Cortical localization of the Gα protein GPA-16 requires RIC-8 function during *C. elegans* asymmetric cell division

Katayoun Afshar[1], Francis S. Willard[2], Kelly Colombo[1], David P. Siderovski[2] and Pierre Gönczy[1,*]

[1]Swiss Institute for Experimental Cancer Research (ISREC), Swiss Federal Institute of Technology (EPFL), CH-1066 Lausanne, Switzerland
[2]Department of Pharmacology, Lineberger Comprehensive Cancer Center and Neuroscience Center, The University of North Carolina, Chapel Hill, NC 27599-7365, USA
*Author for correspondence (e-mail: pierre.gonczy@isrec.unil.ch)

Accepted 16 August 2005

Development 132, 4449-4459
Published by The Company of Biologists 2005
doi:10.1242/dev.02039

Summary

Understanding of the mechanisms governing spindle positioning during asymmetric division remains incomplete. During unequal division of one-cell stage *C. elegans* embryos, the Gα proteins GOA-1 and GPA-16 act in a partially redundant manner to generate pulling forces along astral microtubules. Previous work focused primarily on GOA-1, whereas the mechanisms by which GPA-16 participates in this process are not well understood. Here, we report that GPA-16 is present predominantly at the cortex of one-cell stage embryos. Using co-immunoprecipitation and surface plasmon resonance binding assays, we find that GPA-16 associates with RIC-8 and GPR-1/2, two proteins known to be required for pulling force generation. Using spindle severing as an assay for pulling forces, we demonstrate that inactivation of the Gβ protein GPB-1 renders GPA-16 and GOA-1 entirely redundant. This suggests that the two Gα proteins can activate the same pathway and that their dual presence is normally needed to counter Gβγ. Using nucleotide exchange assays, we establish that whereas GPR-1/2 acts as a guanine nucleotide dissociation inhibitor (GDI) for GPA-16, as it does for GOA-1, RIC-8 does not exhibit guanine nucleotide exchange factor (GEF) activity towards GPA-16, in contrast to its effect on GOA-1. We establish in addition that RIC-8 is required for cortical localization of GPA-16, whereas it is not required for that of GOA-1. Our analysis demonstrates that this requirement toward GPA-16 is distinct from the known function of RIC-8 in enabling interaction between Gα proteins and GPR-1/2, thus providing novel insight into the mechanisms of asymmetric spindle positioning.

Key words: *C. elegans* embryos, Spindle positioning, G protein

Introduction

Asymmetric divisions play a crucial role in the generation of cell diversity. During development in particular, asymmetric divisions often give rise to daughter cells that differ not only in fates, but also in sizes (for a review, see Horvitz and Herskowitz, 1992). In animal cells, such unequal divisions rely on the eccentric position of the mitotic spindle because the cleavage furrow is specified so as to bisect the spindle by the end of anaphase (for a review, see Rappaport, 1971).

The one-cell stage *C. elegans* embryo has emerged as an attractive model to analyze the mechanisms of cell-intrinsic spindle positioning during unequal cell division (for a review, see Schneider and Bowerman, 2003). In the wild type, in response to anteroposterior (AP) polarity cues, the spindle is displaced towards the posterior by the end of anaphase, resulting in unequal cleavage into a larger anterior and a smaller posterior blastomere. Asymmetric spindle displacement results from unbalanced cortical force generators acting on astral microtubules and pulling on spindle poles (Grill et al., 2001; Grill et al., 2003). Because more force generators are active on the posterior cortex, there is a larger net force pulling on the posterior spindle pole (Grill et al., 2003).

Although the molecular nature of cortical force generators is not known, their activity relies on two α subunits of the heterotrimeric G-proteins: GOA-1 and GPA-16 (see Fig. S1 in the supplementary material). These components act in a partially redundant manner, as pulling forces are decreased in only a modest manner in embryos lacking either GOA-1 or GPA-16 (Afshar et al., 2004), still allowing asymmetric spindle elongation and unequal cleavage (Gotta and Ahringer, 2001). By contrast, simultaneous inactivation of GOA-1 and GPA-16 results in an extreme decrease of pulling forces (Colombo et al., 2003), yielding symmetric spindle elongation and equal first cleavage (Gotta and Ahringer, 2001). Conversely, inactivation of Gβγ results in excess pulling forces (Afshar et al., 2004). Furthermore, the triple inactivation of GOA-1, GPA-16 and Gβγ also yields an equal first cleavage, as in *goa-1/gpa-16(RNAi)* embryos (Gotta and Ahringer, 2001; Tsou et al., 2003), indicating that Gβγ dampens Gα-dependent force generation in one-cell stage embryos.

A phenotype analogous to that of *goa-1/gpa-16(RNAi)* is observed after inactivation of GPR-1/2 (Colombo et al., 2003; Gotta et al., 2003; Srinivasan et al., 2003), a GoLoco protein which acts as a guanine nucleotide dissociation inhibitor (GDI) for GOA-1 (Afshar et al., 2004; Gotta et al., 2003) or of LIN-

5, a coiled-coil protein that physically interacts with GPR-1/2 (Lorson et al., 2000; Srinivasan et al., 2003). GPR-1/2 and LIN-5 are present at the cortex of one-cell stage embryos (Colombo et al., 2003; Gotta et al., 2003; Srinivasan et al., 2003). During mitosis, cortical GPR-1/2 distribution is asymmetric, with a slight enrichment at the posterior (Colombo et al., 2003; Gotta et al., 2003; Tsou et al., 2003). This raises the possibility that GPR-1/2 is responsible for the larger net pulling force exerted on the posterior spindle pole. RIC-8, which acts as a guanine nucleotide exchange factor (GEF) for GOA-1 (Afshar et al., 2004; Hess et al., 2004), is also required for pulling force generation (Afshar et al., 2004). Co-immunoprecipitation experiments and biochemical analyses indicate that RIC-8 is required for the interaction between GPR-1/2 and GOA-1, raising the possibility that RIC-8 acts before GPR-1/2 in the GOA-1 activation cycle (Afshar et al., 2004). Moreover, inactivation of the Gβ subunit GPB-1 alleviates the requirement for RIC-8 during spindle positioning, suggesting that RIC-8 promotes generation of GOA-1 free from Gβγ, thus making it available for binding to GPR-1/2 (Afshar et al., 2004) (for a review, see McCudden et al., 2005).

Whereas GOA-1 is known to be present at the cortex of early embryos (Afshar et al., 2004; Gotta and Ahringer, 2001; Miller and Rand, 2000), the subcellular distribution of GPA-16 has not been investigated. Moreover, although yeast two-hybrid assays indicate that GPA-16 can physically interact with RIC-8 (Afshar et al., 2004) and GPR-1/2 (Li et al., 2004), the latter interaction was not detected in two other studies (Colombo et al., 2003; Gotta et al., 2003), and whether such interactions occur in *C. elegans* embryos is not known. Furthermore, the consequence of putative interactions of GPA-16 with RIC-8 and GPR-1/2 has not been investigated. In addition, the nature of the partial redundancy between GPA-16 and GOA-1 has not been addressed. For example, GPA-16 and GOA-1 could each be essential for activation of separate pathways that trigger distinct effectors, which together ensure force generation. Alternatively, GPA-16 and GOA-1 could each contribute to partial activation of the same pathway.

We investigated these issues in this study. We report that GPA-16 is present predominantly at the cell cortex and that it interacts with RIC-8 and GPR-1/2, both in vitro and in vivo. We show that GPA-16 and GOA-1 become entirely redundant after GPB-1 inactivation, suggesting that the two Gα proteins can activate the same pathway. Furthermore, we establish that GPR-1/2 acts as a GDI for GPA-16, whereas RIC-8 does not act as a GEF for GPA-16. Importantly, we find that RIC-8 is required for GPA-16 cortical localization and that this novel requirement is distinct from its known role in enabling interaction between Gα proteins and GPR-1/2.

Materials and methods

Nematode strains and RNAi

Wild-type (N2), *ric-8(md303)* and *ric-8(md1909)* (Miller et al., 2000), *goa-1(sa734)* (Robatzek and Thomas, 2000), and *gpa-16(it143)* (Bergmann et al., 2003) mutant strains of *C. elegans* were cultured at 16°C according to standard procedures (Brenner, 1974). *ric-8* and *gpa-16* mutant animals were typically shifted to 20°C >36 hours prior to analysis. We found that the spindle positioning phenotype of *gpa-16(it143)* mutant embryos is not markedly different when homozygous mutant animals are raised at 20°C or at 25°C (data not shown). Nevertheless, we performed spindle severing experiments on *gpa-16(it143)* mutant embryos at 25°C to ensure maximal inactivation.

Bacterial RNAi feeding strains were as described (Afshar et al., 2004; Colombo et al., 2003). The conditions for RNAi by feeding were as follows, starting with L3/L4 larvae: *gpa-16*, *goa-1*, *gpb-1* and *gpr-1/2*: 48-60 hours at 20°C; *ric-8*: 40-44 hours at 20°C. RNAi-mediated inactivation of *ric-8* in *ric-8(md1909)* mutant animals was performed as described (Afshar et al., 2004). For experiments where two genes were inactivated with RNAi, appropriate controls were performed in parallel to ensure the efficiency of each RNAi condition.

Microscopy and spindle severing

Time-lapse DIC microscopy was performed capturing 1 image every 5 seconds (Gönczy et al., 1999). Spindle severing experiments and measurement of peak velocities were performed essentially as before (Grill et al., 2001), using a Leica LMD microscope equipped with a pulsed N2 laser (λ=337 nM).

Antibody production and use

For generating GPA-16 antibodies, the full-length *gpa-16* cDNA (Colombo et al., 2003) was cloned into pGEX-6P-2. GST-GPA-16 was expressed, purified from inclusion bodies, run on an SDS-PAGE gel and injected into a rabbit (Eurogentec). Antibodies were strip-purified against GST-GPA-16, eluting with 0.1 M glycine (pH 2.5). Affinity-purified antibodies were dialyzed against PBS and kept at –20°C in 50% glycerol.

