Locomotion defects, together with Pins, regulates heterotrimeric G-protein signaling during *Drosophila* neuroblast asymmetric divisions

Fengwei Yu,[1,4] Hongyan Wang,[1] Hongliang Qian,[1] Rachna Kaushik,[2] Mary Bownes,[3] Xiaohang Yang,[2] and William Chia[1,5]

[1]Temasek Lifesciences Laboratory and Department of Biological Sciences, National University of Singapore, Singapore 117604; [2]Institute of Molecular and Cell Biology, Proteos, Singapore 138673; [3]Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh, EH9 3JR United Kingdom

Heterotrimeric G proteins mediate asymmetric division of *Drosophila* neuroblasts. Free Gβγ appears to be crucial for the generation of an asymmetric mitotic spindle and consequently daughter cells of distinct size. However, how Gβγ is released from the inactive heterotrimer remains unclear. Here we show that Locomotion defects (Loco) interacts and colocalizes with Gαi and, through its GoLoco motif, acts as a guanine nucleotide dissociation inhibitor (GDI) for Gαi. Simultaneous removal of the two GoLoco motif proteins, Loco and Pins, results in defects that are essentially indistinguishable from those observed in *Gβ13F* or *Gγ1* mutants, suggesting that Loco and Pins act synergistically to release free Gβγ in neuroblasts. Furthermore, the RGS domain of Loco can also accelerate the GTPase activity of Gαi to regulate the equilibrium between the GDP- and the GTP-bound forms of Gαi. Thus, Loco can potentially regulate heterotrimeric G-protein signaling via two distinct modes of action during *Drosophila* neuroblast asymmetric divisions.

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Asymmetric cell division is a universal mechanism used to generate cellular diversity during development. The *Drosophila* embryonic central nervous system (CNS) derives largely from neural progenitors called neuroblasts (NBs). NBs delaminate from the neuroectoderm and undergo asymmetric cell division along the apical/basal axis to give rise to two daughters of distinct fate and size. The larger apical daughter cell retains a NB identity and undergoes repeated asymmetric divisions, whereas the smaller basal daughter differentiates into a ganglion mother cell (GMC) that divides only once to generate two neurons/glia (Campos-Ortega 1997). Three well-characterized features of the NB asymmetric divisions (Jan and Jan 2001; Knoblich 2001; Wodarz and Huttner 2003) are (1) asymmetric localization and segregation of cell fate determinants and their adaptor proteins Numb/Partner of Numb (Pon), Prospero (Pros)/Miranda (Mira) into the basal GMC; (2) reorientation of the mitotic spindle along the apical/basal axis at metaphase; (3) generation of an apically biased asymmetric mitotic spindle (Kaltschmidt et al. 2000; Kaltschmidt and Brand 2002) and the displacement of the spindle toward the basal cortex during ana/telophase as well as asymmetric formation of astral microtubules (MTs) (Giansanti et al. 2001), which lead to the generation of two unequal-sized daughter cells.

These features of the NB asymmetric division are controlled by an apically localized complex of proteins that include the *Drosophila* homologs (Doe and Bowerman 2001) of the conserved Par3 (Bazooka, Baz)/Par6 (DmPar6)/aPKC (DaPKC) protein cassette first identified in *Caenorhabditis elegans* (Kemphues 2000), the novel protein Inscuteable (Insc), Gαi, a subunit of heterotrimeric G proteins (Schaefer et al. 2001; Yu et al. 2003), and an evolutionarily conserved molecule, Partner of Insc (Pins) (Parmentier et al. 2000; Schaefer et al. 2000; Yu et al. 2000) that acts as a guanine nucleotide dissociation inhibitor (GDI) for Gαi. Loss of single members of the apical complex, such as *baz* or *pins*, results in defective basal protein localization and spindle misorientation in mitotic NBs up to metaphase, although these defects can be partially corrected late in mitosis, a phenomenon called telophase rescue (Schober et al. 1999; Peng et al. 2000). However, unlike basal protein localization and

Corresponding authors.
[4]E-MAIL fengwei@tll.org.sg; FAX 65-6-872-7007.
[5]E-MAIL wchia@tll.org.sg; FAX 65-6-872-7007.
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spindle orientation, the generation of an asymmetric spindle and its displacement toward the basal cortex are largely unaffected, and NBs lacking one component of the apical complex usually divide like wild-type NBs to produce two unequal-sized daughter cells. Simultaneous disruption of the two redundant apical pathways, Baz/DaPKC and Pins/Gαi, prevents the formation of an asymmetric spindle, and two daughter cells of similar size are produced (Cai et al. 2003).

Heterotrimeric G proteins have been shown to be involved in controlling distinct microtubule-dependent processes in one-cell embryos of *C. elegans* (Gotta and Ahringer 2001). Gβγ is important for correct centrosome migration around the nucleus and spindle orientation, while Gα subunits, GOA-1 and GPA-16, are required for asymmetric spindle positioning. Recent studies have shown that the GoLoco-motif-containing proteins, GPR1/2, act as GDIs for GOA-1 and GPA-16 to translate polarity cues, mediated by the asymmetrically localized Par proteins, into asymmetric spindle positioning in the *C. elegans* zygote (Colombo et al. 2003; Gotta et al. 2003; Srinivasan et al. 2003). In *Drosophila* NBs, heterotrimeric G proteins Gβ13F and Gγ1 are required for the asymmetric localization/stability of the apical components and, hence, the formation of an asymmetric spindle. This is likely to be achieved through the generation of free Gβγ since depletion of Gβγ function by overexpression of wild-type Gαi/Gαo (Schaefer et al. 2001; Yu et al. 2003) or loss of *Gβ13F* or *Gγ1* function (Fuse et al. 2003; Izumi et al. 2004) can lead to the generation of a symmetric and centrally placed mitotic spindle, and NBs frequently divide to produce daughter cells of similar size (henceforth referred to as "similar-sized divisions,", defined below). Thus, generation of free Gβγ is crucial for NB asymmetric divisions. However, it is not clear whether Gβγ mediates spindle geometry independently of the Gα subunit(s) or alternatively by controlling the localization of Gα subunit(s) and/or the GoLoco proteins. Pins has previously been shown to act as a GDI to facilitate the dissociation of Gβγ from heterotrimers by binding to and stabilizing the GDP-bound form of Gαi (GDP-Gαi) (Schaefer et al. 2001). However, paradoxically, loss of *pins* function does not produce the severe spindle defects seen in the *Gβ13F* or *Gγ1* mutant NBs, suggesting that the absence of the Pins GDI activity does not prevent the generation of free Gβγ. Similarly, loss of *Gαi*, while causing defects in spindle orientation and the localization of the basal proteins up to metaphase, like *pins* loss of function, also does not cause the severe spindle asymmetry defects seen in *Gβ13F* or *Gγ1* mutant NBs; however, it remains possible that additional Gα subunits may be involved in this process.

Here we show that *locomotion defects* (*loco*), a gene previously shown to be required for glial cell differentiation and dorsal–ventral patterning (Granderath et al. 1999; Pathirana et al. 2001), encodes a novel component of the NB apical complex that exhibits both guanine nucleotide dissociation inhibitor (GDI) and GTPase-activating protein (GAP) activities for Gαi. Loco interacts with GDP-Gαi through its GoLoco motif (Siderovski et al. 1999) and forms a complex with Gαi in vivo. Loco colocalizes with Gαi and Pins at the apical cortex of NBs throughout mitosis and is required for the asymmetric localization/stabilization of Pins/Gαi. Analyses of various double-mutant NBs suggest that Loco, like Pins and Gαi, functions redundantly with the Baz/DaPKC pathway in regulating spindle geometry. Interestingly, loss of both *loco* and *pins* functions leads to similar-sized divisions in the majority of NBs, similar to that seen in either *Gβ13F* or *Gγ1* mutants, suggesting that activation of Gβγ is mediated in a redundant manner by both Loco and Pins. Our data therefore provide functional support for the idea that the activation of heterotrimeric G-protein signaling through the generation of free Gβγ, crucial for NB asymmetric divisions, can occur via a receptor-independent mechanism by using multiple GDIs that functionally overlap. Moreover, we show that Loco can, through its RGS domain (De Vries and Gist Farquhar 1999), also function as a GAP to regulate the balance between GDP-Gαi and GTP-Gαi. Hence, both the GDI and GAP functions of Loco are important for NBs to regulate the activities of Gαi and Gβγ.

