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Asymmetrically Distributed *C. elegans* Homologs of AGS3/PINS Control Spindle Position in the Early Embryo

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Summary

Background: Spindle positioning during an asymmetric cell division is of fundamental importance to ensure correct size of daughter cells and segregation of determinants. In the *C. elegans* embryo, the first spindle is asymmetrically positioned, and this asymmetry is controlled redundantly by two heterotrimeric Gα subunits, GOA-1 and GPA-16. The Gα subunits act downstream of the PAR polarity proteins, which control the relative pulling forces acting on the poles. How these heterotrimeric G proteins are regulated and how they control spindle position is still unknown.

Results: Here we show that the Gα subunits are regulated by a receptor-independent mechanism. RNAi depletion of *gpr-1* and *gpr-2*, homologs of mammalian AGS3 and *Drosophila* PINS (receptor-independent G protein regulators), results in a phenotype identical to that of embryos depleted of both GPA-16 and GOA-1; the first cleavage is symmetric, but polarity is not affected. The loss of spindle asymmetry after RNAi of *gpr-1* and *gpr-2* appears to be the result of weakened pulling forces acting on the poles. The GPR protein(s) localize around the cortex of one-cell embryos and are enriched at the posterior. Thus, asymmetric G protein regulation could explain the posterior displacement of the spindle. Posterior enrichment is abolished in the absence of the PAR polarity proteins PAR-2 or PAR-3. In addition, LIN-5, a coiled-coil protein also required for spindle positioning, binds to and is required for cortical association of the GPR protein(s). Finally, we show that the GPR domain of GPR-1 and GPR-2 behaves as a GDP dissociation inhibitor for GOA-1, and its activity is thus similar to that of mammalian AGS3.

Conclusions: Our results suggest that GPR-1 and/or GPR-2 control an asymmetry in forces exerted on the spindle poles by asymmetrically modulating the activity of the heterotrimeric G protein in response to a signal from the PAR proteins.

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Introduction

Asymmetric cell divisions are important for generating cell diversity in all eukaryotes [1]. During asymmetric cell division, the mitotic spindle must be positioned along the axis of polarity to ensure the correct segregation of determinants to the two daughter cells. However, how spindle positioning is controlled and how it is coordinated with cell polarity in animal cells is still unknown. Asymmetric spindle positioning occurs during the first division of *C. elegans*, and this is an excellent model system because the transparency of the embryos allows direct visualization of the process.

In *C. elegans*, the first mitotic spindle is initially set up in the center, but from metaphase through anaphase it is displaced to the posterior. Because the cleavage furrow bisects the spindle, this displacement results in an asymmetric cleavage. The spindle itself has an asymmetric morphology, with the anterior aster being round and the posterior one flat. The PAR proteins are asymmetrically localized along the A-P axis and control the overall embryonic polarity, including spindle asymmetry. PAR-3, PAR-6, and PKC-3 are enriched at the anterior cortex, whereas PAR-1 and PAR-2 are enriched at the posterior cortex of the embryo (reviewed in [2]). Asymmetric spindle positioning in the one-cell embryo depends on differential pulling forces on the spindle poles [3]. By severing the central spindle with a laser microbeam, Grill et al. [3] showed that stronger pulling forces are exerted on the posterior pole compared to the anterior one. PAR proteins control the asymmetry of these pulling forces. In *par-2* and *par-3* mutants, in which the spindle remains central, these forces are equal at both poles. In *par-3* mutants, in which both the anterior pole and the posterior pole are flat like the posterior one in the wild-type, strong forces are exerted on both poles. In contrast, in *par-2* mutants, in which both anterior and posterior asters are round like the anterior one in the wild-type, weak forces are exerted on both spindle poles.

It has been previously shown that two Gα subunits of heterotrimeric G proteins (GOA-1 and GPA-16) control asymmetric spindle positioning in the early *C. elegans* embryo [4, 5]. Based on amino acid sequence similarity, GOA-1 belongs to the Gαi/o class of Gα genes. GPA-16 does not belong to any of the known classes [6], but it is the most closely related Gα to GOA-1 in *C. elegans*. When *goa-1* and *gpa-16* are simultaneously inhibited by RNAi, the mitotic spindle in the one-cell embryo remains central, which results in a symmetric cleavage. However, embryonic polarity, as assayed by the localization of P granules and PAR proteins, is normal in these embryos. This uncoupling of spindle asymmetry from general polarity suggests that Gα might be responsible for translating embryonic polarity into asymmetric mitotic spindle behavior in response to a signal from the PAR proteins. However, it is not known how these Gα subunits are regulated.

Heterotrimeric G proteins are classically coupled to

seven-transmembrane receptors (7-TM, also called GPCRs, G protein-coupled receptors), which activate them after receiving signals from the environment or neighboring cells (for a review, see [7]). Activation results in the exchange of GDP by GTP in the Gα subunit, leading to a conformational change that causes dissociation of Gα-GTP from Gβγ. Both Gα-GTP and Gβγ can regulate effectors. *C. elegans* embryos are encased in an impermeable chitin eggshell, so there are no cells that could send a signal to a GPCR at the one-cell stage. In addition, in P1 and P2 cell spindle orientation, which is controlled by Gβγ [4, 8], is cell autonomous [9], suggesting that this event may occur independently of a classical GPCR at the cell surface. Interestingly, it has been demonstrated in mammalian systems that receptor-independent activation of heterotrimeric G proteins can occur [10–15]. One of the proteins responsible for this receptor-independent activation is called AGS3 (activator of G protein signaling). AGS3 has N-terminal TPR domains (protein-protein interaction motifs) and C-terminal GPR (or GoLoco) domains, which are implicated in binding Gα [10–15]. This gene family also includes *Drosophila pins* (*partner of inscuteable*) [16–20], implicated in spindle asymmetry in neuroblasts. Here we show that *C. elegans* divergent members of the AGS3 and PINS protein family most likely control spindle positioning by regulating the forces exerted on the spindle poles downstream of the PAR proteins.