For revealing RIC-8 following immunoprecipitation with GPA-16 antibodies (Fig. 2A, Fig. 5E), we generated RIC-8 antibodies by first cloning the full length *ric*-8 cDNA into a pGEX derivative. Bacterially expressed GST-RIC-8 was purified from inclusion bodies, purified on an SDS-PAGE gel and injected into a rabbit (Eurogentec). Antibodies were column affinity purified against His_6-RIC-8 bound to a HiTrap NHS Hp column (Pharmacia), dialyzed and stored as above. These antibodies (see Fig. S4 in the supplementary material) were used following immunoprecipitation with GPA-16 antibodies in place of previously described antibodies (Miller et al., 2000) (which were used in the case of Fig. 2B) because they are more sensitive.

Fixation and staining of embryos for indirect immunofluorescence was as described (Afshar et al., 2004). The following primary antibodies were used: 1:200 mouse anti-α tubulin (DM1A, Sigma), 1:300 rabbit anti-GPA-16 (this study), 1:300 rabbit anti-GOA-1 (Afshar et al., 2004) and 1:150 rabbit anti-GPR-1/2 (Colombo et al., 2003). Secondary antibodies were 1:500 goat anti-mouse conjugated to Alexa-488 (Molecular Probes) and 1:1000 goat anti-rabbit conjugated to Cy3. Slides were counterstained with ~1 μg/ml Hoechst 33258 (Sigma) to detect DNA. Approximately 1 μm optical slices were collected on an LSM510 Zeiss confocal microscope and processed in Adobe Photoshop. Quantification of GPR-1/2 signal was performed on images collected on a Zeiss Axioplan 2 with at 12-bit Diagnostic Instrument Spot RT Camera controlled by Metamorph software (Universal Imaging). Average cortical intensities were determined in a ~5 μm-long region between the EMS and ABp blastomeres, after subtraction of the average cytoplasmic signal from a neighboring region.

Generation of embryonic extracts, immunoprecipitation (using ~3 μg of GPA-16 antibodies without addition of guanine nucleotides, unless specified otherwise) and western blot analysis were performed as described (Afshar et al., 2004). All primary antibodies for western blot analysis were used at 1:1000, except the new RIC-8 antibodies, which were used at 1:2000. For secondary antibodies, we used 1:10,000 HRP-conjugated goat anti-rabbit (Amersham), except when revealing GPR-1/2, where HRP-conjugated Protein A (Amersham) was used at 1:2000 in place of secondary antibodies because the heavy chain of immunoglobulins is similar in size to GPR-1/2.

Protein purification

Purification of GOA-1 (amino acids 28-351) and His_6-RIC-8 was as described (Afshar et al., 2004). The RIC-8 open reading frame (ORF) was cloned into pGEX4TEV2 (Kimple et al., 2004) and GST-RIC-8 purified by chromatographic methods as previously described (Willard and Siderovski, 2004). We have previously observed aggregation of RIC-8 during purification (Afshar et al., 2004); accordingly chromatography buffers were supplemented with 400 mM NaCl and 5-10% (v/v) glycerol. GST-GPR-1/2 (amino acids 374-476) was cloned into pGEX4TEV2 and purified using the methods described above.

All baculoviral and insect cell culture reagents were obtained from Invitrogen. DNA encoding GPA-16 (amino acids 5-357) was cloned into pFastBacHTb and recombinant bacmid DNA was generated using the Bac-to-Bac method. Insect cell transfection and viral amplification were performed by the Tissue Culture Core Laboratory (University of Colorado Cancer Center). Protein was expressed by infecting Hi5 cells (1.0×10^6 cells/ml) grown in Express Five SFM (containing 16.5 mM L-glutamine) with baculovirus at a multiplicity of infection of 1.0. After incubation at 27°C for 2 days, cells were pelleted by centrifugation at 3000 ***g***, and resuspended in buffer N [50 mM Tris/HCl (pH 7.5), 100 mM NaCl, 5% (v/v) glycerol, 10 mM imidazole, 50 μM GDP, 20 mM NaF, 30 μM $AlCl_3$, 5 mM $MgCl_2$, 0.5% (w/v) sodium cholate and 1 mM 2-mercaptoethanol]. His_6-GPA-16 was then purified using Ni^{2+}-affinity chromatographic methods as described (Willard and Siderovski, 2004). Protein-containing fractions were pooled and subjected to HiTrapQ (Amersham) anion exchange chromatography. The eluent was 20 mM Tris/HCl (pH 8.0), 10 mM NaCl, 5% (v/v) glycerol, 10 μM GDP and 1 mM DTT; protein was eluted with a linear gradient of 0-300 mM NaCl over 20 column volumes. Protein fractions were analyzed for the presence GPA-16 by SDS-PAGE/Coomassie Blue staining, immunoblot with α-His_6 antibodies (Covance) and GTPγS binding. His_6-GPA-16 containing fractions were pooled and concentrated using a Vivaspin 30 kDa cutoff centrifugal filter (Sartorius).

Biochemical assays

[^{35}S]-GTPγS binding assays were performed as described (Afshar et al., 2004). Rates of GTPγS binding were calculated using a one-site exponential model (Prism v4.0; GraphPad Software). Protein-protein interactions were measured by surface plasmon resonance (SPR) spectroscopy as described (Kimple et al., 2004). Eluent buffer [20 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM $MgCl_2$, 0.005% (v/v) NP-40] was supplemented with GDP (50 μM), GTPγS (50 μM), or GDP·AlF_4^- (50 μM GDP, 20 mM NaF, 30 μM $AlCl_3$). GOA-1 and GPA-16 were diluted to 2 μM in the GDP, GTPγS, and GDP·AlF_4^- buffers, and incubated for 30 minutes at 25°C to ensure full nucleotide loading. Nucleotide-locked Gα proteins (2 μM) were injected (KINJECT, 30 μl, 300 s dissociation time, 5 μl/minute) over an anti-GST antibody-conjugated CM5 sensor chip, loaded with GST (1500 RU), GST-RIC-8 (800 RU) and GST-GPR-1 (amino acids 374-476) (1400 RU). Background binding to the GST surface was subtracted from all sensorgrams (BIAevaluation v3.0; Biacore).

Results

GPA-16 is present at the cortex of early embryonic blastomeres

We sought to determine the subcellular distribution of GPA-16 in early *C. elegans* embryos. We raised and affinity-purified antibodies which recognize a major species of the expected size in wild-type embryonic extracts that is vastly diminished in *gpa-16(RNAi)* embryonic extracts (Fig. 1A). These antibodies detect a strong and uniform signal at the cell cortex of early embryos (Fig. 1B,C). This distribution corresponds to bona fide GPA-16 because it is significantly diminished in *gpa-16(RNAi)* embryos (Fig. 1D). These antibodies also label the cytoplasm, with a slight enrichment in the vicinity of microtubule asters, but this aspect of the signal is barely diminished in *gpa-16(RNAi)* embryos (Fig. 1D), suggesting that it is not specific or corresponds to a particularly stable pool of GPA-16. In summary, GPA-16 is present predominantly at the cortex of one-cell stage embryos.

GPA-16 interacts with RIC-8 and GPR-1/2 in vivo

We investigated whether GPA-16 is present in a complex with RIC-8 and GPR-1/2 in *C. elegans* embryos. As shown in Fig. 2A (lane 7), we found that GPA-16 antibodies co-immunoprecipitate both RIC-8 and GPR-1/2 from wild-type embryonic extracts. Furthermore, we found that the interaction between GPA-16 and RIC-8 is severely compromised in embryonic extracts derived from *ric-8(md1909)* or *ric-8(md303)* mutant animals (Fig. 2A, lane 8 and data not shown). These observations establish that GPA-16 normally associates with both RIC-8 and GPR-1/2 in vivo.

We next addressed the order in which GPA-16 interacts with RIC-8 and GPR-1/2 by conducting co-immunoprecipitation experiments in embryonic extracts depleted of RIC-8 or GPR-1/2. These experiments established that the interaction between GPA-16 and RIC-8 is not altered in *gpr-1/2(RNAi)* embryonic extracts (Fig. 2A, lane 11, compare with lane 7). By contrast, the interaction between GPA-16 and GPR-1/2 is essentially abolished in *ric-8(md1909)* or *ric-8(md303)* embryonic extracts (Fig. 2A, lane 8, compare with lane 7 and data not shown). We conclude that RIC-8 is required for efficient assembly of a complex containing GPA-16 and GPR-1/2.

These findings prompted us to test whether RIC-8 may activate GPA-16 by supporting stable levels of Gβγ-free GPA-16. If this were the case, then inactivation of Gβγ might enable GPA-16 to interact with GPR-1/2 in the absence of RIC-8. Accordingly, we found that the interaction between GPA-16 and GPR-1/2 is partially restored in *ric-8(md1909) gpb-1(RNAi)* embryos (Fig. 2A, lane 10, compare with lane 8). Therefore, as for GOA-1 (Afshar et al., 2004), inactivation of Gβγ alleviates the need for RIC-8 to permit association of GPR-1/2 with GPA-16.

GOA-1 and GPA-16 become completely redundant during asymmetric cell division when GPB-1 is inactivated

As our findings suggest that generation of both GOA-1 and GPA-16 free from Gβγ is important for asymmetric spindle positioning, we investigated the nature of the partial redundancy between the two Gα proteins. We reasoned that if GPA-16 and GOA-1 are essential for distinct pathways, an excess of GPA-16 liberated from Gβγ should not compensate for loss of GOA-1. Similarly, an excess of GOA-1 liberated from Gβγ should not compensate for loss of GPA-16.