Results

Loco, a GoLoco motif protein, interacts with GDP-Gαi and can function as a GDI

In *Drosophila* NBs, the activation of heterotrimeric G-protein signaling can in principle occur via a receptor-independent mechanism through the release of Gβγ from the inactive heterotrimer GDP-GαiGβγ, which is facilitated by the binding of Pins as a GDI to GDP-Gαi (Schaefer et al. 2001). The GoLoco motif of Pins should therefore play a critical role through its GDI function to complex with GDP-Gαi and generate free Gβγ. However, previous studies have shown that inactivation of Gβγ by either loss of function of *Gβ13F* or *Gγ1* or overexpression of wild-type Gαi/Gαo leads to delocalization/destabilization of both apical pathway components and the generation of similar-sized daughter cells in the majority of telophase NBs, whereas loss of *pins* function has relatively mild effects, for example, producing similar-sized daughters (defined as telophase NBs from stage 10 embryos in which the ratio of the GMC/NB diameter is $\geq$0.8; for wild-type NBs, GMC/NB = 0.43 ± 0.08) from only a small proportion of NB divisions (15%) (Cai et al. 2003; Fuse et al. 2003; Yu et al. 2003; Izumi et al. 2004). We reasoned that if a GDI-mediated receptor-independent mechanism were to be responsible for G-protein activation in NBs, then other unidentified GDI(s) must exist that can activate Gβγ activity even in the absence of *pins* function. We therefore searched the annotated *Drosophila* genome and identified only three GoLoco-motif-containing proteins, namely, Pins, Loco, and RapGAP2. Further analysis indicated that while RapGAP2 appears not to be expressed in NBs (R. Kaushik, unpubl.), Loco plays a key role and is asymmetrically localized in mitotic NBs.

There exist at least four alternatively spliced forms of Loco protein that all include a common core region containing a RGS domain, two Ras-like Raf-binding domains (RBDs), and a GoLoco motif (Fig. 3A, below). Database searches further revealed that two homologs of *Drosophila* Loco, RGS12 and RGS14, exist in vertebrates (Kimple et al. 2001), suggesting that *loco* is duplicated in vertebrates during evolution. We have confirmed a previously reported (Granderath et al. 1999) interaction between Gαi and the GoLoco motif of Loco in yeast two-hybrid assays. We further observed that GαiQ205L, a presumably constitutively active (GTP-bound) form, fails to interact with the GoLoco motif of Loco in yeast two-hybrid assays, suggesting that the GoLoco motif of Loco preferentially binds to GDP-Gαi (Fig. 1A). These observations were further confirmed using GST pull-down assays. ^{35}S-labeled Gαi can interact with GST-GoLoco but not with GST alone, whereas ^{35}S-labeled GαiQ205L cannot interact with GST alone and interacts very poorly with GST-GoLoco (Fig. 1B).

To show that the physical interaction between Gαi and Loco reflects an in vivo interaction, we made use of a transgenic fly strain that can be induced by heat shock to express Loco-C2 fused with two tandem Flag epitopes at its C terminus. Loco-Flag, when induced at low levels, colocalizes with Pins and Gαi as apical cortical crescents in NBs (data not shown; see also Fig. 2A–D). In coimmunoprecipitation (CoIP) experiments, when the immunocomplex was precipitated using anti-Gαi antibody (Schaefer et al. 2001), Loco-Flag can be detected by an anti-Flag antibody, only from HS but not non-HS embryonic extracts; endogenous Pins, detected using an anti-Pins antibody (Yu et al. 2002), CoIPs with Gαi from both HS and non-HS embryonic extracts (Fig. 1C). Although Gαi can CoIP both Loco and Pins, Loco-Flag can CoIP only Gαi but not Pins from HS embryonic extracts (Fig. 1D), suggesting that Loco and Pins do not simultaneously complex with the same Gαi molecule. To test whether the GoLoco motifs of Loco and Pins can act as GDIs, we carried out in vitro GDI assays. The GoLoco motifs of Loco and Pins decrease the rate of exchange of GDP for GTP on Gαi (Fig. 1E), indicating that both Pins and Loco can act as GDIs for Gαi.

Loco colocalizes with and depends on Pins and Gαi for its apical localization

To ascertain the subcellular localization of Loco, we generated anti-Loco antibodies against two regions of the core domain shared by all Loco isoforms (amino acids 357–636 and 564–731 of Loco-C1). These two antibodies were found to be specific for Loco since identical immunofluorescence signals were seen in wild-type embryos and these signals were absent in embryos depleted for both maternal and zygotic *loco* (Fig. 3P). Loco localizes as a crescent to the apical cortex as early as late interphase (Fig. 2A′). From prophase onward, Loco forms an apical crescent and segregates into the apical daughter cell at telophase (Fig. 2B′–D′), colocalizing with Pins (Fig. 2A–D) and Gαi (Fig. 2E,E′) in mitotic NBs.

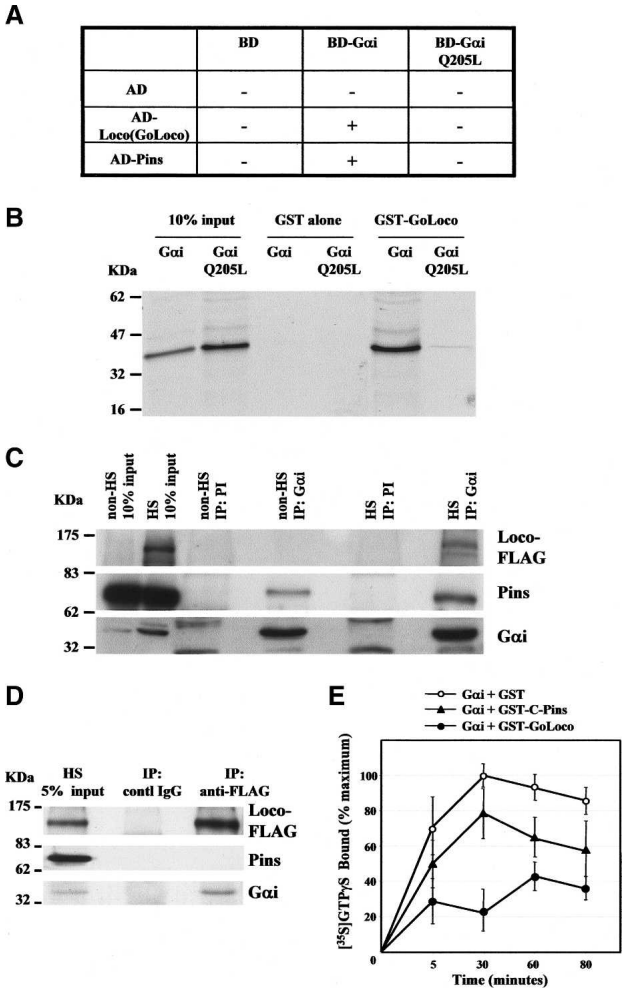


Figure 1. Loco complexes with Gαi through a direct interaction and can function as a GDI for Gαi. (*A*) The GoLoco motif of Loco interacts with wild-type Gαi but not GαiQ205L, the GTP-bound form of Gαi, in yeast two-hybrid assays. (+) Positive interaction; (–) lack of interaction. (*B*) In GST pull-down assays, GST-GoLoco can pull down wild-type Gαi but not GαiQ205L. (*C*,*D*) Loco and Pins can both complex with Gαi, but not with the same Gαi molecule simultaneously. Heat-shocked (HS) or non-HS embryos were collected from transgenic flies carrying *hs-loco-c2*. CoIP experiments were carried out using preimmune serum (PI) or anti-Gαi antibody with either non-HS or HS embryo extracts. The immunocomplexes were blotted with anti-Flag, anti-Pins, and anti-Gαi antibodies, respectively. (*C*) Anti-Gαi can pull down both Pins and Loco in vivo. (*D*) Loco and Pins cannot be found in the same protein complex. The immunoprecipitation was carried out by using either a control IgG or anti-Flag antibody in HS embryo extracts. Loco-Flag can only pull down Gαi but not Pins. (*E*) Both Loco and Pins possess GDI activity toward Gαi. GDI assays were carried out by measuring the rate of [^{35}S(GTPγS binding by Gαi in time-course experiments in the presence of GST alone, GST-C-Pins (amino acids 378–658), or GST-GoLoco (amino acids 564–731 of Loco-C1).