Results

Disruption of GPR-1 and GPR-2 Function Results in Loss of Spindle Asymmetry

The *C. elegans* genome contains three genes with homology to mammalian AGS3. F32A6.4 (*ags-3*) is most similar and has the same overall organization, with six TPR domains, involved in protein-protein interaction, and three GPR domains, which are Gα binding motifs. The other two genes, F22B7.13 (*gpr-1*) and C38C10.4 (*gpr-2*), have sequence similarity in the TPR domains and one GPR domain (Supplemental Figure S1, available with this article online). RNAi of *ags-3* results in embryos with wild-type development (our unpublished data). Because *gpr-1* and *gpr-2* are 97% identical at the nucleotide level, RNAi of either is predicted to target both genes. We found that RNAi of *gpr-1* and *gpr-2* results in 100% embryonic lethality (n = 250; [21]). To try to distinguish whether both proteins or only one is required for viability, we injected dsRNA to their 3′ untranslated regions, which would be expected to be gene specific based on the lower sequence conservation. However, in light of the fact that neither dsRNA nor the combination of the two gave embryonic lethality, these regions may be too short for efficient RNAi (our unpublished data). We therefore do not know whether the genes show functional redundancy or whether only one of the two genes is active in the early embryo. For simplicity, we will refer to the active gene as *gpr-1/2*.

gpr-1/2(RNAi) embryos showed a number of defects identical to those observed in *Gα(RNAi)* embryos. In this paper we focus on the defects observed during the first embryonic division. In *gpr-1/2(RNAi)* embryos, as in *Gα(RNAi)* embryos, the first mitotic spindle fails to rock as it elongates and is not displaced posteriorly (inset in Figure 1, Supplemental Figure S2, and Table 1), resulting in a symmetric, rather than an asymmetric, first cleavage. In addition, spindle morphology is symmetric, with both the anterior and posterior asters having a round morphology similar to a wild-type anterior one (Figures 1C and 1D). Despite the fact that the first cleavage is symmetric in size as in *par* mutants, polarity appears to be normal; polarity markers such as PAR-2, PAR-3, PAR-6, and P granules are correctly localized in these embryos (Figure 2 and our unpublished data).

GPR-1/2 Controls Spindle-Pulling Forces

We next examined spindle-pulling forces in *gpr-1/2 (RNAi)* embryos by using two indirect measures. Elongation of the mitotic spindle during anaphase B is mostly due to pulling forces exerted on the spindle poles [22, 23]. Thus, the length of the spindle at anaphase should correlate with the intensity of the pulling forces. We measured anaphase spindle length in wild-type, *par-2, par-3*, *Gα(RNAi)*, and *gpr-1/2(RNAi)* embryos as an indirect measure of pulling force. As previously shown, in *par-2(RNAi)* embryos, where forces are weak, the spindle is shorter than in the wild-type or in *par-3* mutants (Table 1 and [24]). We find that the anaphase spindle in *gpr-1/2(RNAi)* embryos is shorter than in wild-type or *par-2(RNAi)* embryos (Table 1). Spindle length is short in *Gα(RNAi)* embryos (Table 1 and [24]). Spindle length is also short in *gpr-1/2(RNAi);par-3(it71)* and *Gα(RNAi); par-3(it71)* embryos (Table 1), indicating that *gpr-1/2* and Gα are epistatic to *par-3*.

These results suggest that GPR-1/2 might be involved in controlling the forces exerted on the spindle poles. We investigated this possibility by using a spindle breakage assay. The spindle midzone can be removed by RNAi of a *C. elegans* kinesin that is related to MCAK [3]. In wild-type and in *par-3* mutant embryos, the forces exerted on the poles are sufficient to provoke spindle rupture after *mcak(RNAi)* treatment. However, the weak forces in *par-2* mutants are not sufficient for spindle rupture and the spindle remains intact [3]. As previously reported, *mcak(RNAi)* caused spindle rupture in wild-type animals (Figures 3B and 3E, n = 10; [3]). In 20 out of 20 *gpr-1/2(RNAi);mcak(RNAi)* double RNAi embryos, the spindle did not break, similar to the case with *par-2* mutants (Figure 3C; [3]; our unpublished data). Staining of *gpr-1/2(RNAi);mcak(RNAi)* embryos with anti-tubulin antibodies confirmed that the central spindle was intact (Figure 3F). CeMCAK protein was undetectable in *gpr-1/2(RNAi);mcak(RNAi)* embryos, indicating that depletion was efficient (our unpublished data). The failure in spindle breakage and the short anaphase spindle suggests that the forces exerted on both spindle poles in *gpr-1/2(RNAi)* embryos are weak or absent, leading to a short symmetric spindle.

GPR-1/2 Acts as a Guanine Dissociation Inhibitor for Gαo

How does GPR-1/2 activate heterotrimeric G protein signaling? In mammalian and *Drosophila* cells, the GPR domain binds the GDP bound conformation of Gαi and