To test whether this is the case, we investigated the extent of pulling forces using laser microbeam-mediated spindle severing experiments, analyzing the resulting spindle pole movements with time-lapse differential interference contrast (DIC) microscopy (Grill et al., 2001). In these experiments, we used *goa-1(sa734)*, a deletion allele (Robatzek and Thomas, 2000), *gpa-16(it143)*, a strong reduction of function allele that

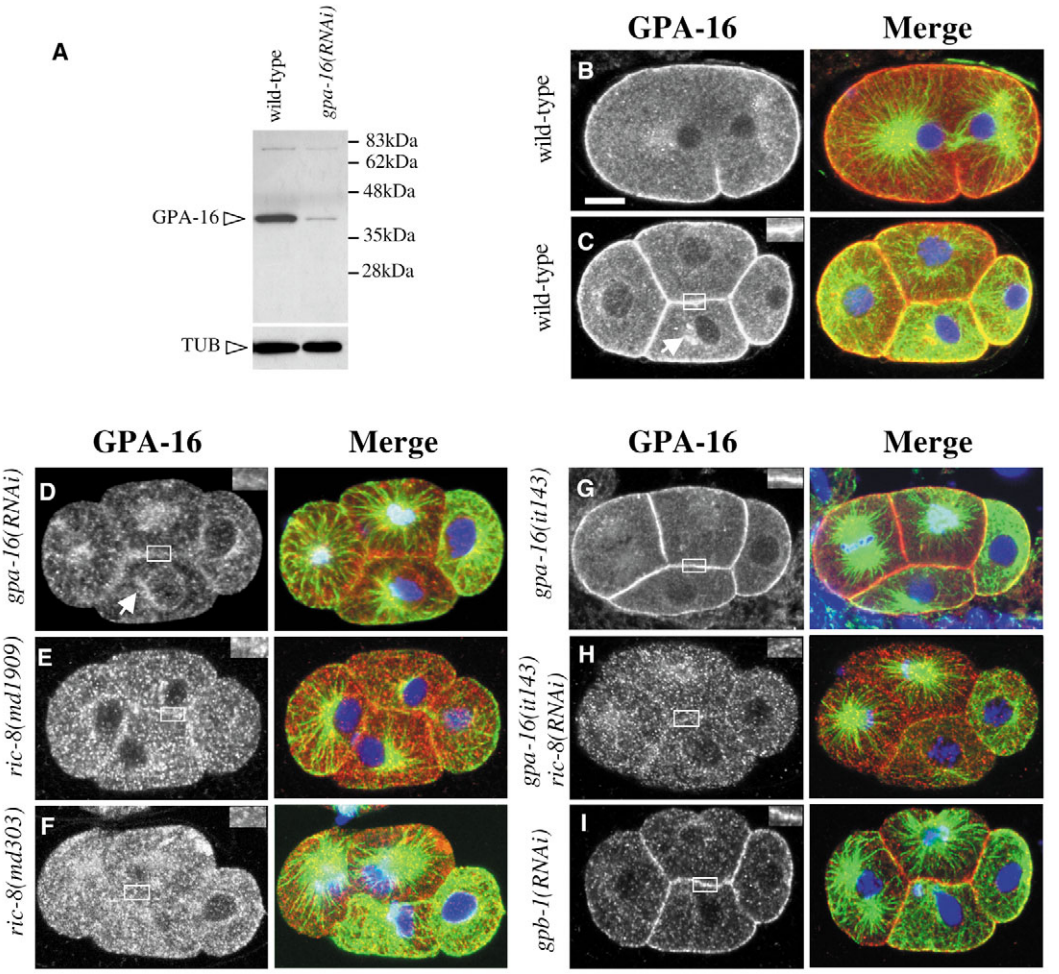

Fig. 1. GPA-16 distribution in early embryos. (A) Western blot analysis using GPA-16 antibodies on wild-type or *gpa-16(RNAi)* embryonic extracts. The blot was reprobed with α-tubulin antibodies as a loading control (bottom). (B-H) Wild-type embryos (B, one-cell stage late telophase; C, four-cell stage), as well as four-cell stage embryos of the indicated genotypes stained with antibodies against GPA-16 (red) and α-tubulin (green); DNA is shown in blue. Left panels show GPA-16 staining alone, right panels the merge of the three signals. Rectangles highlight a region of the cortex at the ABp/EMS boundary to ease comparison of GPA-16 levels; this region does not exhibit the variability in staining intensity sometimes observed on the cortex facing the outside. Insets represent ~2.5× magnified view of the approximate region indicated by the rectangles. Analogous distributions are observed in one-cell stage embryos (data not shown). Arrows in C,D indicate signal around microtubule asters, which persists in *gpa-16(RNAi)* embryos. In this and other figures, anterior is leftwards, posterior is rightwards. Scale bar: 10 μm.

results in a G202D substitution in the switch II region of the GTPase domain (Bergmann et al., 2003), and RNAi-mediated inactivation of *goa-1* or *gpa-16*. We inactivated *gpb-1* using RNAi, because *gpb-1* homozygous mutant animals die as larvae (Zwaal et al., 1996).

The outcome of the spindle severing experiments is summarized in Fig. 3 and Table S1 in the supplementary material. After spindle severing in wild-type one-cell stage embryos, the peak velocity of the anterior spindle pole is ~0.64 μm/second, whereas that of the posterior spindle pole is ~1.1 μm/second, reflecting the imbalance of net pulling forces acting on the two spindle poles (see also Afshar et al., 2004; Grill et al., 2001). Inactivation of either *goa-1* or *gpa-16* results in diminished peak velocities of both spindle poles (see also Afshar et al., 2004). In contrast, *gpb-1(RNAi)* results in high peak velocities of both spindle poles (~1.0 μm/second) (see also Afshar et al., 2004). Importantly, we found that *goa-1(sa734) gpb-1(RNAi)* or *goa-1(RNAi) gpb-1(RNAi)*, and *gpa-16(it143) gpb-1(RNAi)* or *gpa-16(RNAi) gpb-1(RNAi)* embryos exhibit a phenotype indistinguishable from that of *gpb-1(RNAi)* embryos, in that peak velocities of both spindle poles are high. This is not due to upregulation of GOA-1 or GPA-16 protein in the absence of the other Gα subunit (Fig. 2B, lanes 2 and 4). As expected from previous reports (Gotta and Ahringer, 2001; Tsou et al., 2003), we found, in addition, that following triple inactivation of *goa-1 gpa-16* and *gpb-1*, peak velocities are analogous to those observed in *goa-1/gpa-16(RNAi)* embryos (Colombo et al., 2003), indicating that Gβγ normally dampens Gα-dependent force generation.

An examination of GPR-1/2 cortical distribution in early two-cell stage embryo corroborated the outcome of the spindle severing experiments (Fig. 4; see also Figs S2 and S3 in the supplementary material for a quantitative assessment in four-cell stage embryos). Cortical GPR-1/2 is essentially absent in

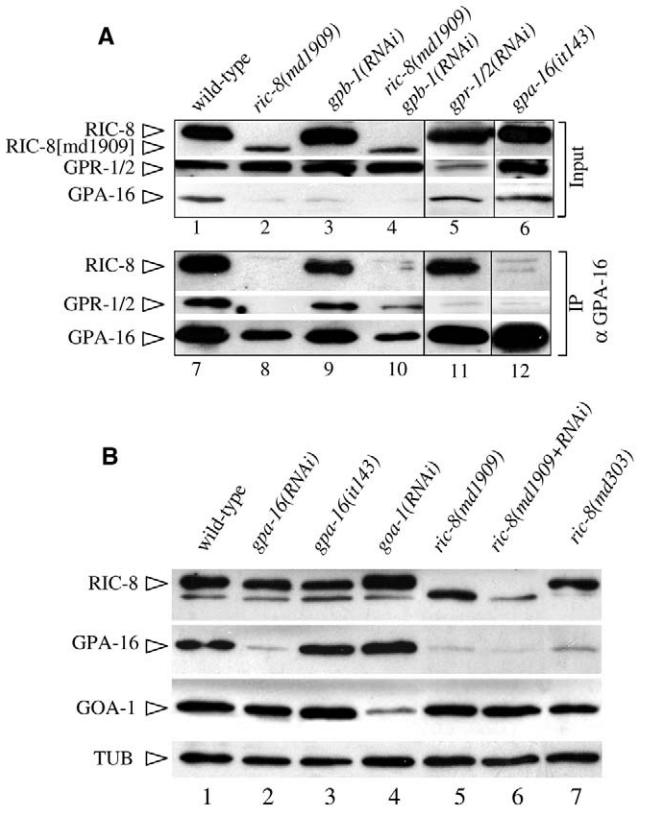

Fig. 2. RIC-8 is required for interaction between GPA-16 and GPR-1/2, and for normal GPA-16 protein levels. (A) Embryonic extracts of the indicated genotypes were immunoprecipitated with GPA-16 antibodies and analyzed by western blot using RIC-8, GPR-1/2 or GPA-16 antibodies, as indicated on the left by arrowheads. The top panel shows inputs (1/50 of starting materials). Whereas GPA-16 antibodies co-immunoprecipitate both RIC-8 and GPR-1/2, RIC-8 or GPR-1/2 antibodies do not co-immunoprecipitate GPA-16 (data not shown). Lanes 1-5 and 7-11 are from the same experiment; lanes 6 and 12 from a different experiment performed with appropriate controls. GPA-16 levels are diminished in inputs from the *gpb-1(RNAi)* embryonic extract. Quantifications of the intensity of the GPA-16 band from four experiments, using the α-tubulin or the GPR-1/2 band as loading control, indicate that the amount of GPA-16 in *ric-8(md1909)* and in *gpb-1(RNAi)* embryos is essentially identical [ratio of *gpb-1(RNAi)* versus *ric-8(md1909)*:1.15; s.d.=0.4]. RIC-8 is truncated in *ric-8(md1909)* mutant embryos (Afshar et al., 2004) and is present at a lesser abundance than in the wild type. (B) Western blot analysis of embryonic extracts of the indicated genotypes using sequentially antibodies against GPA-16, GOA-1 and RIC-8, as well as α-tubulin as a loading control, as indicated on the left with arrowheads. RIC-8 antibodies also detect a minor non-specific species, which co-migrate with the truncated protein in *ric-8(md1909)* mutant embryos (Afshar et al., 2004).