To test whether asymmetric localization of Loco is dependent on other key players for NB asymmetric divisions, we examined Loco distribution in various mutants including *insc* as well as *pins*, *Gαi*, *baz*, and *Gβ13F* (for

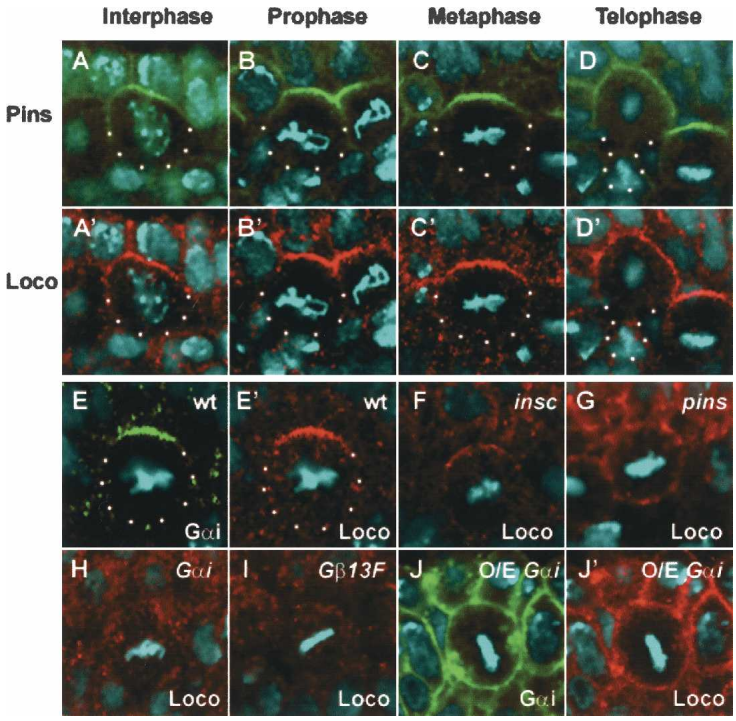


Figure 2. Loco colocalizes with Pins and Gαi at the apical cortex in wild-type NBs, and its asymmetric localization requires *pins*, *Gαi*, and *Gβ13F*. Pins (*A–D*, green) and Loco (*A′–D′*, red) colocalize at the apical cortex in NBs from late interphase to telophase. Loco (*E′*, red) also colocalizes with Gαi (*E*, green) during mitosis. In *insc* mutants, Loco can be observed as apical crescents with reduced intensity (*F*), while in *pins* mutants, apical localization of Loco is disrupted and Loco is uniformly distributed around the cortex (*G*). Loco is cytoplasmic in NBs lacking *Gαi* (*H*) or *Gβ13F* GLCs (*I*). Overexpressed Gαi is distributed uniformly around the cortex (*J*, green) and causes cortical localization of Loco (*J′*, red). DNA is in cyan. Apical is up.

which both maternal and zygotic components were removed). In *insc* NBs, Loco was observed as an apical crescent of reduced intensity (75%, $n = 48$) (Fig. 2F) or is undetectable (25%, $n = 48$) (data not shown). Similar results were seen in *baz* NBs (data not shown). Loco is uniformly distributed around the cortex in *pins* metaphase NBs (100%, $n = 20$) (Fig. 2G); while in *Gαi* NBs, Loco is unable to be localized to the cortex and shows cytosolic localization (100%, $n = 29$) (Fig. 2H). Similar to that seen in *Gαi* NBs, Loco is distributed in the cytosol with no obvious cortical signal in *Gβ13F* NBs (100%, $n = 32$) (Fig. 2I). When wild-type Gαi is overexpressed, Loco (Fig. 2J′) as well as Gαi (Fig. 2J) and Pins (data not shown) become uniformly distributed around the cell cortex (100%, $n = 20$). When Insc is overexpressed in epithelial cells, Loco is recruited from the basolateral to the apical cortex (data not shown), similar to Pins (Yu et al. 2000).

Taken together, these data indicate that Loco is a novel component of the apical complex and its asymmetric localization/stability requires other apical components as well as Gβ13F; its cortical localization requires Gαi, and its apical localization requires Pins.

Loco is required for asymmetric localization of Gαi and Pins and acts in parallel with the Baz/DaPKC pathway to mediate asymmetric daughter cell size

Given that no embryos could be obtained from germline clones (GLCs) using previously described loss-of-function alleles of *loco* and analyses of zygotic loss-of-function embryos revealed no obvious defects in NB asymmetric division, we carried out imprecise excisions using a P-element, EY04589, which is inserted 310 bp upstream of the start point of *loco-c1* transcription (Bellen et al. 2004); three new alleles, $loco^{P452}$, $loco^{P283}$, and $loco^{P237}$, were isolated that delete either partially or entirely the core region of the *loco* protein isoforms (Fig. 3A). The detailed molecular lesions associated with these alleles are given in Materials and Methods. These alleles do not show zygotic loss-of-function defects for NB divisions. Both $loco^{P283}$ and $loco^{P452}$ homozygotes are viable and display severe locomotion defects, similar to homozygotes of *Gαi* and *pins* null mutants, suggesting that they may share similar function. To obtain *loco* mutant embryos that lack both maternal and zygotic components, we crossed mutant mothers homozygous for the alleles $loco^{P283}$ or $loco^{P452}$ or *trans*-heterozygous for the alleles $loco^{P283}$ and $loco^{P237}$ to heterozygous $loco^{P283}$, $loco^{P452}$, or $loco^{P237}$ males. Immunofluorescence confirmed that those resultant embryos are antigen-minus (Fig. 3P), suggesting that both $loco^{P283}$ and $loco^{P452}$ are strong, possibly null alleles. Embryos derived from either $loco^{P283}/loco^{P283}$ or $loco^{P283}/loco^{P237}$ mothers display indistinguishable phenotypes in NB asymmetric divisions, suggesting that $loco^{P283}$ is an amorphic allele. We henceforth refer to $loco^{P283}$ embryos lacking both maternal and zygotic components as *loco* mutants. In this study all phenotypic analyses described for single- and double-mutant combinations were performed using embryos lacking both maternal and zygotic components.

