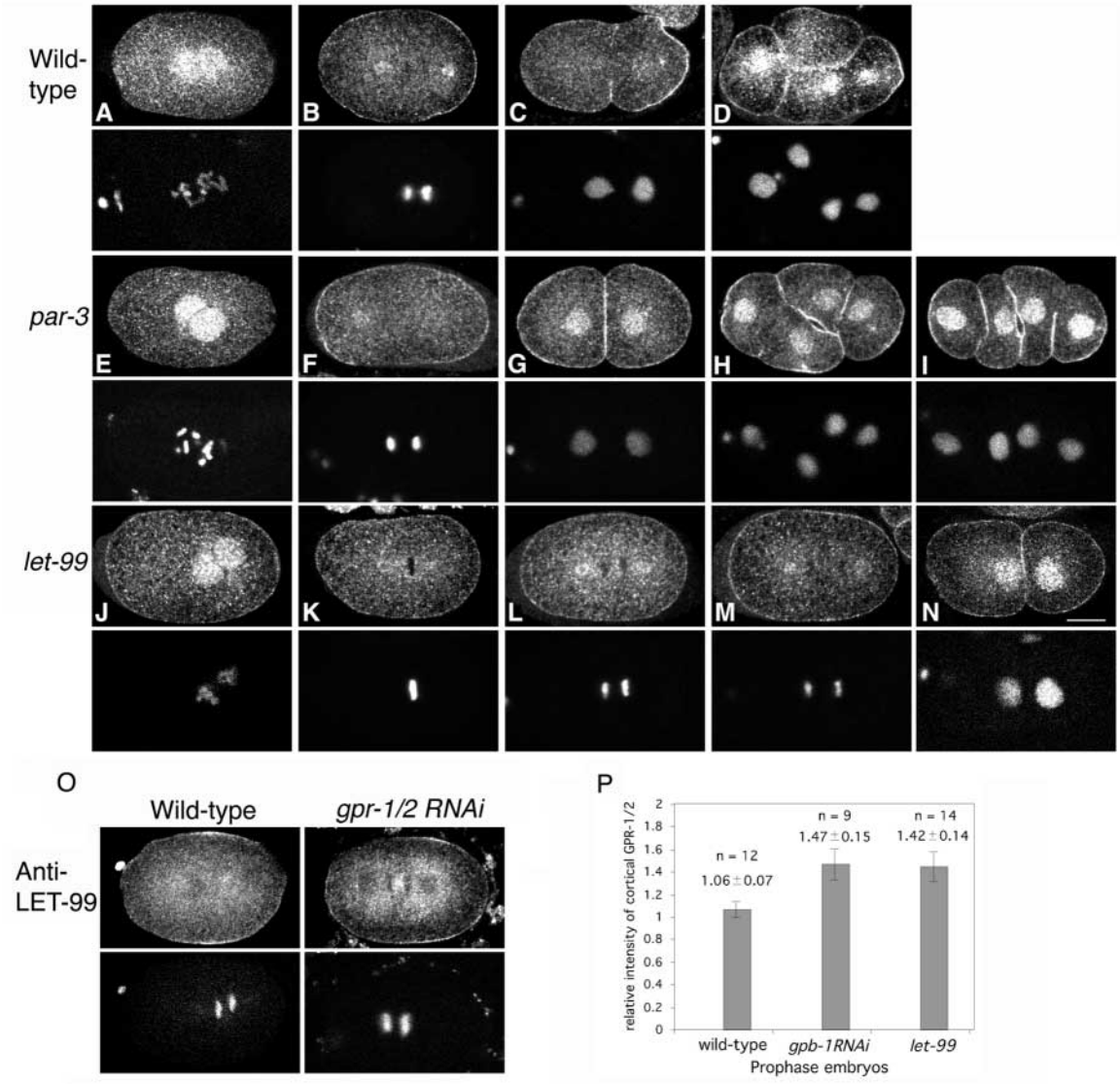

Fig. 4. GPR-1/2 asymmetry depends on PAR-3 and LET-99. (A-N) Confocal images of wild-type embryos (A-D), *par-3* embryos (E-I) and *let-99* embryos (J-N) stained with anti-GPR-1/2 antibodies (top panels) and DAPI (bottom panels). (A,E,J) One-cell prophase embryos. (K) One-cell metaphase embryos. (B,F,L,M) One-cell late anaphase embryos. (C,G,N) Two-cell interphase embryos. (D,H,I) Four-cell interphase embryos. (O) Confocal images of wild-type and *gpr-1/2(RNAi)* anaphase embryos stained with anti-LET-99 antibodies. Scale bar: 10 μm. (P) Quantification of relative intensity of GPR-1/2 staining in wild-type, *gpb-1(RNAi)* and *let-99(or81)* one-cell prophase embryos.

the cell periphery away from cell contact regions (*n*=17; Fig. 4H,I).