goa-1/gpa-16(RNAi) embryos (Fig. 4B, compare with 4A) (Colombo et al., 2003; Gotta et al., 2003; Tsou et al., 2003), as well as following triple inactivation of *goa-1 gpa-16* and *gpb-1* (data not shown). By contrast, we found that cortical GPR-1/2 appears merely diminished compared with wild type in *goa-1(RNAi)* or *gpa-16(RNAi)* embryos (Fig. 4C,D). Similar results were obtained with *goa-1(sa734)* and *gpa-16(it143)* (data not shown). Therefore, GPA-16 and GOA-1 each contribute to GPR-1/2 cortical targeting. As anticipated from the fact that *gpb-1(RNAi)* embryos in which either *goa-1* or *gpa-16* is also inactivated exhibit an indistinguishable phenotype from *gpb-1(RNAi)* embryos, we found that cortical GPR-1/2 is similar to wild type in such doubly inactivated embryos (compare Fig. 4F with 4C and 4A, as well as Fig. 4G with 4D and 4A).

We conclude that GPA-16 and GOA-1 become completely redundant for GPR-1/2 cortical localization and asymmetric spindle positioning when Gβγ is inactivated. This suggests that excess GPA-16 liberated from Gβγ can compensate for a loss of GOA-1, and vice versa. Therefore, instead of being essential for distinct pathways, GPA-16 and GOA-1 appear to each contribute to partial activation of the same pathway.

GPR-1/2 is a GDI for GPA-16, but RIC-8 is not a GEF for GPA-16

Next, we set out to determine whether RIC-8 and GPR-1/2 exhibit the same biochemical activity towards GPA-16 as they do toward GOA-1. To this end, we first conducted surface plasmon resonance (SPR) binding assays to investigate the nucleotide dependency of the interaction between GPA-16 and GPR-1/2, as well as that between GPA-16 and RIC-8.

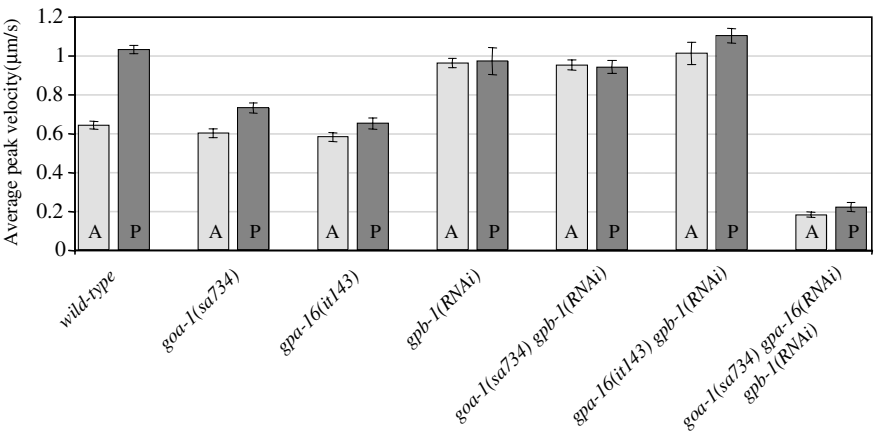

Fig. 3. GPA-16 and GOA-1 become entirely dispensable for generation of pulling forces following GPB-1 depletion. Average peak velocities±s.e.m. of anterior (A) and posterior (P) spindle poles after spindle severing in one-cell stage embryos of the indicated genotypes. Actual values are given in Table S1 in the supplementary material. We found an analogous outcome when examining the movements of centrosomes during centration/rotation prior to mitosis: whereas centration/rotation is gradual, as in wild type, in embryos compromised for either *goa-1* or *gpa-16* function, it is abrupt and accompanied by back and forth movements in all genotypes in which *gbp-1* function is compromised (see Movies 1-7 in the supplementary material). Values for wild type, *goa-1(sa734)* and *gpb-1(RNAi)* are from Afshar et al. (Afshar et al., 2004).

Recombinant GPA-16 was injected over SPR surfaces after pre-incubation with either GDP, the non-hydrolyzable GTP analogue GTPγS, or GDP·AlF_4^- to mimic the transition state of GTP hydrolysis. We found that a GST fusion protein encompassing the GoLoco motif of GPR-1/2 binds exclusively to GPA-16·GDP (Fig. 5A). We found also that a surface of immobilized GST-RIC-8 binds robustly to GPA-16·GDP, but to a much lesser extent to GTPγS-bound or AlF_4^--activated GPA-16 (Fig. 5B).

We conducted [^{35}S]GTPγS radioligand binding assays to determine the effect of GPR-1/2 and of RIC-8 on the kinetics of GTPγS binding to GPA-16. As shown in Fig. 5C, we found that a peptide encompassing an extended GPR-1/2 GoLoco motif exhibits GDI activity towards GPA-16, in a manner analogous to its effect on GOA-1 (Afshar et al., 2004). Unexpectedly, we found that the presence of RIC-8, even in a 20-fold molar excess, does not alter the kinetics of GTPγS binding to GPA-16 (Fig. 5D). SPR-binding assays established that this preparation of RIC-8 binds robustly to GOA-1·GDP (Fig. 5E), as shown previously (Afshar et al., 2004). Moreover, a twofold molar excess of the same preparation of RIC-8 significantly accelerates [^{35}S]GTPγS binding to GOA-1 (Fig. 5F), consistent with previous results (Afshar et al., 2004; Hess et al., 2004). Taken together, these findings lead us to conclude that in vitro, RIC-8 acts as a GEF towards GOA-1 but not towards GPA-16.

We reasoned that if RIC-8 also does not act as a GEF towards GPA-16 in vivo, it could remain associated with GPA-16 even after spontaneous nucleotide exchange has occurred, especially in light of the slight binding of RIC-8 to GPA-16·GTPγS and GPA-16·GDP·AlF_4^- observed in vitro (see Fig. 5B). Compatible with this view, we found that GPA-16 antibodies co-immunoprecipitate RIC-8 equally well in the presence of excess GDP or GTPγS (Fig. 5G). By contrast, GOA-1 antibodies co-immunoprecipitate RIC-8 preferentially in the presence of excess GDP (Fig. 5H) (see also Afshar et al., 2004). As expected, we found also that GPA-16 antibodies co-immunoprecipitate GPR-1/2 only in the presence of excess GDP (Fig. 5E), as is the case with GOA-1 antibodies (Afshar et al., 2004).

Overall, our findings indicate that whereas GPR-1/2 acts as a GDI towards both GOA-1 and GPA-16, RIC-8 exhibits GEF activity towards GOA-1, but not GPA-16.

RIC-8 ensures normal cortical localization and protein levels of GPA-16

Despite RIC-8 not acting as a GEF towards GPA-16, RIC-8 is required for GPA-16 function during asymmetric cell division, as RIC-8 inactivation results in a phenotype analogous to inactivating both GOA-1 and GPA-16 (Afshar et al., 2004). To begin investigating how RIC-8 exerts its requirement towards GPA-16, we examined the distribution of GPA-16 in *ric-8* mutant embryos. Strikingly, we found that GPA-16 distribution at the cortex is extremely diminished when RIC-8 function is compromised (Fig. 1E,F, compare with 1C). Moreover, western blot analysis revealed a severe reduction in GPA-16 protein levels in *ric-8* mutant embryonic extracts (Fig. 2B, lanes 5-7, compare with lane 1).