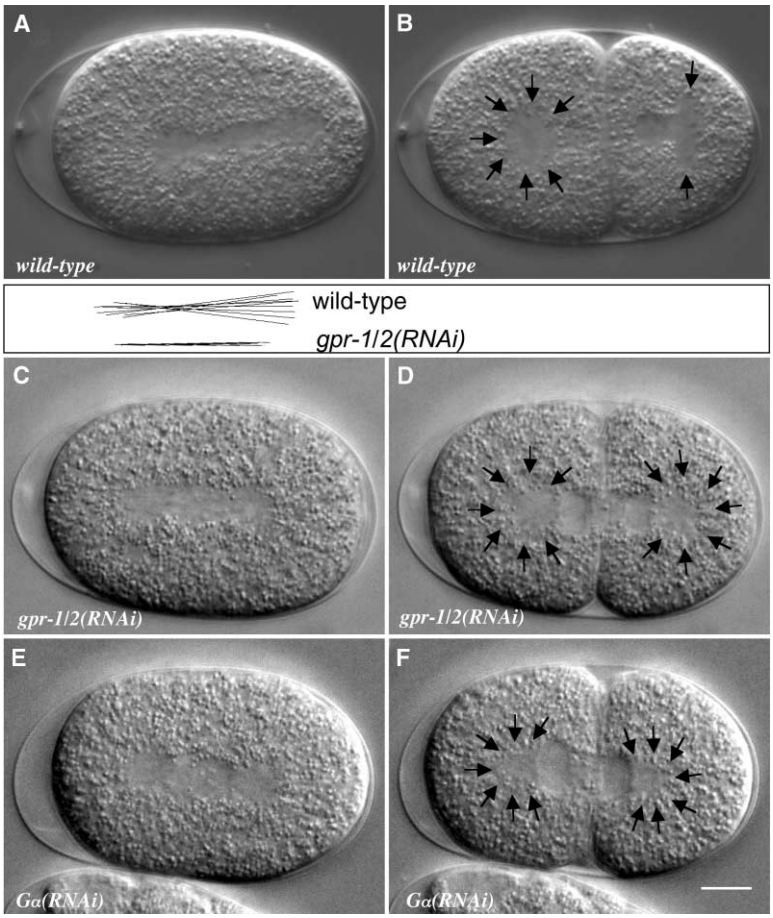

Figure 1. Early Defects in *gpr-1/2(RNAi)* Embryos

(A and B) Wild-type. (A) Rocking and displacement to the posterior of the mitotic spindle is observed. (B) The posterior aster is disc shaped, and the first cleavage is asymmetric, with the posterior cell smaller than the anterior.

(C and D) *gpr-1/2(RNAi)* embryos. (C) Spindle rocking and posterior displacement are not observed (n = 17), similar to the situation in (E) *Gα(RNAi)* embryos (n = 10, [4]). (D) The posterior aster is round, as is the anterior one, and the first cleavage is symmetric, also as in (F) *Gα(RNAi)* embryos. The inset between panels (A) and (B) and panels (C) and (D) shows a schematic representation of the positions of the mitotic spindle in the wild-type and in *gpr-1/2(RNAi)*, taken from images in Figure S2. Arrows in panels (B), (D), and (F) mark the asters. Posterior, right. The scale bar in (F) represents 10 μm.

inhibits nucleotide exchange for GTP (GDI activity) [12–15, 19, 25]. The GPR domain in AGS3 and related proteins appears to be selective for the Gαi subgroup and has a lower affinity for Gαo G proteins. Because GOA-1 is the *C. elegans* ortholog of mammalian Gαo [6], and because *gpr-1* and *gpr-2* are divergent members of the *ags3* gene family, we investigated whether the GPR proteins in *C. elegans* actually regulate the activity of GOA-1. Both GPR-1 and GPR-2 bound to GOA-1 in a two-hybrid assay, but neither of them bound to GPA-16 (our unpublished data). To further address this issue, we examined the binding of purified Gα and GPR proteins and the influence of the GPR motif on the guanine nucleotide binding properties of GOA-1 (see Experimental Procedures). Consistent with the GPR domain acting as a GDI, the GPR domain of GPR-1/2 binds to the GDP bound form of GOA-1 but not to the GTP bound form (Figure 4A). In addition, both the *C. elegans* GPR peptide and a GPR consensus peptide significantly inhibited exchange of GDP for GTP in GOA-1 and in a mammalian Gαi that was used as positive control (Figure 4B). Because we were unable to purify His-tagged GPA-16 under native conditions, we could not investigate the activity of GPR on GPA-16. Taken together, these results show that GPR-1/2 is indeed a GDP dissociation inhibitor for GOA-1. Because Gα and GPR-1/2 have positive roles in spindle positioning, this suggests that GOA-1/GPR-1/2 is an active signaling complex.

GPR-1/2 Shows Posterior Enrichment in the One-Cell Embryo

The distribution of GOA-1 is symmetric in the one-cell embryo (our unpublished data), but forces are asymmetric. To investigate whether an asymmetry in the distribu-

Table 1. Spindle Length and Position of First Cleavage

	Wild-Type n = 13	*par-2(RNAi)* n = 10	*par-3(it71)* n = 7	*gpr-1/2(RNAi)* n = 17	*Gα(RNAi)* n = 10	*gpr-1/2(RNAi); par-3(it71)* n = 10	*Gα(RNAi); par-3(it71)* n = 11
Spindle Length[a]	45.8 ± 2.7	42.5 ± 1.6	45.7 ± 2.4	37.9 ± 2.9	38.0 ± 1.8	38.3 ± 1.5	38.8 ± 1.5
Position of First Cleavage[b]	56.0 ± 1.6	49.9 ± 0.7	50 ± 0.0	52.2 ± 1.9	51.2 ± 1.0	49.5 ± 1.0	49.0 ± 1.8

Both the spindle length and the position of first cleavage were calculated as percentage of egg length. n is the number of embryos analyzed.
[a] Spindle pole separation was calculated at anaphase (by dividing spindle length by embryo length).
[b] Anterior is 0%.