In the majority of *loco* mutant NBs, Pins is no longer apical but rather shows uniform cortical distribution with some cytosolic signal (90%, $n = 90$) (Fig. 3B–E). Occasionally, weak crescents of Pins were observed in in-

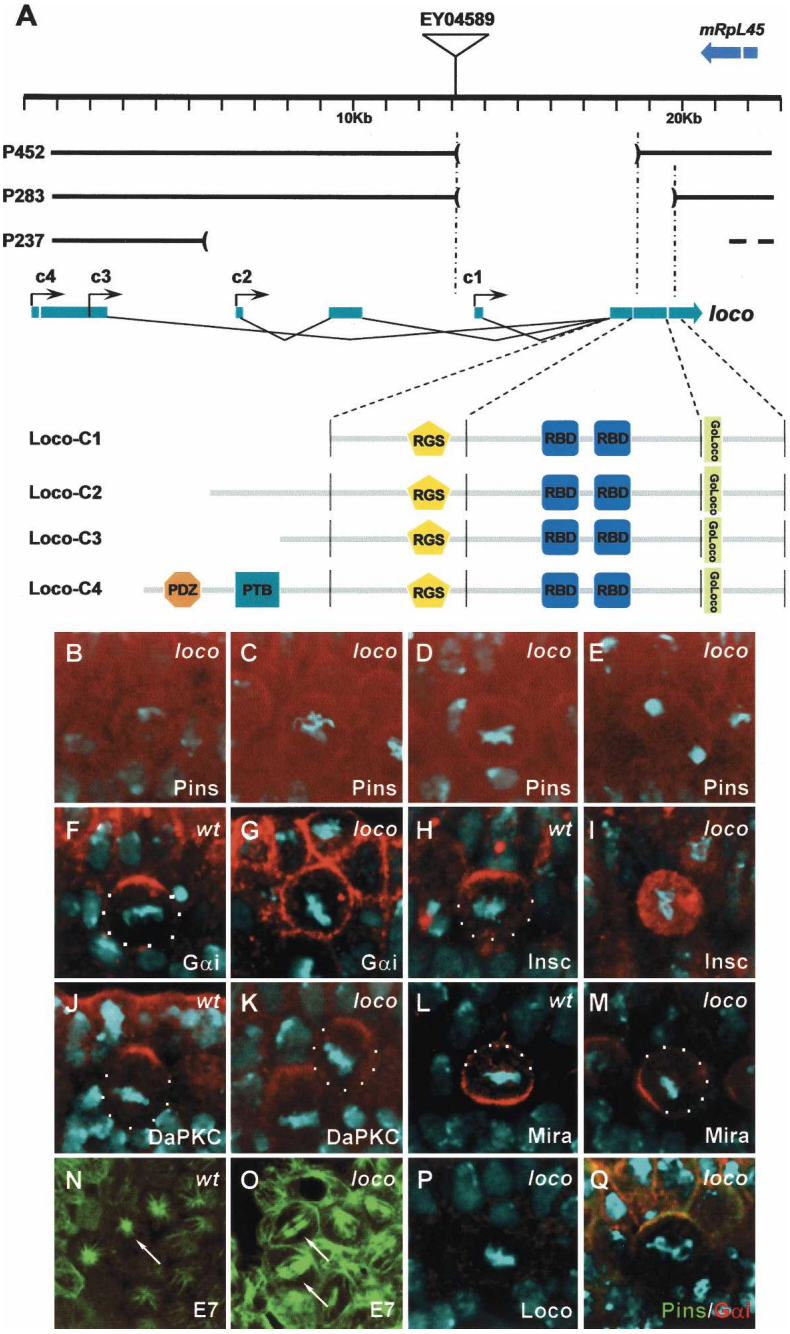


Figure 3. Loco is required for NB asymmetric divisions. (*A*) Schematic representation of four alternatively spliced forms of Loco and three *loco* alleles used for this study. The extent of each deletion is indicated by the parentheses. (*B–K*) Loco is required for proper localization of other apical proteins. Pins (red) is distributed around the cell cortex and in the cytosol from interphase to telophase in *loco* mutants (*B–E*). Gαi (red), which is normally localized at the apical cortex in wild-type NBs (*F*), is distributed uniformly around the cortex in *loco* mutants (*G*). In 12% of interphase/prophase *loco* mutant NBs, apical Pins (green) and Gαi (red) crescents could be observed with weak intensity (*Q*, showing a prophase NB). Insc (red) is apically localized in wild-type NBs (*H*), while Insc (red) is cytosolic in *loco* mutants (*I*). Compared with that seen in wild-type NBs (*J*), DaPKC (red) remains apical in the majority of NBs, although its intensity is drastically reduced in *loco* mutants (*K*). Mira (red), basally localized in wild-type NBs (*L*), can be mislocalized in *loco* NBs at metaphase (*M*). (*N,O*) Spindle reorientation in cells of mitotic domain 9 is defective in *loco* embryos, as indicated by anti-α-tubulin staining (green). The spindle axis of wild-type domain 9 cells is perpendicular to the surface (*N*), while in *loco* mutants it is often aligned parallel to the surface (*O*). No Loco protein can be detected in *loco* mutant embryos (*P*). DNA is in cyan. Apical is up in panels *B–M*.

terphase/prophase NBs (12%, $n = 43$), where Pins colocalizes with Gαi (Fig. 3Q). When detected using a specific antibody raised against full-length Gαi (see Materials and Methods), Gαi shows uniform cortical localization in both *pins* (100%, $n = 19$) (Schaefer et al. 2001; data not shown) and *loco* mutant metaphase NBs (100%, $n = 25$) (Fig. 3G); Insc is cytoplasmic (67%, $n = 45$) (Fig. 3I); DaPKC (86%, $n = 50$) (Fig. 3K) and Baz (data not shown) remain asymmetrically localized in the majority of *loco* mutant NBs, although the intensity of the crescents was dramatically reduced, a phenotype also seen in NBs lacking *pins*, *Gαi*, or *Gβ13F* function. Similar to that seen in *pins* or *Gαi* mutants, in *loco* mutants the basal proteins Mira/Pros and Pon/Numb can be mislocalized relative to the overlying ectoderm at metaphase (52%, $n = 21$) (Fig. 3M; data not shown). Gβ13F remains uniformly cortical, similar to that seen in wild-type NBs (data not shown). Mitotic spindle orientation is also disturbed in *loco* mutants; in cells of mitotic domain 9, mitotic spindle that normally rotates by 90° to align along the apical/basal axis in wild type (Fig. 3N) often fails to reorientate (Fig. 3O).

Wild-type NBs normally divide to give rise to a large apical NB and a smaller basal GMC (Fig. 4A,E). The great

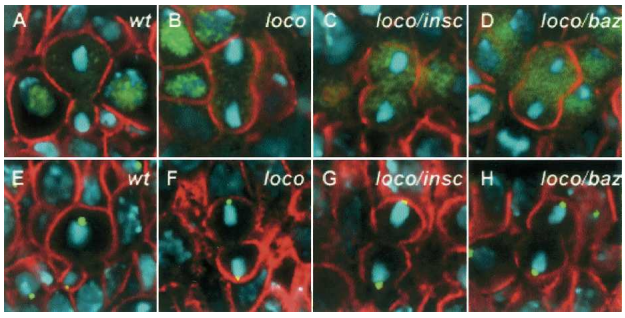


Figure 4. Loco acts redundantly with the Baz/DaPKC/DmPar-6/Insc pathway to regulate spindle displacement and asymmetry, as well as daughter cell size difference. In wild-type telophase NBs (*A*,*E*), the mitotic spindle (deduced from positions of the centrosomes) (*E*) is apically biased and spindle displacement occurs toward the basal cortex to give rise to two daughter cells of unequal size. In *loco* mutants (*B*,*F*), 10% of telophase NBs generate two daughter cells of similar size. *loco/insc* double-mutant NBs (*C*,*G*) show similar-sized divisions in all telophase NBs (100%, see text). (*D*,*H*) Similarly, removal of *baz* function in *loco* NBs increases the frequency of similar-sized divisions to full expressivity. (*F–H*) In similar-sized divisions, the mitotic spindle is symmetric and both centrosomes lie in close vicinity of the cell cortex. NBs were marked by Asense, which is cytosolic green in *A–D*; BP106, a plasma membrane marker, in red (*A–H*); and CNN, a centrosome marker in green in *E–H*. DNA is in cyan. Apical is up.

majority of *loco* mutant NBs divide asymmetrically to produce daughters of different size like wild-type NBs (data not shown). However, similar to *pins* or *Gαi* mutants, a small proportion of *loco* mutant NBs undergo similar-sized division (10%, $n = 69$) (Fig. 4B,F). Previous studies have suggested that two redundant pathways, the Pins/Gαi and the Baz/DaPKC/(DmPar6/Insc) pathways, act redundantly to control daughter cell size difference (Cai et al. 2003). We analyzed the relative size of the two daughter cells in double mutants of *loco/insc* or *loco/baz RNAi*. In all dividing NBs, similar-sized divisions were observed in *loco/insc* (100%, $n = 42$) (Fig. 4C,G) and *loco/baz RNAi* (97%, $n = 31$) (Fig. 4D,H) double mutants. In addition, spindle displacement and asymmetry are both disrupted in these double mutants, as revealed by anti-centrosomin (CNN) staining (Fig. 4F–H).