Next, we asked if the PAR-dependent asymmetric enrichment of GPR-1/2 is mediated through the *let-99* gene, as LET-99 functions in spindle positioning and is asymmetrically localized in response to PAR-3 (Tsou et al., 2002). In *let-99* embryos, where the PAR proteins are distributed normally (Rose and Kemphues, 1998a), the posterior enrichment of GPR-1/2 was no longer observed. Instead GPR-1/2 were uniformly distributed around the entire cortex of P_0 (*n*=19; Fig. 4J-M). Interestingly, unlike in *par-3* embryos, the fluorescence intensity of the cortical GPR-1/2 during prophase appeared higher than in wild-type embryos. This change in GPR staining during prophase appeared similar to that seen in *Gβ* mutant embryos (Fig. 4P). In early interphase of the two-cell stage, no polar enrichment was seen in P_1 (*n*=17; Fig. 4N), and the staining intensity of the AB cell cortex appeared similar to that of the P_1 cortex. These data indicate that the asymmetry of GPR-1/2 localization requires both PAR-3 and LET-99. Conversely, in *gpr-1/2(RNAi)* embryos, LET-99 is asymmetrically enriched at the cortex of P lineage cells as in wild-type embryos (Fig. 4O; *n*=14). Together these results support the hypothesis that LET-99 acts upstream of, or at the level of Gα/GPR-1/2 signaling.

LET-99 functions antagonistically to Gα/GPR-1/2 signaling pathway in the P lineage

We have shown that LET-99 is required for asymmetric GPR-1/2 localization. However, the cortical localizations of LET-99 and GPR-1/2 in cells do not overlap but instead are somewhat reciprocal at anaphase. In addition, the hyperactive and dynein-dependent nuclear and spindle oscillations exhibited by *let-99*

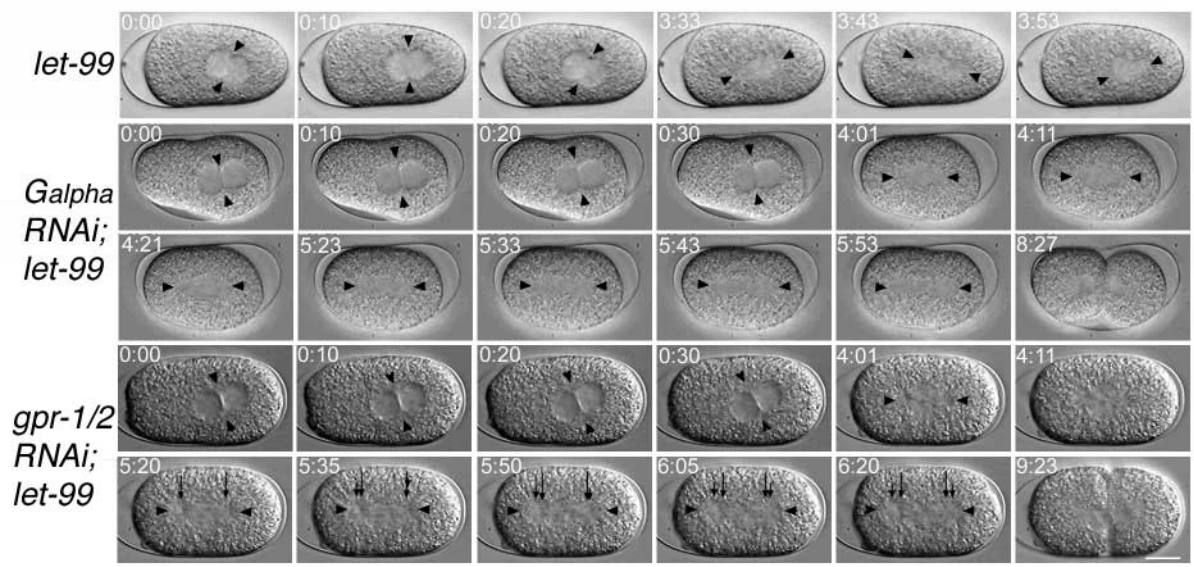

Fig. 5. Hyperactive nuclear and spindle movements of *let-99* embryos are suppressed in *Gα; let-99* and *gpr-1/2; let-99* double mutants. DIC images of live *let-99, Gα(RNAi); let-99* and *gpr-1/2(RNAi); let-99* one-cell embryos recorded by time-lapse video microscopy. Centrosomes are marked as in Fig. 2. Note the lack of hyperactive nuclear rocking, and lack of asymmetric anaphase movements. Relative time points are indicated. Scale bar: 10 μm.

mutants (Tsou et al., 2002) are similar to those shown here for Gβ mutant embryos, which appear to be due to excess Gα/GPR-1/2 activity (Fig. 4K). Together, these observations suggest that *let-99* functions antagonistically to Gα/GPR-1/2 signaling.