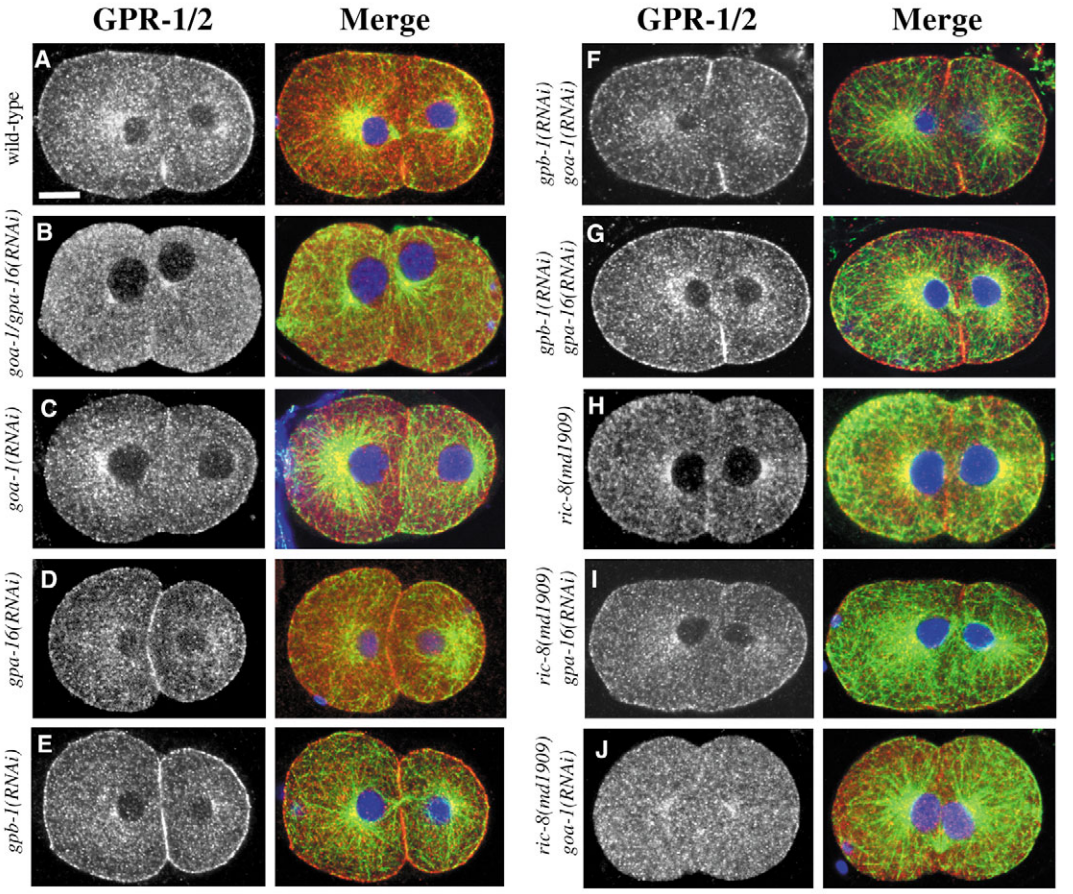

Fig. 4. Cortical GPR-1/2 distribution. Early two-cell stage embryos of the indicated genotypes stained with antibodies against GPR-1/2 (red) and α-tubulin (green); DNA is shown in blue. Left panels show GPR-1/2 staining alone, right panels the merge of the three signals. See also Figs S2 and S3 in the supplementary material for four-cell stage embryos and corresponding quantifications of GPR-1/2 cortical distribution. Scale bar: 10 μm.

By contrast, RIC-8 is not required for normal GOA-1 distribution (Afshar et al., 2004) or protein levels (Fig. 2B, lanes 5-7, compare with lane 1), indicating specificity in its requirement for GPA-16. Furthermore, RIC-8 distribution and levels are not affected in *gpa-16(RNAi)* embryos (Fig. 2B, lane 2; data not shown), indicating that this requirement is not reciprocal. Overall, we conclude that RIC-8 is required for normal cortical localization and protein levels of GPA-16.

Compromising the function of GOA-1 and RIC-8 is more detrimental than compromising that of GPA-16 and RIC-8

The above observations suggest that compromising RIC-8 function may be more detrimental to embryos depleted of GOA-1 than to embryos depleted of GPA-16, because GPA-16 levels are already severely compromised in the absence of RIC-8. To test this prediction, we performed time-lapse DIC microscopy of one-cell stage embryos. We compromised *goa-1* and *gpa-16* by RNAi, rather than through the use of mutants for the following reasons. First, using time-lapse DIC microscopy and spindle severing, we found that the spindle positioning phenotypes in *goa-1(RNAi)* and the deletion allele *goa-1(sa734)* are indistinguishable (see Table S1 in the supplementary material). Moreover, *ric-8(md1909) goa-1(RNAi)* embryos already have a penetrant spindle positioning phenotype. Second, null allele of *gpa-16* are not available (Bergmann et al., 2003), and we found using spindle severing that the impairment of force generation in *gpa-16(RNAi)* embryos is as severe as that in *gpa-16(it143)* embryos (see Table S1 in the supplementary material).

In the wild type, asymmetric spindle positioning is accompanied by transverse oscillations of the posterior spindle pole that reflect the extent of pulling forces and results in unequal cleavage (Fig. 6A; see Movie 8 in the supplementary material). In *goa-1(RNAi)* or *gpa-16(RNAi)* embryos, transverse oscillations are dampened, but asymmetric spindle positioning is nevertheless achieved, resulting in unequal cleavage (Fig.

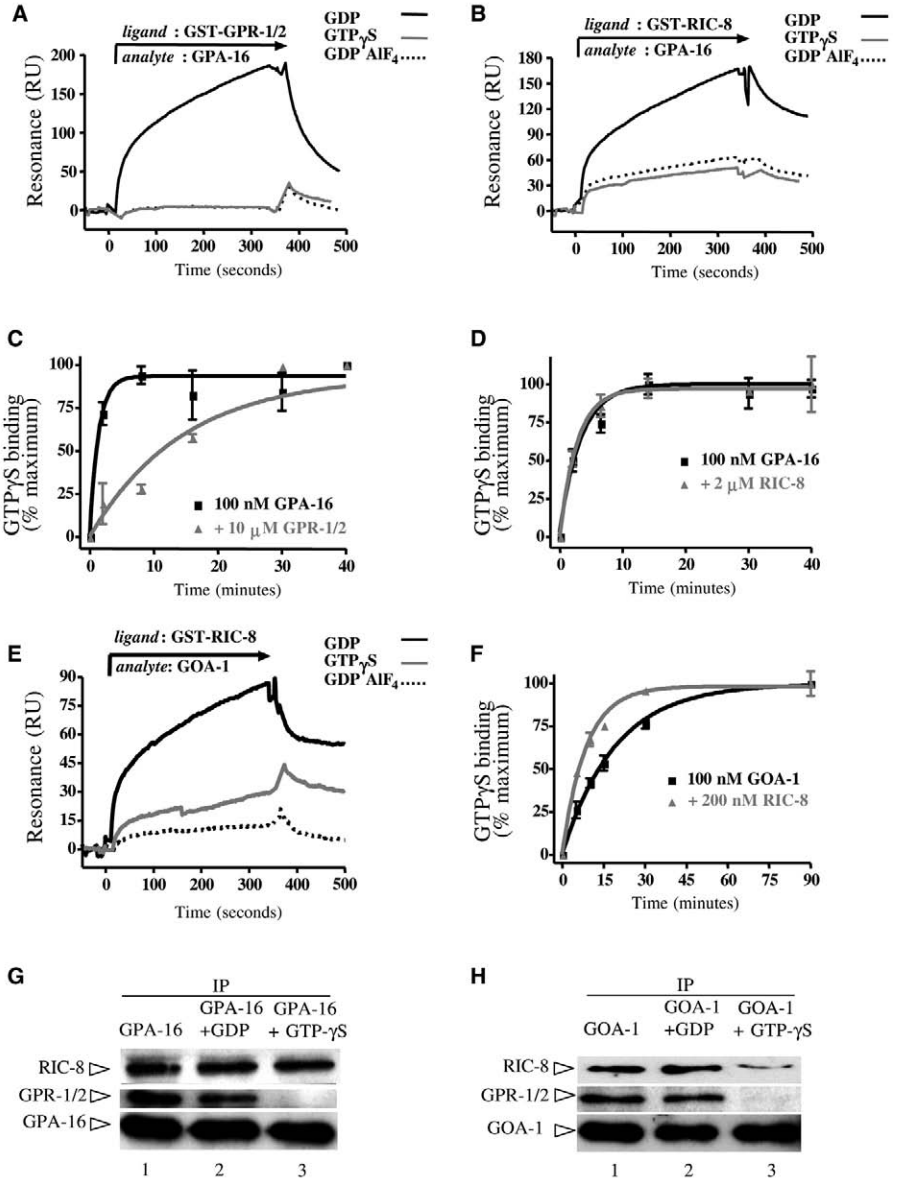

Fig. 5. GPR-1/2 is a GDI for GPA-16; RIC-8 is not a GEF for GPA-16. (A,B,E) Surface plasmon resonance was used to analyze the binding of GPA-16 to GST-GPR-1/2 (amino acids 374-476) (A) and to GST-RIC-8 (B), as well as of GOA-1 to GST-RIC-8 (E). GST fusion proteins were immobilized on a GST antibody biosensor surface. 'Analyte' (30 μl of 2 μM GPA-16 or GOA-1), in each of the indicated nucleotide bound states, was injected over the biosensor surface. Non-specific binding to GST was subtracted from each curve. (C) Time-course of [^{35}S]GTPγS binding to 100 nM GPA-16 in the presence or absence of 10 μM GPR-1/2 peptide (amino acids 423-461). Results are the mean±s.e.m. of duplicate samples. Observed association rate constants (with 95% confidence intervals in parentheses) were: GPA-16, 0.73 (0.44-1.0) minutes^{-1}; GPA-16+GPR-1/2, 0.069 (0.050-0.087) minutes^{-1}. (D,F) Time-course of [^{35}S]GTPγS binding to 100 nM GPA-16 (D) or 100 nM GOA-1 (F) in the presence or absence of 2 μM RIC-8 (D) or 200 nM RIC-8 (F). Results are the mean±s.e.m. of duplicate samples. Observed association rate constants (with 95% confidence intervals in parentheses) were: GPA-16, 0.32 (0.24-0.40) minutes^{-1}; GPA-16+RIC-8, 0.35 (0.26-0.43) minutes^{-1}; GOA-1, 0.052 (0.044-0.061) minutes^{-1}; GOA-1+RIC-8, 0.12 (0.093-0.14) minutes^{-1}. (G,H) Co-immunoprecipitation of the same wild-type embryonic extracts with GPA-16 (G) or GOA-1 (H) antibodies either alone (lanes 1) or in the presence of 100 μM GDP (lanes 2) or 100 μM GTP-γS (lanes 3). The co-immunoprecipitated material was detected using RIC-8, GPR-1/2, GPA-16 or GOA-1 antibodies, as indicated on the left with arrowheads. A previous study reported that spindle positioning in *ric-8(md1909) goa-1(RNAi)* embryos is similar to that of *ric-8(md1909)* embryos (Couwembergs et al., 2004); the difference with our results may reflect the use of distinct RNAi conditions.

6B,C; see Movies 9 and 10 in the supplementary material) (Miller and Rand, 2000). The same is true in *ric-8(md1909)* mutant embryos and *ric-8(md1909) gpa-16(RNAi)* embryos (Fig. 6D,E; see Movies 11 and 12 in the supplementary material). By contrast, in *ric-8(md1909) goa-1(RNAi)* embryos, the spindle remains centrally located and sometimes even drifts towards the anterior, resulting in equal cleavage (Fig. 6F; see Movie 13 in the supplementary material). An analogous behavior is observed in *goa-1/gpa-16(RNAi)* embryos (Fig. 6G,H; see Movie 14 in the supplementary material) (Gotta and Ahringer, 2001).