Taken together, *loco* loss of function displays defects similar to those seen in *pins* or *Gαi* mutants, and Loco acts redundantly with the Baz/DaPKC pathway to regulate spindle displacement and asymmetry, as well as daughter cell size difference.

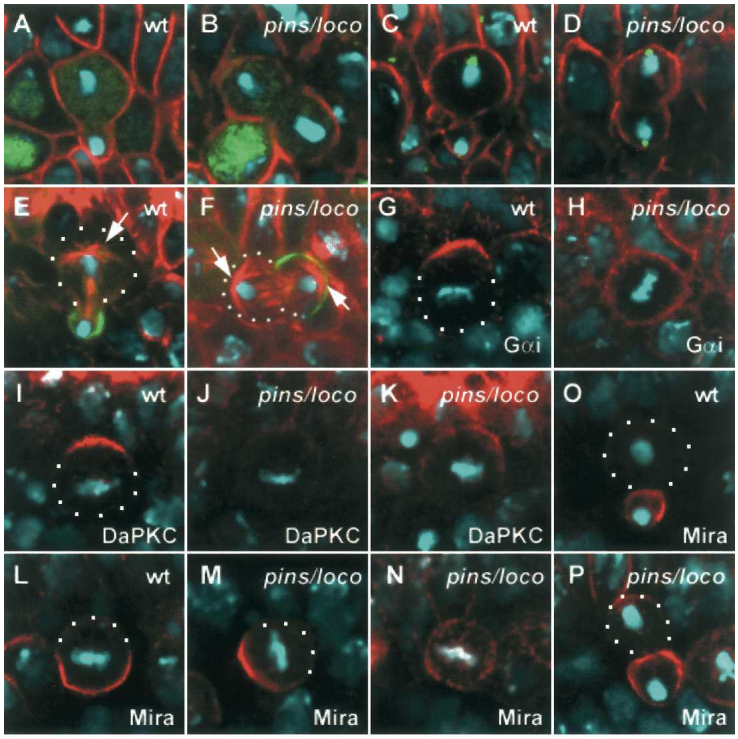
Loco acts to activate Gβγ activity in conjunction with Pins

Given that the frequency of similar-sized divisions in *pins* mutants is much lower than that observed in GLCs of either *Gβ13F* or *Gγ1* (Cai et al. 2003; Fuse et al. 2003; Izumi et al. 2004), we hypothesized the existence of an additional molecule with GDI activity that could activate Gβγ signaling in the absence of Pins. Loco is an obvious candidate for this role, given its function as a GDI for Gαi and its role in NB division. To test our hypothesis, we generated embryos derived from double GLCs of *loco* and *pins* and compared their phenotypes with those of *Gβ13F* GLCs. In double GLCs of *pins* and *loco*, the majority of NBs undergo symmetric divisions to generate two similar-sized daughter cells in stage 10 mutant embryos (60%, $n = 73$) (Fig. 5B); the cleavage plane is placed near the middle of the two centrosomes and the spindle is positioned symmetrically with both centrosomes lying in close proximity to the cell cortex (Fig. 5D), as revealed by anti-Centrosomin (CNN) staining, suggesting that spindle displacement and asymmetry are frequently disrupted in telophase NBs in the absence of both *pins* and *loco*. Astral microtubules, which are normally associated only with the apical centrosome in wild-type NBs (Fig. 5E), can emanate from both centrosomes in *loco/pins* double mutant NBs (Fig. 5F). These defects are strikingly similar to those observed in *Gβ13F* mutants (Fuse et al. 2003; Yu et al. 2003), suggesting that free Gβγ might be depleted by excessive GDP-Gαi around the NB cortex when both GDIs are removed simultaneously. Consistent with this, in double GLCs of *pins* and *loco,* Gαi shows uniform cortical localization in mitotic NBs (100%, $n = 30$) (Fig. 5H, cf. wild type in G), colocalizing with Gβ13F (data not shown). In *loco/pins* double-mutant NBs, DaPKC is either nearly undetectable in most NBs (71%, $n = 31$) (Fig. 5J) or shows some degree of asymmetric localization on the cell cortex in NBs when it is detectable (Fig. 5K), similar to that seen in *Gβ13F* mutants (Fuse et al. 2003; Yu et al. 2003). Miranda is mislocalized (Fig. 5M) or delocalized (Fig. 5N) in a minority of metaphase NBs (40%, $n = 40$), but nevertheless segregates exclusively to one of the daughter cells during telophase in the great majority of *loco/pins* mutant NBs (Fig. 5P), suggesting that, similar to *Gβ13F* NBs, the Baz/DaPKC function is not totally lost in *loco/pins* mutant NBs.

These data indicate that maternal and zygotic depletion of both *loco* and *pins* produce phenotypes that share all of the features seen in the loss of Gβγ function. A detailed quantitation of the relative sizes of the NB daughters for various mutants further supports this view (Fig. 5Q). Our data suggest that Loco and Pins have overlapping functions as GDIs to release free Gβγ, which, in turn, initiates downstream signaling.

Ectopic expression of Loco can drive Pins off the apical cortex

To ascertain the effects of overexpressing Loco on NB asymmetric divisions, we expressed the Loco-C1 isoform under the control of a strong maternal driver, mata-*gal4* VP16 V32. Under these conditions, anti-Loco immunofluorescence in NBs appears more intense than in wild type (Fig. 6C); two types of Loco distribution were observed, uniformly cortical (25%, $n = 64$) (Fig. 6A) or apically enriched (75%, $n = 64$) (Fig. 6B). In either case, Loco colocalizes with Gαi in mitotic NBs (Fig. 6A′,B′). Strikingly, ectopic expression of Loco leads to cytoplasmic



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Genotype	GMC (μm)	NB (μm)	GMC/NB
wild type (n=65)	4.1 ± 0.6	9.6 ± 1.1	43%
pins (n=42)	4.5 ± 0.6	9.4 ± 1.2	48%
loco (n=38)	4.4 ± 0.7	9.8 ± 1.0	45%
pins/loco (n=73)	5.7 ± 0.9	7.3 ± 1.1	78%
Gβ13F (n=30)	5.6 ± 0.8	7.0 ± 1.2	80%

Figure 5. Loco acts to activate Gβγ activity in conjunction with Pins. (*A–F*) Confocal images of triple-labeled telophase NBs (BP106, a membrane marker, red [*A–D*]; DNA, cyan [*A–F*]; Asense, a NB marker, cytosolic green [*A,B*]; CNN, a centrosome marker, green [*C,D*]; α-tubulin, red [*E,F*]; and Miranda, green [*E,F*]) showing unequal size divisions in wild-type (*A,C,E*) and similar-sized divisions in *loco/pins* double mutants (*B,D,F*). NBs from *pins/loco* double GLC embryos show high frequencies of similar-sized divisions (*B*) (63%, see text) in which the mitotic spindle is symmetric, as judged from CNN staining (cf. wild-type [*C*] and double mutants [*D*]). (*E*) In wild-type NBs astral microtubules are associated only with the apical centrosome; they grow out robustly and form a prominent, cap-like structure (arrow). (*F*) However, in *loco/pins* NBs that undergo similar-sized divisions, two astral microtubule caps are formed, one over each centrosome (arrows). Gαi, apical in wild-type NBs (*G*), is cortically localized in *pins/loco* double mutants (*H*). In *loco/pins* double mutants, DaPKC is nearly undetectable in 71% of NBs (*J*) and show weak crescents in the rest of the NBs (*K*), compared with that in wild-type NBs (*I*). Forty percent of *loco/pins* NBs ($n = 40$) show mislocalization (*M*) or cortical localization (*N*) of Mira at metaphase; however, as in wild type (*O*), Mira segregates to one of the daughter cells at telophase (*P*). DNA is in cyan. Apical is up. (*Q*) Quantitation of the daughter cell sizes and their ratios in wild-type and various mutant NBs. *n* is the number of telophase NBs scored. The diameters of GMCs (the relatively smaller cell) and NBs (the relatively large cell) in telophase NBs of stage 10 embryos were measured for each genotype. The data are means ± SD. GMC/NB is the ratio of the diameter of GMC relative to its sibling NB.