To test this hypothesis, we examined *Gα(RNAi); let-99(or81)* and *gpr-1/2(RNAi); let-99(or81)* double mutant embryos during the first cell cycle. In both *Gα(RNAi); let-99* and *gpr-1/2(RNAi); let-99* double mutant embryos, the phenotype resembled that of *Gα(RNAi)* embryos alone. In particular, the *let-99* centration defects and nuclear and metaphase rocking phenotypes were completely suppressed (Fig. 5; Table 1), consistent with the idea that the hyperactive nuclear movements observed in *let-99* embryos are due to an excess of Gα/GPR-1/2 signaling. During anaphase in *Gα(RNAi); let-99* and *gpr-1/2(RNAi); let-99* double mutant embryos, the spindle poles did not exhibit oscillations, and spindle elongation and first cleavage were symmetric as seen in *Gα(RNAi)* embryos (Fig. 5). Two-cell double mutant embryos also showed Gα phenotypes, such as mispositioned nuclei (Fig. 5). Interestingly, the slightly asymmetric spindle elongation movements observed during anaphase in *gpr-1/2(RNAi)* embryos were not observed in *gpr-1/2(RNAi);let-99* double mutant embryos (compare Fig. 2 with Fig. 5, Table 1). The remaining asymmetry in *gpr-1/2(RNAi)* embryos suggests that although force generation is greatly reduced in these embryos, the spindle is still responding to an asymmetric cue. The loss of asymmetry in *gpr-1/2(RNAi);let-99* embryos thus suggests that LET-99 is part of that asymmetric cue.

To test further if *let-99* functions antagonistically to the Gα/GPR-1/2 pathway, we asked whether a partial loss of *let-99* function could suppress any aspects of Gα loss of function. The *gpa-16(it143ts)* allele is a strong loss-of-function mutation in one of the partially redundant Gαs [previously known as *spn-1(it143)* (Bergmann et al., 2003)]. We found that *it143/it43; let-99/+* worms raised at 25°C produced embryos with a hatch rate of 53% (*n*=459) compared with a hatch rate of 30% (*n*=526) for *it143/it143* worms alone. The decrease in embryonic lethality is consistent with the hypothesis that *let-99* functions antagonistically to the Gα/GPR-1/2 signaling pathway. These genetic results and the loss of GPR-1/2 asymmetry in *let-99* embryos support the hypothesis that Gα/GPR-1/2 functions to upregulate forces and LET-99 serves as an asymmetric cue that negatively regulates force generation in response to PAR polarity.

GPR-1/2 and LET-99 are asymmetrically localized in opposite patterns at the EMS/P_2 boundary in response to MES1/SRC-1 signaling

To see if Gα/GPR-1/2 and LET-99 are also involved in the asymmetric division of EMS cells, we first determined the localization of both proteins. Strikingly, in EMS cells from prophase to prometaphase, when nuclear rotation normally occurs, GPR-1/2 were asymmetrically enriched at the EMS/P_2 boundary (Fig. 6B,C,G,I; *n*=33). As we previously reported, there is no cortical LET-99 band in the EMS cell. However, we observed a cell cycle dependent change in LET-99 localization at cell contacts. In contrast to the enrichment of GPR-1/2 at prophase, LET-99 was greatly reduced at the EMS/P_2 boundary compared with other cell boundaries during the same stage (Fig. 6E,F,H,I; *n*=25), even though LET-99 was initially present at this boundary during interphase (Fig. 6D). To test whether GPR-1/2 and LET-99 asymmetries depend on either of the signaling pathways known to function in EMS spindle orientation, we examined their localization in mutant embryos defective in either the MES-1/SRC-1 or the Wnt signaling pathways. In *mom-5* mutant embryos (MOM-5 is the Frizzled receptor in the Wnt pathway), GPR-1/2 and LET-99 asymmetries were still observed at the EMS /P_2 boundary (Fig. 6J, *n*=9 and 11 embryos respectively), just as in wild type. However, in *mes-1* mutant embryos, GPR-1/2 were no longer enriched at the EMS/P_2 boundary (Fig. 6J, *n*=14). Similar results were recently reported for GPR-1/2 asymmetry by others