We found in addition that inactivation of *gpa-16* in *ric-8(md1909)* mutant embryos does not further decrease cortical GPR-1/2 compared with *ric-8(md1909)* embryos (Fig. 4H, compare with 4I). By contrast, inactivation of *goa-1* in *ric-8(md1909)* mutant embryos results in extremely diminished levels of cortical GPR-1/2, comparable with those of *goa-1/gpa-16(RNAi)* embryos (Fig. 4J, compare with 4B). Corroborating these results, we found that inactivation of *goa-1* has a more severe consequence on embryonic lethality of *ric-8* mutant embryos than that of *gpa-16* (data not shown).

Although we cannot formally exclude that these results reflect partial inactivation of *gpa-16*, we view this as unlikely because GPA-16 protein levels are significantly diminished in *gpa-16(RNAi)* embryonic extracts (Fig. 2B, lane 2) and because the *gpa-16(RNAi)* phenotype is as severe as that of the strong reduction of function allele *gpa-16(it143)* (see Table S1 in the supplementary material). Therefore, compromising simultaneously GOA-1 and RIC-8 appears more detrimental to asymmetric spindle positioning than compromising simultaneously GPA-16 and RIC-8, as expected from the fact that GPA-16 cortical localization and protein levels are already severely diminished in the absence of RIC-8.

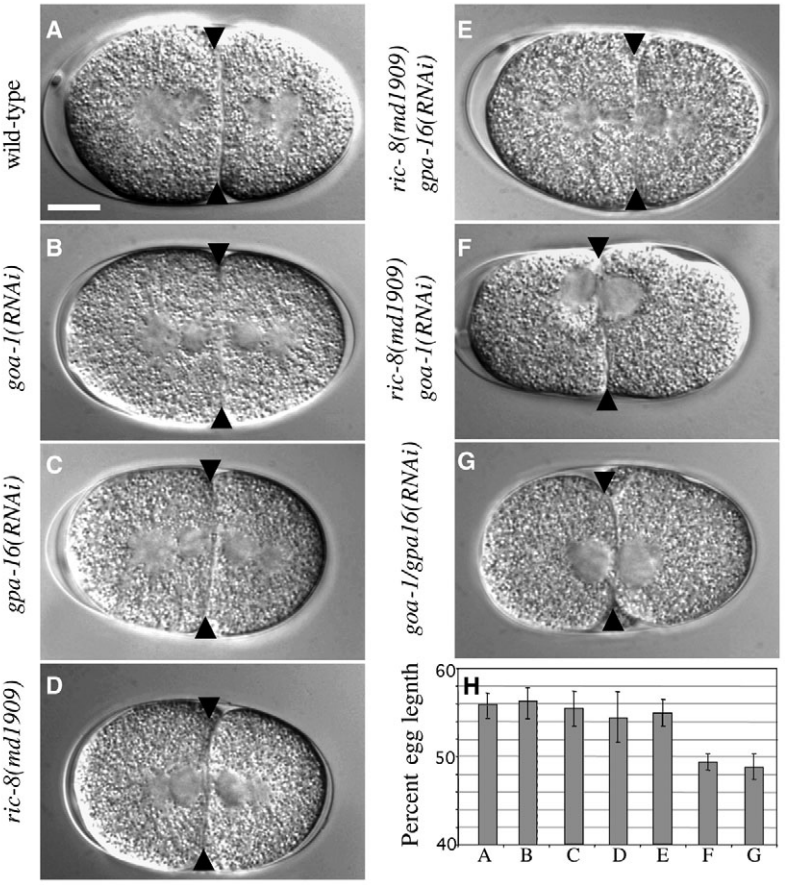

Fig. 6. Compromising the function of GOA-1 and RIC-8 is more detrimental than compromising that of GPA-16 and RIC-8. (A-G) Early two-cell stage embryos of the indicated genotypes (see also Movies 8-14 in the supplementary material). Arrowheads indicate cleavage furrow position. (H) Average cleavage furrow positions along the AP axis (0% egg-length, anterior-most; 100% egg-length, posterior-most), along with standard deviations, for embryos of the genotypes illustrated in A-G. Number of embryos examined for each genotype: A, 10; B, 7; C, 8; D, 7; E, 6; F, 8; G, 8. Scale bar: 10 μm.

The requirement of RIC-8 for normal GPA-16 cortical localization and protein levels is distinct from that enabling interaction between GPA-16 and GPR-1/2

Reduced GPA-16 cortical localization and protein levels in *ric-8* mutant embryos could be due to the lack of interaction between GPA-16 and GPR-1/2 or instead uncover a novel requirement for RIC-8. If the former was the case, then mutations that impair the interaction between GPA-16 and GPR-1/2 should necessarily result in reduced GPA-16 cortical localization and protein levels. Contrary to this prediction, we found that GPA-16 distribution and levels are normal in *gpa-16(it143)* mutant embryos (Fig. 1G and Fig. 2B, lane 3, compare with lane 1) (Bergmann et al., 2003), despite the interaction between GPA-16 and both RIC-8 and GPR-1/2 being severely diminished (Fig. 2A, lane 12, compare with lane 7). In addition, we found that GPA-16 cortical localization and protein levels are compromised in *gpa-16(it143) ric-8(RNAi)* (Fig. 1H; data not shown), indicating that the mutation in *gpa-16(it143)* does not render GPA-16 insensitive to RIC-8. Overall, these findings suggest that the requirement of RIC-8 for ensuring normal GPA-16 cortical localization and protein levels is novel and distinct from its known role in enabling interaction between Gα proteins and GPR-1/2.

RIC-8 is required for cortical localization of GPA-16 and thereby for maintaining normal protein levels

At least two scenarios can be envisaged to explain the dual requirement of RIC-8 in ensuring normal GPA-16 cortical localization and protein levels. First, RIC-8 may be required primarily for normal GPA-16 protein levels, with the lack of cortical localization being a consequence of having insufficient GPA-16 protein in the absence of RIC-8. Alternatively, RIC-8 may be required primarily for cortical localization of GPA-16, with the diminution in protein levels being a consequence of failed GPA-16 localization in the absence of RIC-8.

An examination of GPA-16 distribution in *gpb-1(RNAi)* embryos leads us to favor the latter scenario. We found that GPA-16 protein levels are severely diminished in such embryos (Fig. 2A, lane 3, compare with lane 1), to an extent comparable

with that observed in *ric-8(md1909)* mutant embryos (Fig. 2A, compare lanes 2 and 3). Importantly, in addition, we found that GPA-16 is present at the cortex of *gpb-1(RNAi)* embryos (Fig. 1I). Although we cannot exclude that GPA-16 in *gpb-1(RNAi)* embryos is targeted to the cortex in a manner that does not occur in the wild type, these observations render it unlikely that lack of GPA-16 cortical localization in *ric-8(md1909)* mutant embryos is due merely to diminished protein levels. Therefore, we propose that RIC-8 is required for cortical localization of GPA-16 during asymmetric division of *C. elegans* embryos.

Discussion

Heterotrimeric Gα proteins are crucial for spindle positioning in *C. elegans*, *Drosophila melanogaster* and vertebrate cells (for a review, see Hampoelz and Knoblich, 2004). In the one-cell stage *C. elegans* embryo, the Gα proteins GPA-16 and GOA-1 act in a partially redundant manner to ensure generation of pulling forces during asymmetric spindle positioning. Previous work has focused on the mechanism by which RIC-8 and GPR-1/2 regulate GOA-1 function. Whether similar mechanisms hold for GPA-16 has not been previously addressed.

Distinct regulation of GPA-16 and GOA-1 during asymmetric cell division

In this study, we establish that the interaction of GPA-16 with GPR-1/2 requires RIC-8, and that this requirement is alleviated when Gβγ is inactivated. Moreover, we find that GPR-1/2 acts as a GDI towards GPA-16. Whereas these findings mirror those made with GOA-1 (Afshar et al., 2004), we show also that GPA-16 differs in two important ways from GOA-1 with respect to its relationships with RIC-8. First, RIC-8 does not exhibit GEF activity towards GPA-16, in contrast to its effect on GOA-1. Consistent with our findings, yeast two hybrid experiments indicate that rat Ric8 isoforms interact with both wild type and mutant GTP-ase deficient, $G\alpha_q$ and $G\alpha_o$ (Tall et al., 2003). Interestingly, rat Ric8A also exhibits differential GEF activity towards distinct Gα proteins in vitro (Tall et al., 2003), and our work with GOA-1 and GPA-16 provides the first evidence that such differential activity occurs in a physiological setting.

A second important difference is that RIC-8 is required for normal cortical localization of GPA-16, but not GOA-1. We found also that RIC-8 is needed for efficient cortical localization of GPB-1, but this most likely reflects the requirement of RIC-8 for GPA-16 cortical localization, because a diminution of GPB-1 is observed in *gpa-16(RNAi)* embryos (data not shown). Importantly, we found in addition that GPA-16 cortical localization is not altered in *gpa-16(it143)* mutant embryos, despite the interaction of GPA-16 with RIC-8 and GPR-1/2 being essentially abolished. This indicates that the requirement of RIC-8 for GPA-16 cortical localization is distinct from its known role in ensuring interaction between Gα proteins and GPR-1/2.