distribution of Pins in the great majority of NBs (98%, $n = 46$) (Fig. 6D′), while Insc becomes primarily cytoplasmic (100%, $n = 34$) (Fig. 6E′), although faint cortical crescents can be seen occasionally; DaPKC localizes asymmetrically on the metaphase NB cortex (74%, $n = 34$) (Fig. 6F,F′), but the crescents are broader and less intense compared with wild type; Miranda still asymmetrically localizes and segregates (100%, $n = 20$ of telophase NBs) (data not shown). We have previously shown that Pins cortical localization depends on its association with Gαi (Yu et al. 2002, 2003). The above observations are consistent with the view that excessive levels of Loco can compromise the ability of Pins to localize to the cortex by limiting the availability of GDP-Gαi (see below).

Loco can act as a GAP to regulate the GTPase activity of Gαi through its RGS domain

To determine whether the RGS domain of Loco is able to interact with Gαi and whether this interaction is nucleotide-dependent, bacterially expressed GST or GST-RGS was incubated with in vitro translated ^{35}S-labeled Gαi in the presence of GTPγS, GDP, or GDP + AlF_4^- to mimic the transition state of GTP hydrolysis. While GST-RGS is able to pull down Gαi only to a low extent in the presence of either GDP or GTPγS, the presence of GDP + AlF_4^- strongly promotes the interaction between GST-RGS and Gαi (Fig. 7A, upper panel). These results suggest that the RGS domain of Loco possesses preferential affinity to the transition-state conformation of Gαi during GTP hydrolysis. To ascertain that GST-RGS can interact with endogenous Gαi from embryos, GST-RGS or GST alone was incubated with embryonic extracts. A significant amount of Gαi could be detected by immunoblotting the protein complex bound to GST-RGS, but not in the control (Fig. 7A, lower panel), suggesting that the RGS motif of Loco is likely to interact with Gαi in vivo. Since the RGS domain is able to interact with Gαi, we further carried out GAP assays to test whether the RGS domain can stimulate GTP hydrolysis. In the absence of GST-RGS, Gαi has only weak intrinsic GTPase activity; addition of GST-RGS fusion protein accelerates the GTPase activity of Gαi significantly (Fig. 7B). Taken together, these data indicate that Loco can also act as a GAP for Gαi through its RGS domain, which may, in turn, contribute to the regulation of the balance between GTP-Gαi and GDP-Gαi levels in NBs.

The effects of disturbing the balance of GTP-Gαi and GDP-Gαi on NB asymmetric divisions

To assess the effects of shifting the equilibrium of *Gαi* toward either the GTP- or GDP-bound forms on NB

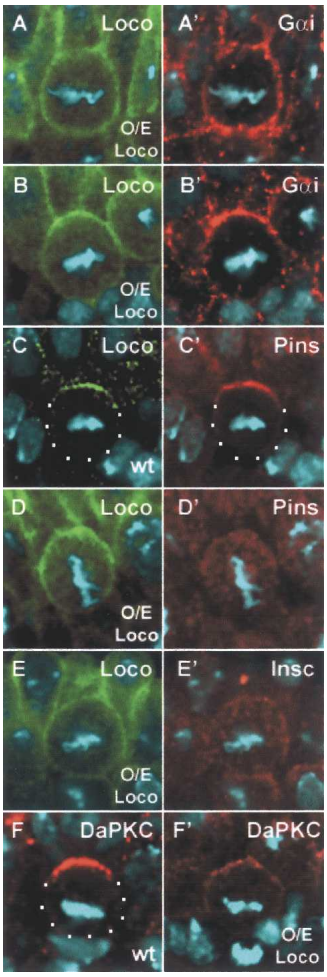

Figure 6. Ectopic expression of Loco leads to a defect in NB asymmetric divisions. Loco (green), when ectopically expressed in NBs, is localized either uniformly around the cell cortex (*A*) or enriched at the apical cortex (*B*). (*A*′,*B*′) In both cases, Gαi (red) colocalizes with ectopically expressed Loco in mitotic NBs. Ectopic Loco (*D*), which shows much stronger intensity than that in wild-type NBs (*C*), leads to delocalization of Pins (*D*′, red). In NBs ectopically expressing Loco (*E*, green), apical localization of Insc is also disrupted (*E*′, red); DaPKC (red) localizes asymmetrically but with reduced intensity (*F*′; wild type, *F*). Note that images were taken at the same gain and processed in parallel. DNA is in cyan. Apical is up.

asymmetric divisions, we overexpressed two mutant versions of Gαi, GαiQ205L and GαiG204A, which represent constitutively GTP-bound and constitutively GDP-bound forms, respectively. Previous studies suggested that overexpression of GαiQ205L perturbs SOP divisions but not NB divisions (Schaefer et al. 2001). We overexpressed GαiQ205L in wild-type NBs using the mata-*gal4* VP16 V32 driver and confirmed that ectopically expressed GαiQ205L is localized primarily around the cell cortex (Fig. 7C; Schaefer et al. 2001). However, interestingly, we observed that whereas Loco remains colocalized with Gαi around the cell cortex in these NBs (Fig. 7D), Pins, which normally forms an intense apical crescent in wild-type control NBs (100%, $n = 42$) (Fig. 7E), is delocalized from the apical cortex (84%, $n = 63$) (Fig. 7F), although a faint apical crescent can be seen occasionally. Similarly, Insc is also delocalized from the apical cortex (87%, $n = 63$) (Fig. 7H, wild-type control), (100% apical, $n = 42$) (Fig. 7G). Mira localization and segregation remain asymmetric in 100% of mitotic NBs ($n = 20$), and 2% of telophase NBs ($n = 60$) divide into similar-sized daughter cells. Delocalization of apical Pins raises the possibility that ectopically expressed GαiQ205L may preferentially bind to endogenous Loco, thereby inhibiting the Loco-mediated hydrolysis of endogenous GTP-Gαi; the effect of this would be delocalization of the Pins/Insc complex at the apical cortex due to a reduction in the levels of GDP-Gαi.

In the above situation, there should still be residual wild-type endogenous GDP-Gαi. To create a more extreme situation, we overexpressed GαiQ205L in a *Gαi* mutant background. Under these conditions, where there should be no GDP-Gαi with all the Gαi in the GTP-bound form, we observed more severe defects in asymmetric protein localization; the low level of Pins that can be detected is cytosolic (Fig. 7J), while Loco (Fig. 7L) and GαiQ205L (Fig. 7I,K) remain uniformly cortically localized. These observations suggest that, in vivo, Pins can associate only with GDP-Gαi; GTP-Gαi in the absence of GDP-Gαi cannot direct Pins to the cell cortex; in contrast to Pins, Loco can be localized to the cortex by either GTP-Gαi or GDP-Gαi (see also the next paragraph). These observations along with the biochemical data support the view that both GTP-Gαi and GDP-Gαi can associate with Loco in vivo, and Loco can act both as a GAP and as a GDI for Gαi. Since Gβγ only binds to GDP-Gαi, in this situation where GTP-Gαi is in excess and GDP-Gαi is absent, Gβγ will remain free and active. Indeed, under these conditions, the ability to generate daughter cell size difference is not adversely affected compared with *Gαi* mutant NBs (data not shown) and Baz localizes asymmetrically (nonuniformly) (80%, $n = 35$ metaphase NBs) but with reduced intensity on the NB cortex (data not shown).