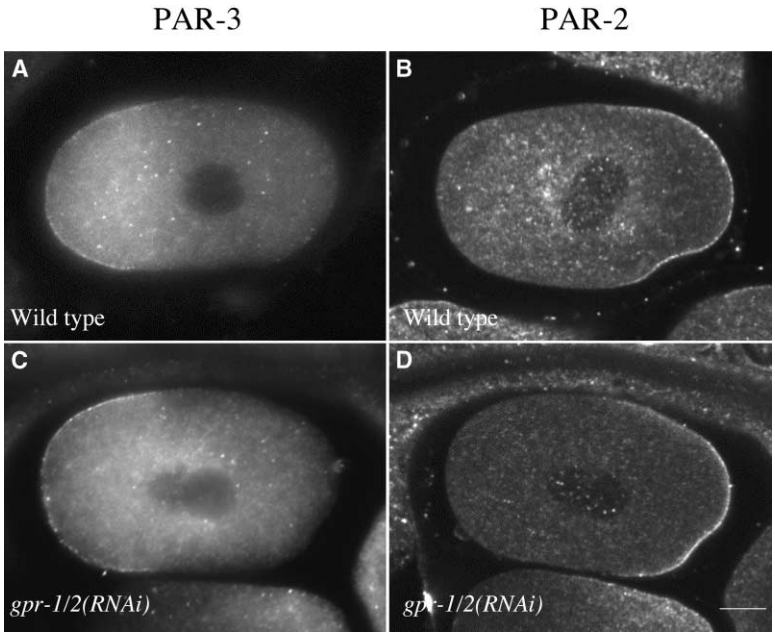

Figure 2. PAR-2 and PAR3 Are Asymmetrically Localized in *gpr-1/2(RNAi)* Embryos

(A and B) PAR-3 and PAR-2 are enriched at the anterior and posterior cortices, respectively, in wild-type embryos (n = 20). (C and D) localization of PAR-3 (C) and PAR-2 (D) is normal in *gpr-1/2(RNAi)* embryos (n = 20 for PAR-3 and n = 23 for PAR-2). Posterior, right. The scale bar in (B) represents 10 μm.

tion of a GOA-1/GPR-1/2 complex could exist, we raised antibodies to full-length GPR-2. These antibodies would be expected to detect both GPR-1 and GPR-2. In most cells, localization of GPR-1/2 is very similar to the localization of GOA-1 and the Gβ subunit GPB-1, at the cell membrane and around the asters of wild-type embryos (Figures 5J–5L). In addition, staining is seen at the poles of the meiotic spindle and on the mitotic spindle, where Gα and Gβ are not found (Figures 5A–5D). Strikingly, GPR-1/2 is enriched at the posterior cortex in one-cell embryos from metaphase to cytokinesis (Figures 5D–5I and our unpublished data). This staining is specific; it is not detectable in *gpr-1/2(RNAi)* embryos (Figures 5M–5O). The strong reduction in staining suggests that RNAi results in a strong loss of GPR-1/2 function, although this may not be a null phenotype. Posterior enrichment supports the idea that asymmetric activation of Gα by GPR-1/2 results in an asymmetry in pulling forces, with stronger forces at the posterior where GPR-1/2 levels are highest.

The asymmetry in pulling forces is under the control of the PAR proteins. A possible mechanism for PAR control of pulling forces is via asymmetric localization of GPR-1/2. If this were the case, we would expect a high level of GPR-1/2 at both anterior and posterior cortices in *par-3* mutants, where pulling forces are high at both poles, and we would expect a low level of GPR-1/2 in *par-2* mutants. Indeed, we found that GPR-1/2 asymmetry is lost in both *par-3* mutant and *par-2(RNAi)* embryos, with strong localization all around the cortex

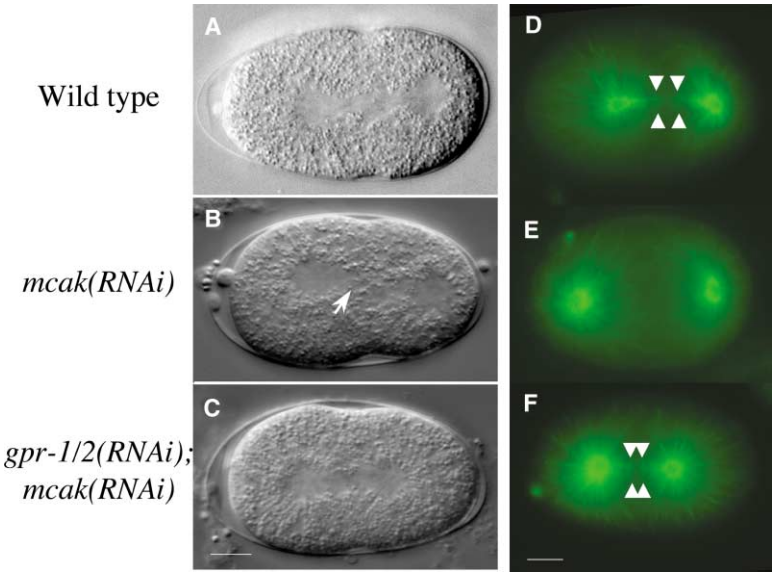

Figure 3. Spindle Rupture Does Not Occur in *gpr-1/2(RNAi)* Embryos

(A) DIC image of a wild-type one-cell embryo at the anaphase-telophase transition. The spindle is visible as an area devoid of lipid droplets.

(B) DIC image of a *mcak(RNAi)* one-cell embryo. The arrow points to the site of rupture of the spindle.

(C) *gpr-1/2(RNAi)* one-cell embryo. The spindle is intact as in wild-type embryos.

(D–F) Mitotic spindle of one cell embryos visualized by anti-tubulin antibodies in (D) wild-type, (E) *mcak(RNAi)*, and (F) *gpr-1/2(RNAi);mcak(RNAi)* embryos. (D and F) Arrowheads point to the spindle midzone. Posterior, right. The scale bar in (C) and (F) represents 10 μm.