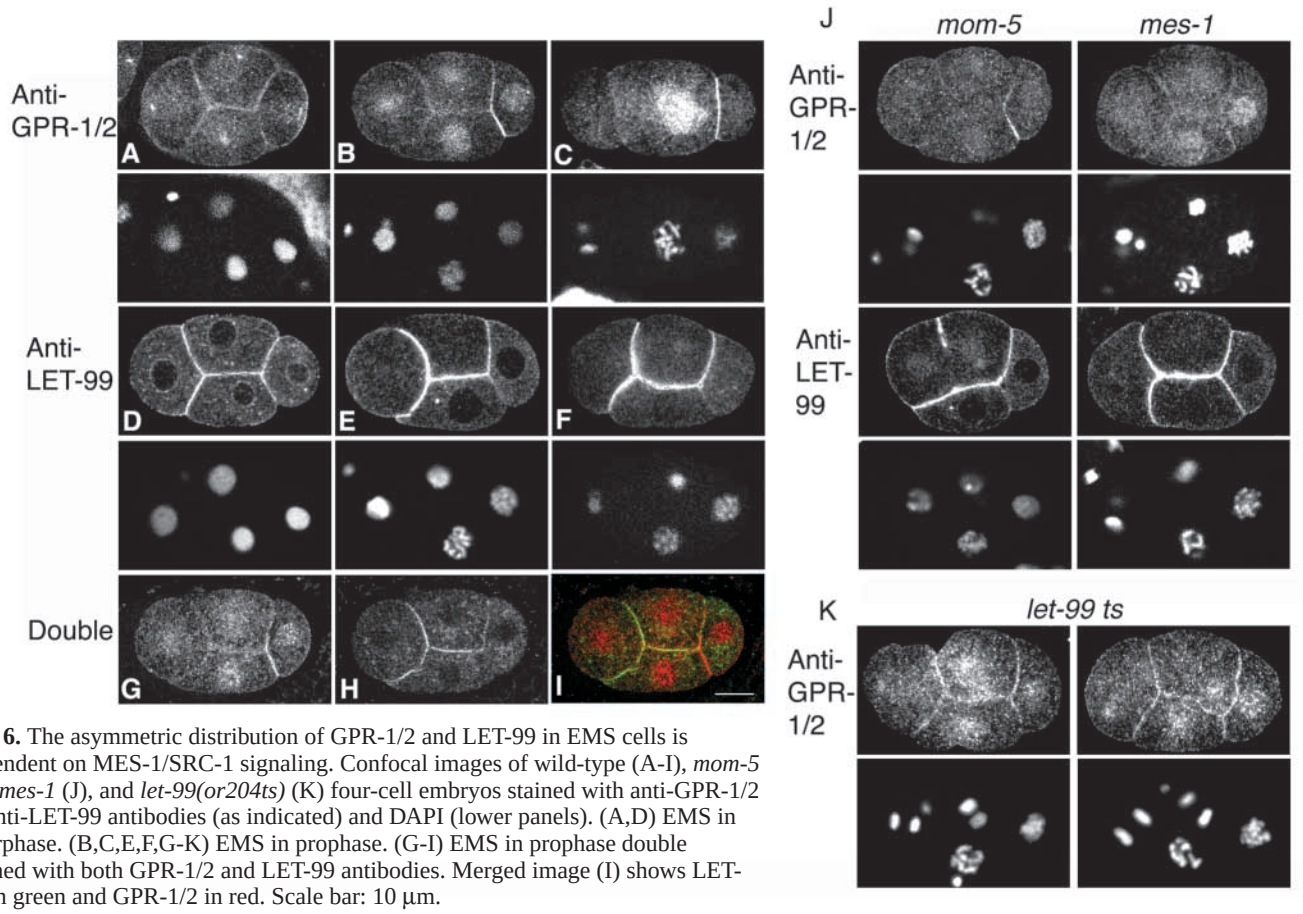

Fig. 6. The asymmetric distribution of GPR-1/2 and LET-99 in EMS cells is dependent on MES-1/SRC-1 signaling. Confocal images of wild-type (A-I), *mom-5* and *mes-1* (J), and *let-99(or204ts)* (K) four-cell embryos stained with anti-GPR-1/2 or anti-LET-99 antibodies (as indicated) and DAPI (lower panels). (A,D) EMS in interphase. (B,C,E,F,G-K) EMS in prophase. (G-I) EMS in prophase double stained with both GPR-1/2 and LET-99 antibodies. Merged image (I) shows LET-99 in green and GPR-1/2 in red. Scale bar: 10 μm.

(Srinivasan et al., 2003). Interestingly, LET-99 was now present at the EMS/P_2 boundary during prophase/prometaphase in *mes-1* mutant embryos (Fig. 6J, *n*=20). Similar results were obtained in *mom-5; mes-1* double mutants (data not shown). Thus, the asymmetric patterns of both LET-99 and GPR-1/2 at the EMS/P_2 cell boundary are MES-1/SRC-1 signaling dependent, which suggests that LET-99 and Gα signaling act downstream of MES-1/SRC-1 to promote spindle orientation in EMS.

To determine if LET-99 is required for the Gα/GPR-1/2 asymmetry in EMS cells, we used a *let-99* temperature-sensitive mutation. Homozygous *let-99(or204ts)* worms were grown at 16°C and shifted to 25°C during the division of P_1. The embryos were observed until prophase of the EMS cell cycle, then fixed and stained for GPR-1/2. Although GPR-1/2 were present at all cell contacts, the enrichment of GPR-1/2 at the EMS/P_2 boundary was not seen in any of the *let-99* embryos (*n*=8, Fig. 6K). This loss of GPR-1/2 asymmetry is unlikely to be due to disruption of P_2 cell fate. Under these conditions, the P_1 spindle was oriented normally as in wild type (Fig. 7K), and division was unequal and produced an EMS and P_2 cell with asynchronous cell cycles (Fig. 6K). Thus, these results suggest that LET-99 is required at the four-cell stage for the asymmetry in GPR-1/2 localization.