Although the presence of GDP-Gαi is necessary for apical Insc/Pins/Gαi localization, excessive GDP-Gαi will prevent the generation of free Gβγ. For example, in Gαi mutant NBs overexpressing GαiG204A, a constitutively GDP-bound form, GαiG204A (Fig. 7M,O), Loco (Fig. 7N), and Pins (Fig. 7P) are all uniformly cortically localized; the majority of NBs divide to produce two daughter cells of similar size (82%, $n = 57$) (Fig. 7O,P), similar to that seen for *Gβ13F* or *Gγ1* mutant NBs, suggesting a failure to activate G-protein signaling.

These data suggest that the balance between GDP-Gαi and GTP-Gαi is important not only to regulate Gβγ activity but also to asymmetrically localize Insc/Pins/Loco.

Discussion

Previous studies have shown that heterotrimeric G-protein components play important roles in NB asymmetric divisions (Schaefer et al. 2001; Fuse et al. 2003; Yu et al. 2003; Izumi et al. 2004). In this study we consider the

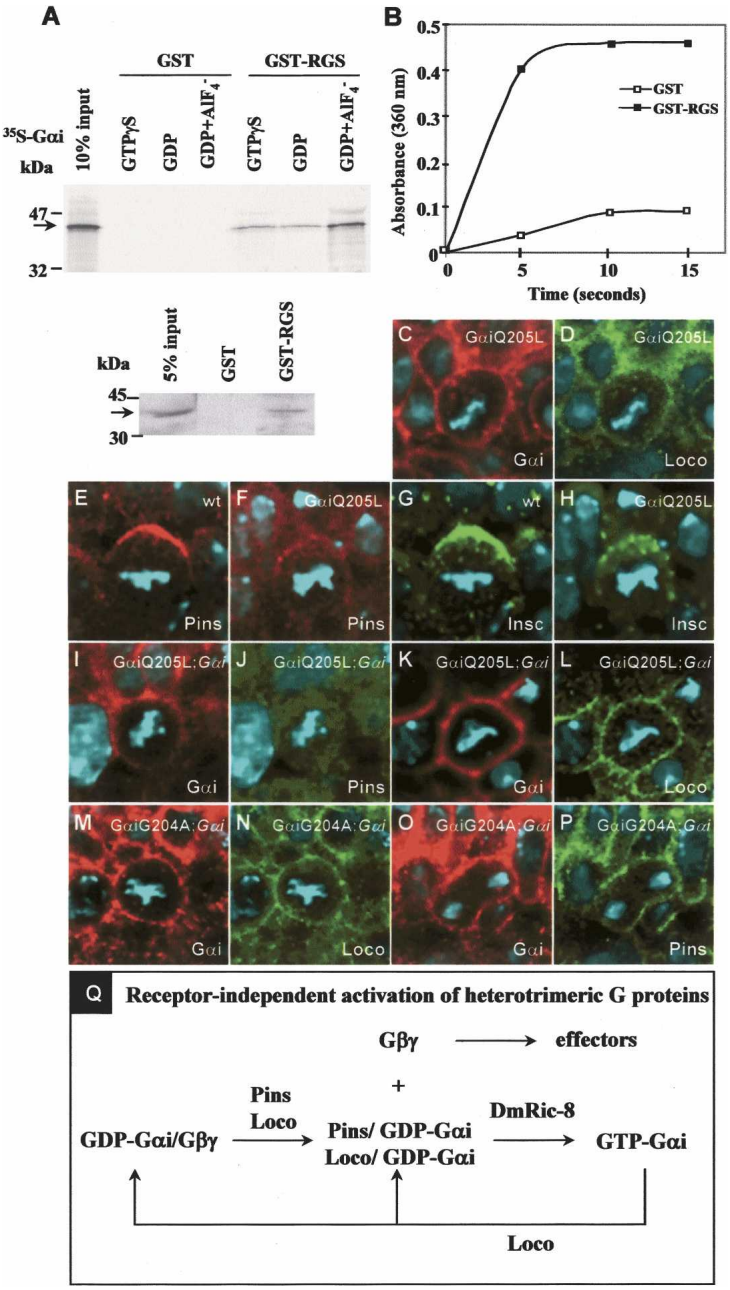

Figure 7. Loco also acts as a GAP to regulate the GTPase activity of Gαi through its RGS domain. (*A*) GST-RGS can also bind to Gαi. (*Upper* panel) The binding assay was carried out between ^{35}S-labeled Gαi and GST alone or GST-RGS. GST-RGS has weak binding activity with Gαi in the presence of GTPγS or GDP but much higher affinity to Gαi in the presence of GDP and AlF_4^-. (*Lower* panel) GST-RGS but not GST alone is capable of complexing with endogenous Gαi (see text). (*B*) Loco exhibits GAP activity for Gαi. GST-RGS can accelerate the GTPase activity of Gαi. (*C–P*) Overexpression of two mutant forms of Gαi in wild-type or *Gαi* mutant backgrounds. In wild-type NBs, Pins (*E*, red) and Insc (*G*, green) are localized as intense apical crescents. In wild-type NBs ectopically expressing GαiQ205L, GαiQ205L is cortically distributed (*C*, red) and colocalizes with Loco (*D*, green). (*F*) Ectopic expression of GαiQ205L leads to disruption of Pins crescents (red) in 84% of NBs. (*H*) Similarly, Insc localization (green) is also disrupted. Note that NBs in panels *E* and *F* are identical to those in panels *G* and *H*, respectively, and those images were taken at the same gain. In *Gαi* NBs ectopically expressing GαiQ205L (*I–L*), GαiQ205L is cortically localized (*I*,*K*, red) and Pins is cytosolic (*J*), while Loco is distributed around the cell cortex (*L*). In *Gαi* NBs ectopically expressing GαiG204A (the GDP-bound form) (*M–P*), GαiG204A is cortically localized during mitosis (*M*,*O*, red); both Loco (*N*, green) and Pins (*P*, green) are localized around the cell cortex. (*Q*) A working model for receptor-independent activation of heterotrimeric G proteins in *Drosophila* NBs. See Discussion.

issues of how heterotrimeric G-protein activation might be mediated during NB asymmetric divisions and the roles that Gβγ, GTP-Gαi, and GDP-Gαi play in this process. We show that Loco is a novel asymmetrically localized component of the NB asymmetric division machinery that possesses both GDI and GAP activities for Gαi. We provide evidence that indicates that the redundant GDI activities of Pins and Loco lead to the generation of free Gβγ, which plays a crucial role for the formation of an asymmetric mitotic spindle and daughter cells of distinct size. Based on loss-of-function phenotype, Gαi appears to play a less important role than Gβγ in this process; however, the proper balance between the levels of GTP- and GDP-bound forms of Gαi, which may be mediated, at least in part, by the GAP activity of Loco, is crucial for the asymmetric localization of Pins and Insc. It is important to note that there may exist additional Gα subunit(s) that might functionally overlap with Gαi in the generation of an asymmetric spindle. Therefore the possibility that Gβγ might mediate asymmetric spindle geometry by regulating the localization Gα subunit(s) (and GoLoco proteins) cannot be excluded at this point.

Multiple GDIs mediate receptor-independent activation of heterotrimeric G proteins during NB asymmetric divisions

Heterotrimeric G proteins are classically known to transmit extracellular signals to targets within the cell

through seven transmembrane, G-protein coupled receptors (GPCRs). Upon ligand binding, GPCR acts as a GEF to stimulate release of GDP from the Gα subunit, which, in turn, is converted to the GTP-bound form. GTP-Gα and Gβγ dissociate and activate their respective effectors to initiate downstream signaling. G-protein signaling is attenuated through the hydrolysis of GTP to GDP by the GTPase activity of Gα, which is accelerated by GAPs, which often contain a RGS domain. GDP-Gα can reassociate with and inactivate Gβγ.