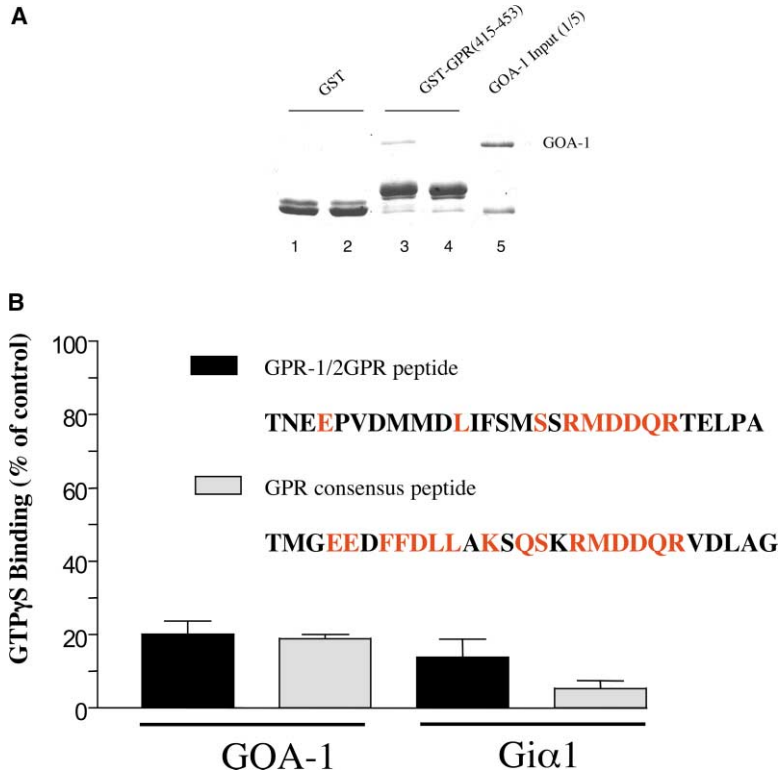

Figure 4. The GPR Domain of GPR-1/2 Binds Preferentially to GOA-1GDP and Inhibits Binding to GTP

(A) SDS-PAGE gel stained with Coomassie Blue. Lanes 1 and 3, His-GOA-1GDP binding to GST and GST-GPR(415–453) respectively; lanes 2 and 4, His-GOA-1GTP binding to GST and GST-GPR(415–453), respectively. Lane 5, 1/5 of input.

(B) GTPγS binding to His-GOA-1 and Giα1 in the presence of GPR peptides. GTPγS (500 nM) binding to Gα subunits was measured in the absence and presence of 100 μM peptides. The red amino acids in the GPR consensus peptide and the GPR-1/2 peptide indicate the core GPR motif as defined by Peterson et al. [12]. However, it should be noted that although not strictly conserved, the amino-terminal half of the GPR-1/2 peptide exhibits general groupings of residues that are similar to those in the GPR consensus peptide. Data are expressed as the percent of specific binding observed in the absence of peptide. The control GTPγS binding value for GOA-1 was about 1.4 pmol of specific binding (total binding = approximately 60,000 cpm, nonspecific binding = approximately 350 cpm). The control GTPγS binding value for Giα1 was about 5 pmol of specific binding (total binding = approximately 300,000 cpm, nonspecific binding = about 2500 cpm). Data are presented as the mean +/− standard error derived from two experiments performed in duplicate.

in *par-3* mutants and weak localization in *par-2(RNAi)* embryos (Figures 5P and 5Q). Thus, a high level of cortical GPR-1/2 is correlated with a strong pulling force.

At the four-cell stage, two cells that contact each other (EMS and P2) also undergo asymmetric cell division, with the spindles being displaced toward the region of contact between the cells [26]. Consistent with the view that GPR-1/2 promotes strong pulling force, we find that there is a high level of GPR-1/2 at this junction, and that this asymmetry is controlled by *par-2, par-3*, and *lin-5* as in the 1-cell embryo (Figure S3).

LIN-5 Is a GPR-1/2 Binding Partner

We performed a two-hybrid screen to find interacting partners of GPR-1/2. Using GPR-1 as a bait, we identified multiple isolates of two interacting proteins, GOA-1 and LIN-5. LIN-5 is a large coiled-coil protein essential for many aspects of cell division, including chromosome alignment at metaphase and sister chromatid separation. It also has a role in meiotic divisions [27]. LIN-5 is localized at the meiotic spindle, on centrosomes, and on kinetochore microtubules. In addition, LIN-5 localizes at the cortex from the two-cell stage onward, but no asymmetry has been reported [27].

lin-5 mutant and *lin-5(RNAi)* embryos display phenotypes very similar to those of *Gα(RNAi)* and *gpr-1/2(RNAi)* embryos; the rocking movement of the spindle is absent, and spindle position is often central. Loss of LIN-5 also leads to defects in chromosome segregation and cytokinesis after two to four divisions, and the embryos arrest early with polyploid nuclei. In *gpr-1/2(RNAi)* and *Gα(RNAi)* embryos, we observe similar phenotypes (our unpublished data and Figure 6, numbers in figure legend). *lin-5(RNAi)* embryos have also been reported to have meiotic defects; in embryos fixed before pronuclear migration, two maternal pronuclei were observed in about 30% (n = 25) of the embryos. We did not observe such meiotic defects in either *Gα(RNAi)* (n = 23) or *gpr-1/2(RNAi)* (n = 25) embryos. Despite the localization of GPR-1/2 on the meiotic spindle, there is no obvious role in meiosis, although this could be due to incomplete inhibition with RNAi. The similarity in phenotype and partial overlap in localization between the proteins suggests that LIN-5 and GPR-1/2 may act together in a subset of roles.

To explore this interaction further, we asked whether GPR-1/2 or LIN-5 localization was affected by depletion of the other protein. The LIN-5 distribution is unaffected in *gpr-1/2(RNAi)* embryos (n = 15; our unpublished data). In contrast, GPR-1/2 is strongly delocalized from the cortex in one cell and later *lin-5(RNAi)* embryos (Figure 5R and Supplemental Figure S3). This suggests that LIN-5 has a role in targeting or anchoring GPR-1/2.