Gα and LET-99 are required for nuclear rotation in EMS cells

If Gα/GPR-1/2 and LET-99 transmit asymmetric MES-1/SRC-1 cues to the machinery that orients the spindle in EMS, then both Gα and LET-99 should be required for nuclear rotation in EMS. Defects in EMS division have been reported previously for *let-99* and G protein mutant embryos (Zwaal et al., 1996; Gotta and Ahringer, 2001; Rose and Kemphues, 1998a). However, because Gα and LET-99 are required for asymmetric cell division of the P_1, it is unknown whether the defects in EMS spindle orientation reflect direct roles for these proteins in spindle orientation or reflect a failure to properly specify the EMS and P_2 cells. To address the role of LET-99 and Gα in EMS, we therefore carried out temperature shift experiments using the *let-99(or204ts)* and the *gpa-16(it143ts)* alleles. *let-99(or204ts)* and *gpa-16(it143ts)* embryos were shifted to 25°C during second cleavage as described above, to ensure proper asymmetric division of the P_1 cell (Fig. 7F,K). In all EMS cells of such *let-99* embryos, nuclear rotation failed and the spindle set up transversely (*n*=8; Fig. 7M-O). Similarly, nuclear rotation failed in 31% of the EMS cells of *gpa-16(it143ts)* embryos shifted up during P_1 cleavage (*n*=13; Fig. 7H-J). The incomplete penetrance of the *gpa-16(it143ts)* phenotype probably reflects partial redundancy with *goa-1*. These results indicate that both Gα signaling and LET-99 are required for proper spindle orientation in EMS cells. Thus, asymmetric divisions specified by both intrinsic cues and extrinsic signaling involve common downstream components.

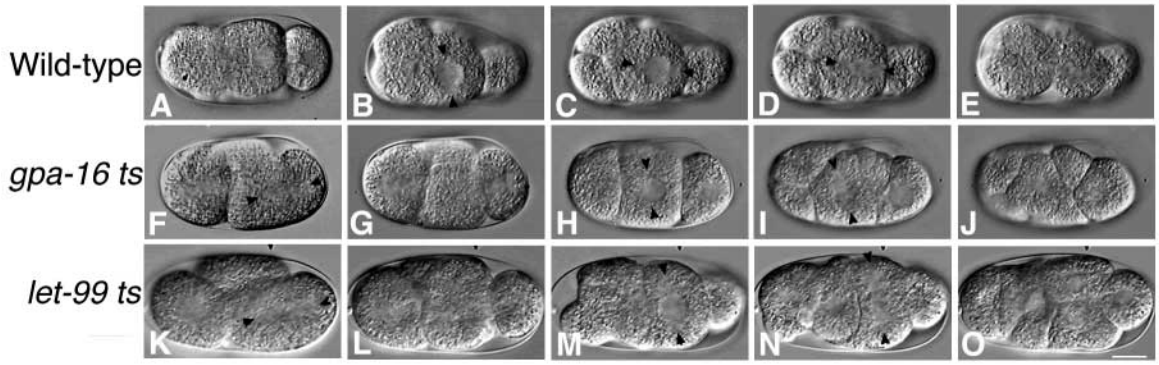

Fig. 7. Gα and *let-99* are required for nuclear rotation in EMS cells. DIC images of live wild-type (A-E), *gpa-16(it143ts)* (F-J) and *let-99(or204ts)* (K-O) embryos recorded by time-lapse video microscopy after shifting to 25°C as described in text. Black arrowheads mark the current position of centrosomes. Note the normal division of P_1 (F,G,K,L), but the absence of EMS nuclear rotation in the mutants. Scale bar: 10 μm.

Discussion

Gα/GPR-1/2, but not Gβγ, are key regulators that control force generation in *C. elegans* embryos

Proper spindle positioning during asymmetric cell division involves polarized nuclear and spindle movements. To produce these movements, the magnitude and polarity of the forces that act on centrosomes and spindle poles are key factors that could be regulated in response to polarity cues. The studies presented here are in agreement with recent reports that concluded that Gα/GPR-1/2 are required for the majority of force generation during anaphase (Srinivasan et al., 2003; Colombo et al., 2003; Gotta et al., 2003). Furthermore, our results provide new evidence for the hypothesis that the Gα/GPR-1/2 signaling pathway is also responsible for the magnitude of force generation during nuclear rotation. In spherical *Gα* mutant embryos, nuclear rotation failed and in *Gα* and *gpr-1/2* mutant embryos less-active spindle movements were observed, suggesting that less force is produced in these mutant cells. By contrast, in *Gβ* mutant embryos, abnormal nuclear and spindle oscillations indicative of hyperactive forces were observed. In addition, the hyperactive movements seen in *Gβ* mutants were completely suppressed when the activities of Gα or GPR-1/2 were removed together with Gβ. Together these results suggest that Gα and GPR-1/2 function together to upregulate force production, and that this force production is needed both for spindle orientation and asymmetric anaphase elongation. Significantly, the suppression of the Gβ phenotypes in these double mutants suggest that Gβ does not activate specific downstream effectors in the one cell, and raises the possibility that other aspects of the Gβ mutant phenotypes are due to excess Gα activity as well.