It will be interesting to investigate the mechanism by which the novel requirement of RIC-8 for GPA-16 cortical localization is exerted. One possibility is suggested by the fact that myristoylation and palmitoylation of Gα subunits is important for their cortical localization (reviewed by Wedegaertner, 1998). In view of this, RIC-8 may be needed for lipid modification of GPA-16. Alternatively, RIC-8 could help fold GPA-16 to make it competent for cortical localization. Yet an alternative possibility is suggested by the fact that Gα subunits in vertebrate cells can redistribute from the cortex to the cytosol in response to agonist stimulation (Allen et al., 2005; Wedegaertner and Bourne, 1994). In this scenario, RIC-8 may be a negative regulator of GPA-16 removal from the cortex. Regardless of the underlying mechanism, our findings uncover a novel function for RIC-8, that of ensuring cortical localization of a Gα protein. By extension, our results raise the possibility that RIC-8 family members modulate G-protein signaling in other organisms in an analogous manner.

Activation mechanism of GPA-16 during asymmetric cell division

Our previous work suggested a model for the activation mechanism of GOA-1 in which RIC-8 GEF activity first generates GOA-1·GTP, after which the intrinsic GTPase activity of GOA-1 converts GOA-1 to the GDP-bound form capable of binding GPR-1/2 (Afshar et al., 2004). Our present findings suggest a simpler model for GPA-16, in which nucleotide exchange does not occur prior to interaction of GPA-16 with GPR-1/2 (Fig. 7). As Ric-8A is inactive on heterotrimer-complexed Gα·GDP (Tall et al., 2003), it is likely that RIC-8 acts on Gβγ-independent GPA-16·GDP. Because RIC-8 does not exhibit GEF activity towards GPA-16, we propose that nucleotide exchange does not occur on GPA-16 prior to interaction with GPR-1/2 and the associated protein LIN-5. The GPA-16·GDP-GPR-1/2-LIN-5 complex may then promote generation of pulling forces along astral microtubules. Such a complex may still be associated with RIC-8, because some GPR-1/2 is present following co-immunoprecipitation with RIC-8 antibodies from wild-type embryonic extracts (Afshar et al., 2004). Spontaneous nucleotide exchange ensues, after which the intrinsic GTPase activity of GPA-16, most

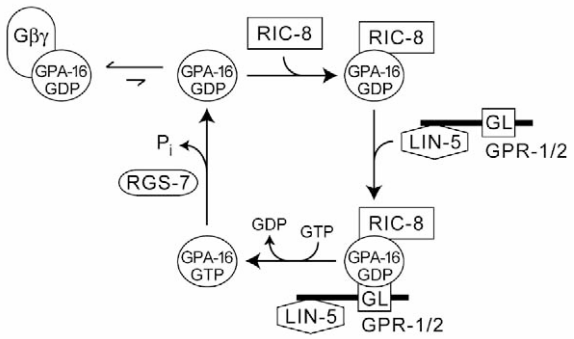

Fig. 7. Working model of GPA-16 activation during asymmetric cell division. RIC-8 binds to GPA-16·GDP, counteracting the formation of the GPA-16/Gβγ heterotrimer; this allows association of GPR-1/2 and LIN-5 with GPA-16. A complex of GPA-16·GDP-GPR1/2-LIN-5 promotes generation of pulling forces on spindle poles. As some GPR-1/2 co-immunoprecipitates with RIC-8 (Afshar et al., 2004), RIC-8 may still be present in this complex, as illustrated. As RIC-8 does not exhibit GEF activity towards GPA-16, RIC-8-independent nucleotide exchange may promote formation of GPA-16·GTP. Co-immunoprecipitation experiments (see Fig. 5G) suggest that RIC-8 can associate with GPA-16·GTP, although this is not illustrated. RGS-7 GAP activity promotes GTP hydrolysis and formation of GPA-16·GDP and is thus is required for the activation cycle (Hess et al., 2004).

probably accelerated by RGS-7 (Hess et al., 2004), terminates the activation cycle.

Could the activation mechanism of GOA-1 in the embryo be simpler than initially envisaged and resemble that proposed here for GPA-16? Whereas GPA-16 does not have a known requirement outside of the germ line and the embryo, GOA-1 is also needed for synaptic transmission in the adult nervous system (Miller and Rand, 2000). Therefore, although inactivation of *ric-8* and of *goa-1* yield opposite phenotypes in the nervous system (Miller et al., 2000), possibly because RIC-8 is also required for activation of the $G\alpha_q$ EGL-30, RIC-8 may act as a GEF towards GOA-1 solely in the context of receptor-dependent signaling. During receptor-independent activation in the one-cell stage embryo, perhaps mechanisms that do not invoke RIC-8 GEF activity are to be considered for both GPA-16 and GOA-1.

Mechanism of partial redundancy: GOA-1 and GPA-16 counter the effect of Gβγ to ensure accurate asymmetric spindle positioning

Our study contributes to understanding the partial redundancy between GOA-1 and GPA-16. We find that simultaneous inactivation of the Gβ subunit GPB-1 and of either GOA-1 or GPA-16 results in the same phenotype as that observed after GPB-1 inactivation alone. Therefore, GOA-1 and GPA-16 become entirely redundant for asymmetric spindle positioning following Gβγ inactivation. These findings illustrate the importance of the balance between Gα proteins and Gβγ during receptor-independent activation of heterotrimeric G proteins. This is reminiscent of the interplay between Gα and Gβγ proteins in modulating receptor-dependent heterotrimeric G protein signaling (for a review, see Gilman, 1987).

Our findings suggest that in wild-type *C. elegans* embryos, the presence of both Gα subunits provides substantial Gα molecules free from Gβγ, which can then associate in a RIC-8-dependent manner with GPR-1/2-LIN-5 to generate appropriate pulling forces. When either GPA-16 or GOA-1 is inactivated, less Gα is available for interaction with GPR-1/2-LIN-5, resulting in lower pulling forces. When GPB-1 is inactivated in addition, no Gβγ-dependent dampening occurs and either GOA-1 or GPA-16 can alone sustain full generation of pulling forces. Interestingly, this is the case in embryos simultaneously compromised for GPB-1 and GOA-1 (see Fig. 3; Table S1), despite GPA-16 protein levels being diminished in the absence of GPB-1. However, we found that the bulk of residual GPA-16 protein in *gpb-1(RNAi)* embryos is present at the cortex (see Fig. 1I), which appears sufficient to recruit GPR-1/2-LIN-5 and generate pulling forces in the absence of GOA-1.

Gα requirement during spindle positioning: beyond *C. elegans*

Whereas two Gα proteins act in concert in one-cell stage *C. elegans* embryos, a single Gα that belongs to the $G\alpha_i$ class is known to be required for proper spindle orientation in *Drosophila* neuroblasts and sensory organ precursor cells (Schaefer et al., 2001). Although a second Gα that belongs to the $G\alpha_o$ class is expressed in these cells, it is not essential for spindle orientation (Yu et al., 2003). However, overexpression of either $G\alpha_o$ or $G\alpha_i$ yields identical spindle positioning defects, possibly owing to depletion of free Gβγ (Yu et al., 2003). Therefore, reminiscent of the situation in *C. elegans*, the balance between Gα proteins and Gβγ is also crucial in *Drosophila*. However, the mechanisms of activation may differ between the two species, as overexpression of $G\alpha_o$ or $G\alpha_i$ in *Drosophila* results in a phenotype independent of the GoLoco protein PINS (Yu et al., 2003). By contrast, Gβγ inactivation in *C. elegans*, which presumably results in excess free Gα proteins, results in a GPR-1/2-dependent phenotype (Tsou et al., 2003).

The requirement for Gα proteins and their regulators in spindle positioning extends to vertebrate cells. The GoLoco motif and RGS domain-containing protein RGS14, which exerts both GDI and GAP activities towards $G\alpha_{i/o}$ subunits, is crucial for spindle assembly in the mouse zygote (Martin-McCaffrey et al., 2005). LGN, a mammalian GoLoco protein more closely related to GPR-1/2 and PINS, is recruited to the cell cortex through association with a $G\alpha_i$ (Du and Macara, 2004). LGN also associates with the coiled-coil protein NuMA, thus targeting it to the cell cortex, where it may function as a spindle positioning effector. It has been suggested that GPR-1/2 and PINS may serve an analogous function, perhaps targeting LIN-5 and Inscuteable, respectively (Du and Macara, 2004; Willard et al., 2004). If this view were correct, our work would indicate that both GPA-16 and GOA-1 are needed to provide sufficient binding sites for GPR-1/2 to ensure efficient LIN-5 cortical recruitment and generation of pulling forces.

We are grateful to Lorenzo Cerutti for advice with sequence analysis, Kenneth Miller for RIC-8 antibodies, Lesilee Rose for *gpa-16(it143)* mutant animals, Christopher McCudden for providing the anti-GST sensor chip, and Jason Snyder for valuable advice on insect cell culture and protein purification. We thank Marie Delattre, Barry Ganetzky, Michael Glotzer and Tu Nguyen-Ngoc for critical reading of the manuscript. Some strains were obtained from the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). F.S.W. is a post-doctoral fellow of the American Heart Association. Supported by grants R01 GM062338 from the NIH (to D.P.S.) and 3100A0-102087 from the Swiss National Science Foundation (to P.G.).

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/20/4449/DC1

References

Afshar, K., Willard, F. S., Colombo, K., Johnston, C. A., McCudden, C. R., Siderovski, D. P. and Gönczy, P. (2004). RIC-8 is required for GPR-1/2-dependent Gα function during asymmetric division of *C. elegans* embryos. *Cell* **119**, 219-230.

Aiyar, A. (2000). The use of CLUSTAL W and CLUSTAL X for multiple sequence alignment. *Methods Mol. Biol.* **132**, 221-241.

Allen, J. A., Yu, J. Z., Donati, R. J. and Rasenick, M. M. (2005). β-adrenergic receptor stimulation promotes G alpha s internalization through lipid rafts: a study in living cells. *Mol. Pharmacol.* **67**, 1493-1504.