Discussion

We previously showed that two Gα subunits control asymmetric spindle positioning in the one-cell embryo. In this paper, we identify GPR-1/2 as a Gα regulator. GPR-1/2 binds to and is a GDP dissociation inhibitor

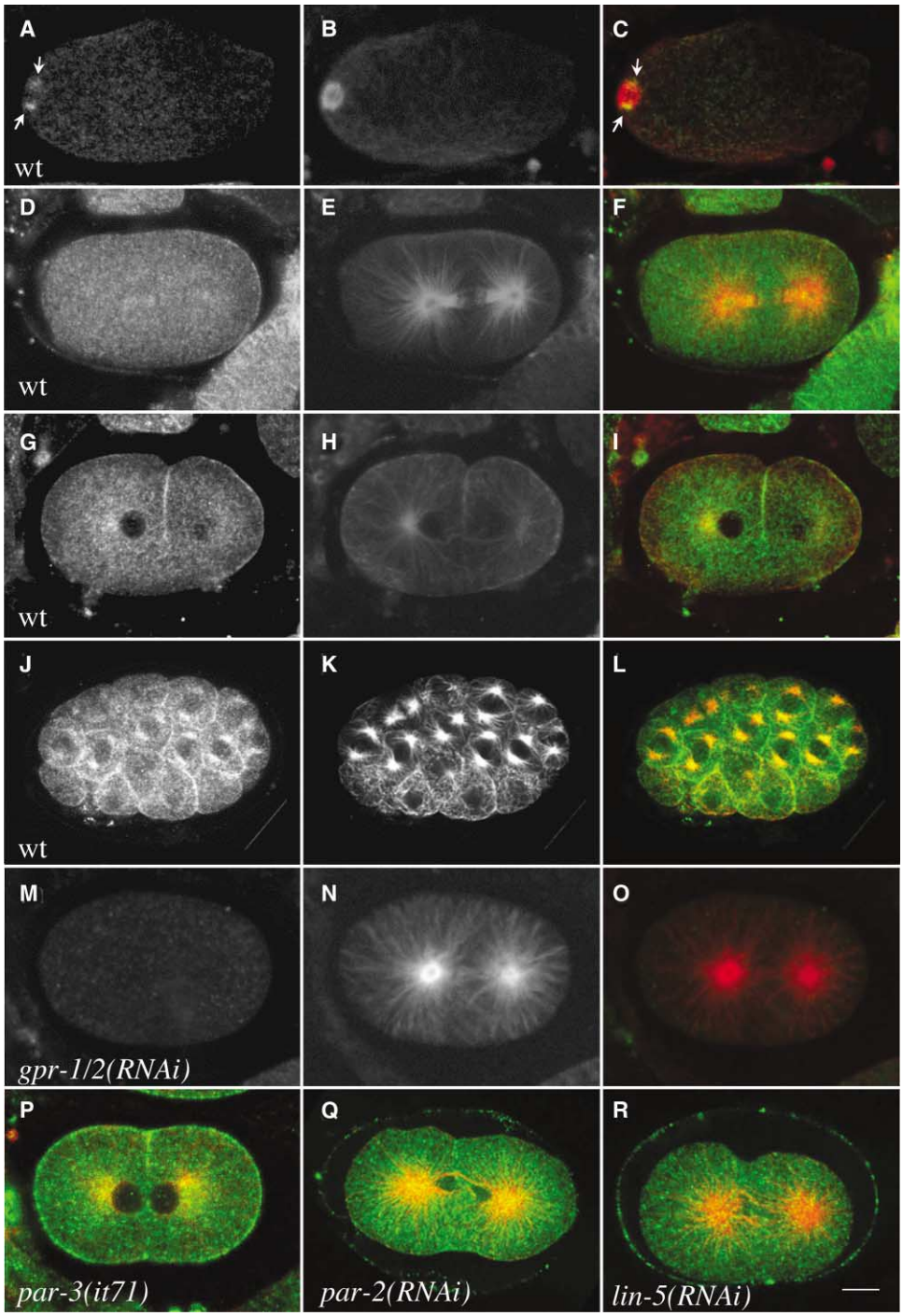

Figure 5. GPR-1/2 Localization in Wild-Type and Mutant Embryos

For (A–M), the left column shows GPR-1/2 localization, the middle column shows tubulin, and the right column is a merge. (A–C) Meiotic spindle. Arrows in (A) and (C) point to GPR-2 staining on the meiotic spindle poles (7/7 embryos). (D–F) Anaphase one-cell embryo; (G–I) telophase/cytokinesis embryo; (J–L) late wild-type embryo; and (M–O) *gpr-1/2(RNAi)* embryo. GPR-1/2 is enriched at the posterior cortex in 90% of the embryos from metaphase to cytokinesis (n = 44). GPR-2 was undetectable in 22 out of 29 *gpr-1/2(RNAi)* embryos, weak in 6/29 embryos, and normal in 1/29 embryos from metaphase. In (P–R), GPR is green, and tubulin is red. (P) *par-3(it71)*; (Q) *par-2(RNAi)*; and (R) *lin-5(RNAi)* embryos. In embryos from metaphase to first cytokinesis, staining was symmetric in 36/36 *par-3(it71)* embryos and strong at both the anterior and posterior cortices in 31/35 embryos. In *par-2(lw32)* embryos, GPR-2 staining was symmetric in 25/25 embryos and weak at both cortices in 19/25 embryos. In 7/8 *lin-5(RNAi)* embryos staining was symmetric and either weak or absent. In one embryo there was slight posterior asymmetry. Posterior, right. The scale bar in panel (R) represents 10 μm.