The G proteins and GPR-1/2 are localized at the cortex and the microtubule asters. The adapter protein LIN-5 forms a complex with Gα and GPR-1/2, and is required for the overall cortical and astral localization of the Gα/GPR-1/2 complex (Srinivasan et al., 2003). Gα signaling at either the cortex or the asters could influence microtubules and their interactions with the cortex, and thus regulate force generation. Several observations support the hypothesis that the cortex is the active site of Gα signaling. Previous work showed that Gα is absent from the asters in *Gβ* mutant embryos but is still present at the cortex (Gotta and Ahringer, 2001). The observation that the phenotype of *Gβ* mutants is opposite to that of *Gα* mutants thus suggests that the asters are not the sites of Gα signaling for spindle positioning. In addition, the cortical localization of GPR-1/2 depends on Gα but not Gβ (Colombo et al., 2003) (this report). Indeed, there appears to be more GPR on the cortex during prophase in *Gβ* mutants, and loss of Gα/GPR activity suppressed the Gβ phenotype. These correlations suggest that the primary site of Gα/GPR-1/2 activity for spindle positioning is at the cortex.

The conclusion that Gα/GPR-1/2 are the key regulators of spindle positioning in *C. elegans* is different from what has been reported in *Drosophila* neuroblast cells (Schaefer et al., 2001). When GTP-Gα was overexpressed in neuroblasts, no spindle orientation phenotype was observed. However, when GDP-Gα was overexpressed, random spindle orientations were seen, similar to those observed in Gβ13F mutants (which lack both Gα and Gβ13F) (Schaefer et al., 2001). It was predicted that GDP-Gα would sequester the free Gβγ, and thus it was concluded that Gβγ is the key signaling molecule that regulates polarity and spindle orientation in neuroblast cells (Schaefer et al., 2001). The difference between *Drosophila* and *C. elegans* could reflect differential usage of conserved molecules. An alternative interpretation is that overexpression of GDP-Gα could result in a gain-of-function spindle orientation phenotype, as we have shown here for loss of Gβ. That is, loss or gain of Gα activity could both produce defects in spindle orientation, which may not be distinguishable without live imaging of the spindle movements.

LET-99 serves as an asymmetric cue that functions antagonistically to the Gα/GPR-1/2 signaling pathway

Forces must also be polarized in response to PAR polarity cues in order to achieve proper spindle positioning. The localization of GPR-1/2 has led to the model that the enrichment of GPR-1/2 at the posterior provides higher pulling forces on the posterior spindle pole, thus mediating anaphase spindle positioning (Srinivasan et al., 2003; Colombo et al., 2003; Gotta et al., 2003). This model does not address a role for GPR in nuclear rotation, however. Posterior enrichment of GPR-1/2 was seen in only some embryos during nuclear rotation (Colombo et al., 2003). Such asymmetry at this time is actually predicted to be counter-productive, as it

would potentially hold the nucleus at the posterior and prevent centration and rotation.

We previously proposed that the asymmetric enrichment of LET-99 in a cortical band provides the asymmetric cue to polarize forces during both rotation and anaphase (Tsou et al., 2002). Loss of LET-99 results in an absence of nuclear rotation and an absence of the normal asymmetric spindle pole movements during anaphase (Rose and Kemphues, 1998a; Tsou et al., 2002). Based on the hyperactive movements of nuclei and metaphase spindles, we proposed that the ultimate effect of LET-99 activity is a downregulation of cortical forces that act on centrosomes. Because LET-99 is enriched in a cortical band that encircles P lineage cells, downregulation of cortical forces in this region during prophase would result in higher net anterior and posterior forces that would produce a rotational movement of the nuclear-centrosome complex (Fig. 8, left). After rotation, the posterior centrosome/spindle pole lies partially underneath the LET-99 band. Downregulation of cortical forces in the LET-99 band region at this stage would affect lateral astral microtubule interactions, producing higher net forces directed towards the posterior and thus asymmetric anaphase spindle elongation (Tsou et al., 2002). The results reported here on the genetic interactions between LET-99 and Gα/GPR signaling are consistent with this model. Loss of LET-99 causes gain of Gα/GPR-1/2-like phenotypes, hyperactive nuclear and spindle movements. These hyperactive movements are completely suppressed in *Gα(RNAi); let-99* or *gpr-1/2(RNAi); let-99* mutant embryos, suggesting that LET-99 opposes Gα/GPR-1/2 signaling. The antagonistic role of *let-99* to Gα/GPR-1/2 signaling is further supported by the observation that partially reducing *let-99* activity suppresses the lethality caused by loss of *gpa-16* activity alone. Finally, the weak asymmetry of spindle positioning that we observed in *gpr-1/2(RNAi)* embryos was no longer observed in *gpr-1/2(RNAi); let-99* double mutant embryos. These results suggest that *let-99* not only functions oppositely to Gα/GPR-1/2 signaling, but also indeed provides an asymmetric cue. Based on these results and the pattern of cortical LET-99 localization, we propose that LET-99 antagonizes Gα/GPR-1/2 signaling, thus downregulating cortical forces asymmetrically during both rotation and anaphase spindle elongation.