Bergmann, D. C., Lee, M., Robertson, B., Tsou, M. F., Rose, L. S. and Wood, W. B. (2003). Embryonic handedness choice in *C. elegans* involves the Gα protein GPA-16. *Development* **130**, 5731-5740.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71-94.

Colombo, K., Grill, S. W., Kimple, R. J., Willard, F. S., Siderovski, D. P. and Gönczy, P. (2003). Translation of polarity cues into asymmetric spindle positioning in *Caenorhabditis elegans* embryos. *Science* **300**, 1957-1961.

Couwembergs, C., Spilker, A. C. and Gotta, M. (2004). Control of embryonic spindle positioning and Gα activity by *C. elegans* RIC-8. *Curr. Biol.* **14**, 1871-1876.

Du, Q. and Macara, I. G. (2004). Mammalian Pins is a conformational switch that links NuMA to heterotrimeric G proteins. *Cell* **119**, 503-516.
Gilman, A. G. (1987). G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* **56**, 615-649.
Gönczy, P., Schnabel, H., Kaletta, T., Amores, A. D., Hyman, T. and Schnabel, R. (1999). Dissection of cell division processes in the one cell stage *Caenorhabditis elegans* embryo by mutational analysis. *J. Cell Biol.* **144**, 927-946.
Gotta, M. and Ahringer, J. (2001). Distinct roles for Gα and Gβγ in regulating spindle position and orientation in *Caenorhabditis elegans* embryos. *Nat. Cell Biol.* **3**, 297-300.
Gotta, M., Dong, Y., Peterson, Y. K., Lanier, S. M. and Ahringer, J. (2003). Asymmetrically distributed *C. elegans* homologs of AGS3/PINS control spindle position in the early embryo. *Curr. Biol.* **13**, 1029-1037.
Grill, S. W., Gönczy, P., Stelzer, E. H. and Hyman, A. A. (2001). Polarity controls forces governing asymmetric spindle positioning in the *Caenorhabditis elegans* embryo. *Nature* **409**, 630-633.
Grill, S. W., Howard, J., Schaffer, E., Stelzer, E. H. and Hyman, A. A. (2003). The distribution of active force generators controls mitotic spindle position. *Science* **301**, 518-521.
Hampoelz, B. and Knoblich, J. A. (2004). Heterotrimeric G proteins: new tricks for an old dog. *Cell* **119**, 453-456.
Hess, H. A., Röper, J.-C., Grill, S. W. and Koelle, M. R. (2004). RGS-7 completes a receptor-independent heterotrimeric G protein cycle to asymmetrically regulate mitotic spindle positioning in *C. elegans*. *Cell* **119**, 209-218.
Horvitz, H. R. and Herskowitz, I. (1992). Mechanisms of asymmetric cell division: two Bs or not two Bs, that is the question. *Cell* **68**, 237-255.
Kimple, R. J., Willard, F. S., Hains, M. D., Jones, M. B., Nweke, G. K. and Siderovski, D. P. (2004). Guanine nucleotide dissociation inhibitor activity of the triple GoLoco motif protein G18: alanine-to-aspartate mutation restores function to an inactive second GoLoco motif. *Biochem. J.* **378**, 801-808.
Li, S., Armstrong, C. M., Bertin, N., Ge, H., Milstein, S., Boxem, M., Vidalain, P. O., Han, J. D., Chesneau, A., Hao, T. et al. (2004). A map of the interactome network of the metazoan *C. elegans*. *Science* **303**, 540-543.
Lorson, M. A., Horvitz, H. R. and van den Heuvel, S. (2000). LIN-5 is a novel component of the spindle apparatus required for chromosome segregation and cleavage plane specification in *Caenorhabditis elegans*. *J. Cell Biol.* **148**, 73-86.
McCudden, C. R., Hains, M. D., Kimple, R. J., Siderovski, D. P. and Willard, F. S. (2005). G-protein signaling: back to the future. *Cell Mol. Life Sci.* **62**, 551-577.
Martin-McCaffrey, L., Willard, F. S., Pajak, A., Dagnino, L., Siderovski, D. P. and D'Souza, S. J. (2005). RGS14 is a microtubule-associated protein. *Cell Cycle* **4**, 953-960.
Miller, K. G. and Rand, J. B. (2000). A role for RIC-8 (Synembryn) and GOA-1 ($G_o\alpha$) in regulating a subset of centrosome movements during early embryogenesis in Caenorhabditis elegans. *Genetics* **156**, 1649-1660.
Miller, K. G., Emerson, M. D., McManus, J. R. and Rand, J. B. (2000). RIC-8 (Synembryn): a novel conserved protein that is required for G(q)α signaling in the *C. elegans* nervous system. *Neuron* **27**, 289-299.
Page, R. D. (1996). TreeView: an application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* **12**, 357-358.
Rappaport, R. (1971). Cytokinesis in animal cells. *Int. Rev. Cytol.* **31**, 169-213.
Robatzek, M. and Thomas, J. H. (2000). Calcium/calmodulin-dependent protein kinase II regulates *Caenorhabditis elegans* locomotion in concert with a G(o)/G(q) signaling network. *Genetics* **156**, 1069-1082.
Schaefer, M., Petronczki, M., Dorner, D., Forte, M. and Knoblich, J. A. (2001). Heterotrimeric G proteins direct two modes of asymmetric cell division in the *Drosophila* nervous system. *Cell* **107**, 183-194.
Schneider, S. Q. and Bowerman, B. (2003). Cell polarity and the cytoskeleton in the *Caenorhabditis elegans* zygote. *Annu. Rev. Genet.* **37**, 221-249.
Srinivasan, D. G., Fisk, R. M., Xu, H. and van den Heuvel, S. (2003). A complex of LIN-5 and GPR proteins regulates G protein signaling and spindle function in *C. elegans*. *Genes Dev.* **17**, 1225-1239.
Tall, G. G., Krumins, A. M. and Gilman, A. G. (2003). Mammalian Ric-8A (synembrin) is a heterotrimeric Gα protein guanine nucleotide exchange factor. *J. Biol. Chem.* **278**, 8356-8362.
Tsou, M. F., Hayashi, A. and Rose, L. S. (2003). LET-99 opposes Gα/GPR signaling to generate asymmetry for spindle positioning in response to PAR and MES-1/SRC-1 signaling. *Development* **130**, 5717-5730.
Wedegaertner, P. B. (1998). Lipid modifications and membrane targeting of Gα. *Biol. Signal. Recept.* **7**, 125-135.
Wedegaertner, P. B. and Bourne, H. R. (1994). Activation and depalmitoylation of $G_s\alpha$. *Cell* **77**, 1063-1070.
Willard, F. S. and Siderovski, D. P. (2004). Purification and in vitro functional analysis of the *Arabidopsis thaliana* regulator of G-protein signaling-1. *Methods Enzymol.* **389**, 320-338.
Willard, F. S., Kimple, R. J. and Siderovski, D. P. (2004). Return of the GDI: the GoLoco motif in cell division. *Annu. Rev. Biochem.* **73**, 925-951.
Yu, F., Cai, Y., Kaushik, R., Yang, X. and Chia, W. (2003). Distinct roles of $G\alpha_i$ and Gβ13F subunits of the heterotrimeric G protein complex in the mediation of *Drosophila* neuroblast asymmetric divisions. *J. Cell Biol.* **162**, 623-633.
Zwaal, R. R., Ahringer, J., van Luenen, H. G., Rushforth, A., Anderson, P. and Plasterk, R. H. (1996). G proteins are required for spatial orientation of early cell cleavages in *C. elegans* embryos. *Cell* **86**, 619-629.

Table S1. Spindle severing experiments

Genotype	*n*	Anterior (μm/second)	Posterior (μm/second)	Student's *t*-test versus *gpb-1(RNAi)* (Anterior)	Student's *t*-test versus *gpb-1(RNAi)* (Posterior)
Wild type	13	0.64±0.07*,†	1.03±0.08*,†	*P*<0.02	*P*<0.2
goa-1/gpa16(RNAi)	15	0.21±0.04‡	0.22±0.03‡	*P*<0.001‡	*P*<0.0011‡
goa-1(sa734)	12	0.60±0.08*	0.73±0.09*	*P*<0.001	*P*<0.02
goa-1(RNAi)	8	0.50±0.09	0.80±0.12	*P*<0.001	*P*<0.05
gpa-16(it143)	10	0.58±0.07	0.65±0.09	*P*<0.001	*P*<0.02
gpa-16(RNAi)	15	0.6±0.07*	0.65±0.07*	*P*<0.001	*P*<0.001
gpb-1(RNAi)	11	0.96±0.08*	0.97±0.23*,§	–	–
goa-1(sa734) gpb-1(RNAi)	11	0.95±0.087	0.94±0.11§	*P*>0.2	*P*>0.2
goa-1(RNAi) gpb-1(RNAi)	10	0.94±0.07	0.90±0.1§	*P*>0.2	*P*>0.2
gpa-16(it143) gpb-1(RNAi)	10	1.01±0.18	1.1±0.12§	*P*>0.2	*P*>0.2
gpa-16(RNAi) gpb-1(RNAi)	11	0.95±0.13	0.9±0.13§	*P*>0.2	*P*>0.2
goa-1(sa732) gpa-16(RNAi) gpb-1(RNAi)	9	0.18±0.04	0.22±0.07	*P*<0.001	*P*<0.001

Average peak velocities±s.d. were estimated as described in the Materials and methods for the anterior and posterior spindle poles.

*Afshar et al., 2004.

†The spindle of wild-type embryos (*n*=9) were severed in this series of experiments, and the values obtained were statistically indistinguishable from those reported in Afshar et al. (*P*>0.2).

‡Colombo et al., 2003.

§The extent of the net pulling forces on the posterior spindle pole are probably underestimated as the mitotic spindle sets up more toward the posterior (see Movies 1-7); as a result, peak velocities on the posterior spindle pole probably cannot be fully developed.