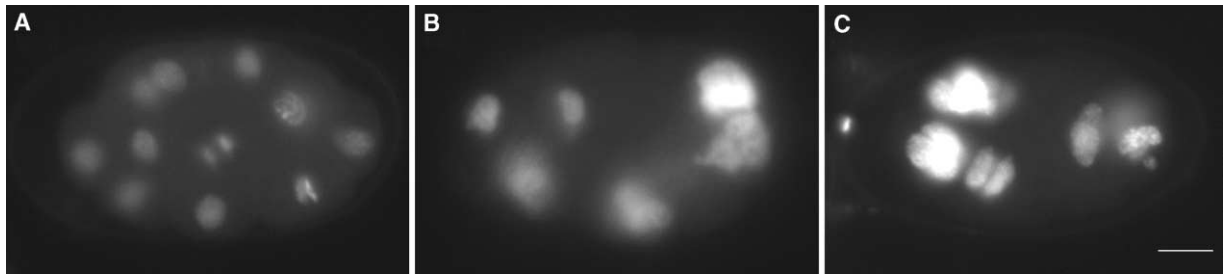

Figure 6. *Gα(RNAi)* and *gpr-1/2(RNAi)* Embryos Have Similar Mitotic Defects

(A) Wild-type, (B) *Gα(RNAi)*, and (C) *gpr-1/2(RNAi)* embryos stained with DAPI to illustrate the early embryonic arrest with polyploid nuclei, similar to the case of *lin-5(RNAi)* embryos [27] (See Figure S3). More than 50 embryos were analyzed in each case. Posterior is right. The scale bar in panel (C) represents 10 μm.

for Gα, and it has a similar role in spindle positioning. RNAi of *gpr-1/2* results in a symmetric short spindle, and pulling forces from the poles are reduced. In wild-type embryos, GPR-1/2 is enriched at the posterior cortex, where forces are higher. These data lead to a model whereby asymmetric distribution of an active cortical complex of GPR-1/2 and GDP-GOA-1 promotes stronger pulling forces at the posterior cortex than at the anterior one.

A GPR-1/2-Gα Complex

Inhibition of GPR-1/2 or of Gα prevents asymmetric spindle positioning. Loss of Gα would be expected to result in constitutive activation of Gβγ as well as a loss of Gα signaling. In previous work, we showed that it is loss of Gα signaling itself that is responsible for the spindle positioning defect in *Gα(RNAi)* embryos [4]. Therefore, Gα has a positive role in spindle positioning. Because loss of GPR-1/2 mimics loss of Gα, and because these proteins bind to each other, we suggest that GPR-1/2 and Gα form an active signaling complex.

AGS3 and PINS have been shown to promote dissociation of Gβγ from GαGDP [11, 19]. In addition, it has been recently shown that binding of GPR domains to GαGDP leads to conformational changes that preclude coincident Gβγ binding to GPR-complexed GαGDP [28]. One possibility is that AGS3, PINS, and GPR-1/2, by promoting dissociation of Gβγ, uncover a site of interaction with effectors in the GαGDP subunit. Such effectors may be different from the effectors that interact with GαGTP. Alternatively, a new site of interaction with effectors could be created in the GPR-1/2-GαGDP complex. How a GPR-1/2-Gα complex would be inactivated is an open question. In classical heterotrimeric G protein signaling, GTP-to-GDP hydrolysis (regulated by RGS proteins) is necessary to allow binding of Gβγ to Gα, which binding in turn leads to inactivation of the signal [7, 29]. Because Gβγ cannot bind to GαGDP complexed with AGS3, it may be that dissociation of AGS3 from GαGDP is regulated by as-yet-unknown mechanisms to allow reassociation with Gβγ. Another possible model is suggested by the finding that RIC-8, which is also required for asymmetric spindle positioning, appears to be a Gα guanine nucleotide exchange factor (GEF) [5, 30]. Requirement for a GEF suggests that the active molecule in spindle positioning could be GαGTP. In this view, binding of GPR-1/2 to GαGDP might be essential for Gα to be recognized by the GEF.

We found that GPR-1/2 binds to GOA-1, but we did not detect binding to GPA-16 in a two-hybrid assay. Because both GOA-1 and GPA-16 are Gα subunits, and because they have functionally redundant roles in spindle positioning in the embryo, they would be expected to be regulated in a similar way. Further work, using in vitro binding to native GPA-16, should help to resolve this issue.

Possible Targets

How could a GPR-1/2-Gα complex control pulling forces? As yet, no targets of such a complex are known. Pulling forces are likely to involve regulation of interactions between astral microtubules (MTs) and the cortex, possibly through regulating the activity or localization of MT motor proteins. A good candidate is the dynein/dynactin complex. Dynein motors function in numerous processes in which MTs generate forces. Such processes include centrosome separation, nuclear migration, and spindle orientation [31]. Inhibition of this complex in the early *C. elegans* embryo results in defects in pronuclear migration, precluding observation of a role in asymmetric spindle positioning. Partial inhibition of dynein heavy chain, p150glued, or of p50/dynactin via RNAi allows pronuclear migration but prevents movement of the pronuclei and associated centrosomes to the center of the cell as well as its rotation onto the anterior-posterior axis, also preventing assay of asymmetric spindle positioning [32, 33]. Future studies should reveal if a member of the dynein/dynactin complex is a direct target of Gα-GPR-1/2.

We identified LIN-5 as a protein that binds to GPR-1/2. Previous work showed that *lin-5* mutant embryos display defects in spindle positioning, a lack of anaphase, and improper chromosome alignment [27]. It was suggested that these defects arise as a result of loss of spindle forces, both in the central spindle (for chromosome alignment) and from the asters (for spindle positioning). The similarity in the loss-of-function phenotype and the partial overlap in localization between LIN-5 and GPR-1/2 suggest that these proteins act together. Consistent with this idea, we found that inhibition of *lin-5* caused delocalization of GPR-1/2 from the cortex. One possibility is that LIN-5, a coiled-coil protein, might

have a structural role as a scaffold for assembly of a signaling complex.

Conserved Function of AGS3 Proteins in Spindle Positioning

In *Drosophila*, a Gα subunit and PINS, a homolog of AGS3, function in asymmetric spindle positioning in neuroblasts [17]. Similar to the work presented here, previous work has proposed that the PINS-GαGDP complex is an active signaling complex. Therefore, regulation of spindle positioning may be a general property of Gα and AGS3-like molecules.