The molecular mechanism by which LET-99 negatively regulates the Gα/GPR-1/2 signaling pathway remains to be elucidated. However, LET-99 is required for normal GPR-1/2 localization, suggesting that it acts upstream or at the level of Gα/GPR-1/2 signaling. Intriguingly, the region with the highest enrichment of GPR at the posterior during anaphase/telophase corresponds to the posterior domain of low LET-99 staining intensity, and this rise in GPR-1/2 does require LET-99. In addition, the staining intensity of the uniform prophase GPR-1/2 localization appeared stronger in *let-99* embryos than in wild type. These results suggest that LET-99 has an inhibitory effect on GPR-1/2 localization. However, the presence of lower levels of GPR-1/2 in the anterior during anaphase is apparently PAR-3 dependent (Colombo et al., 2003) (this report) but LET-99 independent, because this anterior region does not overlap with the band enriched for LET-99.

The models for GPR-1/2 and LET-99 function during anaphase are not mutually exclusive. Indeed, LET-99 could be acting solely through localization of GPR-1/2. However, both

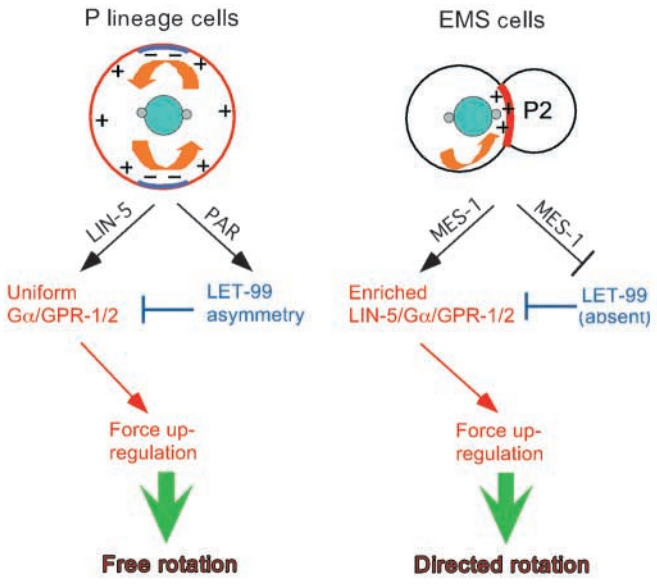

Fig. 8. Models for the roles of Gα/GPR-1/2 and LET-99 in transmitting polarized signals during nuclear rotation in P lineage and EMS cells. LIN-5 is required for cortical localization of GPR-1/2 in P cells and EMS cells, and the enrichment of LIN-5 and GPR-1/2 at the EMS/P_2 boundary is MES-1 dependent (Srinivasan et al., 2003). LET-99 asymmetry (blue band) and its downstream effects are shown in blue. Gα/GPR-1/2 (red outline) and its downstream effects are shown in red. Notice that the Gα/GPR-1/2 levels at the EMS/P_2 boundary are particularly high (thick red line), where LET-99 is absent. Orange arrows indicate the types of nuclear rotation (free versus directed). See text for details.

LET-99 and Gα are also required for polarity-dependent nuclear rotation in the one cell, when asymmetry of GPR-1/2 is not evident, which suggests that the main role of LET-99 may not be localization of GPR-1/2. Rather, we propose that LET-99 antagonizes Gα/GPR-1/2 signaling in addition to, or as part of, its effect on GPR-1/2 localization. One speculative model that fits the current data is that LET-99 directly or indirectly inhibits the association of Gα and GPR-1/2. This would downregulate Gα signaling in the region of the LET-99 band, causing nuclear rotation during prophase. As the cell cycle progresses, the dissociated GPR-1/2 would then be free to reassociate with the posterior cortex where neither PAR-3 nor LET-99 is present at high levels. During anaphase, the inhibition of Gα signaling by LET-99, the enrichment of GPR-1/2 posteriorly, or both could function in asymmetric anaphase spindle elongation. Biochemical experiments and identification of LET-99-interacting proteins will be required to elucidate the molecular mechanism of the interactions of LET-99 with G-protein signaling.

We also found that Gβ function antagonized Gα/GPR signaling. However, it appears that LET-99 acts separately from Gβ. Significantly, *Gβ* mutant embryos exhibit late nuclear rotation and asymmetric oscillations of spindle poles during anaphase, neither of which occurs in *let-99* embryos (this report) (Tsou et al., 2002). The asymmetric localization of GPR-1/2 was also observed in *Gβ* mutants but not in *let-99* mutants. These observations strongly suggest that the cues for polarizing forces that act on centrosomes still exist in *Gβ* mutants but not in *let-99* mutant embryos, and that polarized

force is essential for asymmetric spindle oscillations during anaphase. Although a loss of polarized forces is consistent with the absence of wild-type anaphase spindle pole oscillations in *let-99* embryos, it is unclear why random oscillations similar to prophase nuclear rocking are not seen at anaphase. It is possible that changes in microtubule to cortex interactions during the cell cycle could explain this phenotypic effect. In anaphase, more astral microtubules appear to reach the cortex and these microtubules are more cold stable than prophase microtubules (L. R. DeBella and L.S.R., unpublished results). With a large number of cortical-microtubule contacts during anaphase, the stochastic loss or gain of a few contacts would have little effect on the balance of forces. By contrast, with fewer microtubule contacts during prophase, a similar stochastic effect could lead to a dramatic imbalance of forces and hence cause nuclear rocking. Alternatively, we cannot rule out the possibility that LET-99 functions differently during prophase and anaphase to regulate forces.

G proteins and LET-99 function in spindle positioning in both intrinsically and extrinsically determined asymmetric divisions

We propose that the fundamental roles of Gα signaling and LET-99 are the same in both the P lineage and the EMS cell: Gα upregulates force generation from the cortex on centrosomes, and LET-99 downregulates force generation. Furthermore, we propose that the differences in the spatial and temporal regulation of LET-99 and GPR-1/2 localization provide an explanation for the different types of movements that lead to asymmetric spindle placement and orientation in these two cell types (Fig. 8). In P lineage cells, an intrinsic PAR-dependent mechanism causes 'free nuclear rotation' that occurs in the center of the cell when the extrinsic effects of cell shape are removed (Tsou et al., 2003). Distinct asymmetric elongation movements then position the spindle towards the posterior during anaphase. In these cells, GPR-1/2 is uniformly localized in most cells at the time of rotation, but LET-99 is enriched in a band and antagonizes Gα/GPR-1/2 activity as proposed earlier (Fig. 8, left). In contrast to free central rotation, previous studies have shown that nuclear rotation in the EMS cell occurs directly toward the EMS/P_2 boundary in both intact embryos and in isolated blastomeres (Schlesinger et al., 1999). We showed that in EMS cells LET-99 is not present as a band, but rather is absent from the EMS/P_2 cell contact region, where GPR-1/2 localization is enriched. Both patterns are present at the time of nuclear rotation, and the opposite localization of these two proteins at the EMS/P_2 boundary is consistent with our model that LET-99 antagonizes Gα/GPR-1/2 signaling. The absence of LET-99 would be necessary in order for the enriched GPR-1/2 at the cell boundary to have highest activity. High Gα/GPR-1/2 activity could then cause nuclear rotation directed towards the EMS/P_2 boundary, simultaneously positioning the spindle asymmetrically on the AP axis (Fig. 8, right). Interestingly, we also found that LET-99 is required for the asymmetry of GPR-1/2 at the EMS/P_2 boundary. Although we cannot rule out a role for LET-99 in the ability of the P_2 cell to signal to EMS and thus produce an enrichment of GPR-1/2, LET-99 could function in the EMS cell in a manner analogous to that proposed for the P lineage. That is, the presence of LET-99 at the other cell contacts could inhibit Gα/GPR-1/2 signaling there, further enhancing the asymmetry of Gα/GPR-1/2 signaling. The inhibition could also result in more free GPR-1/2, which would then associate with the EMS/P_2 boundary in response to MES-1 signaling.

In *let-99* mutants, nuclear rotation in the EMS failed completely while in *mes-1* mutants, nuclear rotation failed in only 10% of EMS cells (Bei et al., 2002). Similarly, the *gpa-16* EMS rotation phenotype is stronger than that of *mes-1* mutants. This is not surprising, because *mes-1* mutants cause a loss of asymmetry of LET-99 and GPR-1/2, not a total loss of protein. Similarly, mutations in *par-3* result in symmetric GPR-1/2 and LET-99 localization during prophase in the P lineage, but *par-3*, *let-99* and *gpr-1/2* mutants have different nuclear rotation phenotypes. Nonetheless, the finding that *let-99* and *Gα* mutant EMS cells have stronger defects in rotation suggest that these proteins play a basic role in the interaction between microtubules and the cortex in multiple cells. Consistent with this view, both Gα and *let-99* mutants have defects in nuclear and centrosome positioning in the AB lineage (M.-F.B.T. and L.S.R., unpublished) (Gotta and Ahringer, 2001; Rose and Kemphues, 1998a). In addition, the activity of LET-99 and GPR-1/2, rather than their localization, could also be modulated in the EMS cell in response to Wnt signaling, which acts redundantly with MES-1 to promote EMS spindle orientation.

In summary, our results provide evidence that in *C. elegans* as in *Drosophila*, G-protein signaling is used for spindle positioning in asymmetric divisions that are both intrinsically and extrinsically determined (Knoblich, 2001). Further work to elucidate the molecular mechanisms by which G protein signaling and LET-99 regulate forces on microtubules during asymmetric division will provide insight into both types of divisions.

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