In *Drosophila* neuroblast asymmetric spindle positioning, the PINS-Gα pathway is redundant with the Bazooka(Par-3)/Par-6/PKC3-Inscuteable pathway [17]. Inhibition of either one results in spindle orientation defects; however, neuroblasts still divide asymmetrically. Inhibition of both pathways results in a symmetric neuroblast division. In *C. elegans*, these two pathways are not redundant. For example, in *par-3* mutant embryos, the spindle is symmetrically placed. Furthermore, PAR-3 appears to have a role in generating GPR-1/2 asymmetry by inhibiting its localization to the anterior; a high-level of GPR-1/2 is seen at anterior and posterior cortices in *par-3* mutant embryos. It is also worth noting that although the same proteins are required for controlling similar processes in *C. elegans* embryos and *Drosophila* neuroblasts, the mechanisms that control spindle position might be different. Indeed, in *Drosophila* neuroblasts from the asterless mutant, spindle position and asymmetric cell division occur as in the wild-type [34], suggesting that a functional centrosome and astral microtubules may not be required for this process.

Conclusions

Our results show that a complex between GPR-1/2 and Gα controls the pulling forces exerted on the mitotic spindle in *C. elegans*. Finding the targets of this complex will help to elucidate how GPR/Gα complexes signal and how spindle displacement to the posterior occurs.

Experimental Procedures

Strains, Constructs, and Protein Purification

The Bristol strain N2 was used as the standard wild-type strain. The full-length GOA-1 was cloned into the pDEST15 vector according to the manufacturer's protocol (Gateway cloning technology, Invitrogen). The GPR domain (amino acids 415–453) was amplified from yk75g10 with the primers 5′-GGGGAATTCTAGCGATCGAA AAGAAAGAG-3′ and 5′-GGGGAATTCAGGCGGAATGAATCTGG CAG-3′ and was cloned into the EcoRI site of pGEX-4T-2. Both proteins and GST were purified via standard methods.

Immunofluorescence and Antibodies

Antibody staining was carried out as in [35] with the following modifications: the slides were blocked in PBS, 0.2% Tween, and 1% milk, and all primary and secondary antibodies were diluted in PBS Tween before use. The following primary antibodies were used: rabbit anti-PAR-2 and rabbit anti-CeMCAK. For antibody staining, embryos were fixed at least 24 hr after mothers were injected with dsRNA.

Antibodies to GPR-2 were raised against the full-length protein fused to GST. GPR-2 was amplified from Y. Kohara cDNA yk75g10 with the primers 5′-CGGGTTTCTCGAGAGATGGACGTCTCTTATTA TGATGGC-3′ and 5′-GGGGTTTCTCGAGCTATTTGGACGTCATTGT CACATC-3′, was cut with XhoI, and was cloned into the SalI site of pGEX-5X-1. The protein was expressed in soluble conditions, purified with glutathione agarose beads, and injected into rabbits. Antibodies were purified on a nitrocellulose strip with a His-GPR-2 fusion protein.

RNA Interference

The goa-1 and gpa-16 templates for RNA synthesis were produced by PCR with T3 and T7 oligos as described in [4]. Other templates for RNA synthesis were produced as described in [36]. dsRNA was injected at a concentration of 0.5-1 mg/ml. Embryos from injected mothers were analyzed at least 24 hr after injection.

Analysis of Embryos by DIC 4-D Videomicroscopy

Animals were dissected in a drop of M9 on poly-L-lysine-coated 18 mm × 18 mm coverslips, mounted over an agar pad, and sealed with petroleum jelly. The first cell cycle of the embryos was recorded (12 focal planes every 10 s) with DIC optics on a Leica DMRBE microscope and the Openlab software.

Yeast Two-Hybrid Screen

The two-hybrid screen was performed in yeast strain CG1945 (Clontech system) transformed with pAS2-GPR-1 and a *C. elegans* mixed-stage library (a kind gift of R. Barstead). Two-hybrid assays were performed according to the manufacturer's instruction. A total of 1.0×10^6 colonies were analyzed.

G Protein Binding and GTPγS Binding Assays

Binding of His-tagged GOA-1 to GST-GPR(415–453) was performed as described in [13] with the following modifications: 0.05% NP40 was added, and a 0.5 μM concentration of each protein was used in each assay.

[^{35}S]GTPγS (1250 Ci/mmol) was purchased from Dupont/NEN (Boston, MA). GPR peptides were synthesized and purified at the LSU Health Science Center Core Laboratories, and peptide mass was verified by matrix-assisted laser desorption ionization mass spectrometry. The sequence of the GPR consensus peptide is TMGEEDFFDLLAKSQSKRMDDQRVDLAG; the sequence of the GPR-1/2 peptide is TNEEPVDMMDLIFSMSSRMDDQRTELPA. Guanosine diphosphate (GDP) and guanosine 5′-O-(3- thiotriphosphate (GTPγS) were obtained from Sigma (St. Louis, MO). GTPγS binding assays were conducted as described [12]. Gα proteins (100 nM) were preincubated with GPR peptides or vehicle, and binding assays were initiated by the addition of 0.5 μM GTPγS (GTPγ^{35}S, 4.0×10^4 dpm/pmol). Nonspecific binding of GTPγ^{35}S to Gα protein and/or filters was defined by 100 μM GTPγS. Reactions were incubated in a total volume of 50 μl for 30 min at 24°C. Reactions were terminated by rapid filtration through nitrocellulose filters (S&S BA85) with 4 × 4 ml washes of stop buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM EDTA [pH 7.4], 4°C), and radioactivity bound to the filters was determined by liquid scintillation counting.

Supplemental Data

Additional figures and movies are available with this article online at http://www.current-biology.com/content/supplemental.

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