Analyses of loss of function of *Gβ13F* and *Gγ1* as well as gain of function of Gαi in NBs have provided compelling support for the view that free Gβγ is required for the asymmetric localization/stability of both apical pathway components as well as the generation of asymmetric spindle and daughter cell size. Gαi is required primarily for the asymmetric localization of Pins and makes only a minor contribution in regulating spindle geometry and asymmetric daughter cell size. The mechanism by which heterotrimeric G-protein activation (generation of free Gβγ) is mediated in NBs has been unclear. The fact that no G-protein-coupled receptors (GPCRs) have been implicated in NB asymmetric divisions, the apparent intrinsic polarity exhibited by cultured NBs, as well as the observed GDI activity associated with Pins have raised the possibility that heterotrimeric G-protein activation may occur via a receptor-independent mechanism since GoLoco-containing molecules like Pins should be able to generate free Gβγ from the heterotrimeric complex by competing for binding to GDP-Gαi (Takesono et al. 1999; Natochin et al. 2000; Schaefer et al. 2001). However, loss of *pins* does not cause the majority of NBs to produce daughters of similar size and is therefore inconsistent with a failure to activate G-protein signaling.

This apparent contradiction is resolved by our observations, which indicate that receptor-independent activation of heterotrimeric G-protein signaling may be mediated through the GDI activities of both Pins and Loco. Like Pins, Loco can interact with GDP-Gαi through its GoLoco motif and form an in vivo complex with Gαi. In NBs, Loco colocalizes with Gαi and Pins at the apical cortex throughout mitosis. Removal of maternal and zygotic *loco* leads to delocalization of Pins/Gαi. Analysis of double mutants indicates that Loco functions redundantly with the Baz/DaPKC pathway with respect to the generation of differential daughter size. Simultaneous loss of both *loco* and *pins* results in phenotypic defects essentially indistinguishable to those seen in *Gβ13F* or *Gγ1* loss-of-function NBs. These observations indicate that receptor-independent activation of heterotrimeric G proteins during *Drosophila* NB asymmetric division may be achieved through the actions of the two functionally redundant GDI activities of Pins and Loco (Fig. 7Q).

The GAP activity of Loco and relevance of the equilibrium between GDP-Gαi and GTP-Gαi

In addition to its GDI activity, Loco also possesses a RGS domain that exhibits GAP activity for Gαi in vitro, suggesting that Loco can regulate Gαi via two distinct modes of action, both as a GDI and as a GAP. Our studies suggest that Gβγ, activated by the GDI activity of Pins and Loco, is crucial for NBs to produce daughters of unequal size, while the equilibrium between GDP-Gαi and GTP-Gαi, regulated, at least in part, by the GAP activity of Loco, is required for the localization of Insc/Pins/Loco at the apical cortex in NBs. When the equilibrium is shifted toward GTP-Gαi, that is, when GαiQ205L (the constitutively GTP-bound form) is expressed in the absence of endogenous wild-type Gαi, Pins becomes delocalized/destabilized because it requires binding to GDP-Gαi to localize to the cell cortex; however, the ability to generate an asymmetric spindle and unequal-size daughters is not compromised since Gβγ function should not be compromised. Conversely, when the equilibrium is shifted toward GDP-Gαi, through the ectopic expression of GαiG204A (the constitutively GDP-bound form) in the absence of endogenous wild-type Gαi, free Gβγ fails to be generated and defects similar to those seen in *Gβ13F* or *Gγ1* loss of function result.

While the Loco-associated GAP activity can facilitate the conversion of GTP-Gαi to GDP-Gαi in NBs, how might the reverse reaction be catalyzed without invoking the involvement of a GPCR associate GEF activity? A possible nonreceptor GEF that can fulfill this role may be the *Drosophila* homolog of the mammalian Ric-8A (Synembrin). Mammalian Ric-8A has been shown to act as a nonreceptor GEF for Gαo, Gq, and $G\alpha i_1$ subunits (Tall et al. 2003). Ric-8A is evolutionarily conserved from worm to mammals. More recent reports on *C. elegans* RIC-8 suggest that it is a GEF for the Gα subunits, GOA-1 and GPA-16, to regulate asymmetric divisions in the zygote (Afshar et al. 2004; Couwenbergs et al. 2004; Hess et al. 2004). We also found that the fly homolog, DmRic-8, is able to associate with Gαi and is involved in NB asymmetric divisions (F. Yu, unpubl.). Hence, in principle, a model along the lines schematized in Figure 7Q may explain how heterotrimeric G-protein signaling is regulated during the process of NB asymmetric divisions.

The role of heterotrimeric G proteins in Drosophila *neuroblasts and nematode zygotes*

While receptor-independent activation of heterotrimeric G-protein signaling appears to be a mechanism conserved between fly and nematode, there are clear differences between the two systems. In the nematode zygote, previous studies have suggested that the Gα subunits, GOA-1 and GPA-16, are required for generation of a net pulling force from the posterior cortex that leads to the displacement of the mitotic spindle toward the posterior cortex. Either (possibly both) of the GoLoco/GPR motif proteins, GPR1/2, which are enriched at the posterior pole of the zygote (Colombo et al. 2003; Gotta et al. 2003), can act as GDIs to asymmetrically activate heterotrimeric G-protein signaling. The Gα subunits and GPR1/2 both appear to act downstream of the PAR proteins and their inactivation using RNAi results in identical spindle phenotypes that resemble those seen in

par-2 mutants for which a reduction in cortical spindle forces have been directly demonstrated (Colombo et al. 2003; Gotta et al. 2003). More recently, it has been reported that loss of *ric-8* function also disrupts the movement of the posterior centrosome, suggesting that RIC-8 acts in the same pathway as GPR-1/2 to establish Gα-dependent force generation (Afshar et al. 2004; Couwenbergs et al. 2004; Hess et al. 2004), whereas loss of function of *rgs-7*, encoding a GAP protein for GOA-1, leads to overly vigorous posterior spindle rocking and more exaggerated size difference between two daughter cells, indicating that Gα passes through the GTP-bound state during its activity cycle to regulate the force in one-cell-stage nematode embryos (Hess et al. 2004). In contrast, Gβγ does not appear to regulate spindle displacement in the worm zygote (Srinivasan et al. 2003).

For *Drosophila* NBs, spindle geometry and displacement appear to be regulated to a large extent through Gβγ activation by the GoLoco proteins Loco and Pins. The spindle defects associated with *loco/pins* double loss-of-function NBs resemble those seen in the *Gβ13F* and *Gγ1* mutants. However, it is clear that in *Gβ13F* and *Gγ1* mutants there is a small degree of residual asymmetry in the size of the NB daughters; this residual size difference can be removed by the additional loss of *baz* function (Izumi et al. 2004). There is no evidence implicating a major role for Gαi in spindle asymmetry since loss of *Gαi* has relatively mild effects (Yu et al. 2003). However, the possibility that multiple Gα subunits redundantly regulate NB spindle geometry cannot be ruled out.

Furthermore, in contrast to the *C. elegans* zygote where heterotrimeric G-protein signaling acts downstream of the PAR polarity cues, the precise hierarchical relationship between the heterotrimeric G proteins and the PAR proteins in *Drosophila* NBs is more complex. On the one hand, some observations can be interpreted, at least formally, to suggest that free Gβγ acts upstream of the apical components, since mutations in *Gβ13F* and *Gγ1* cause delocalization of Pins/Loco/Gαi and affect the stability (intensity) of the Baz and DaPKC apical crescents (Yu et al. 2003). However, reduced levels of Baz and DaPKC can nevertheless asymmetrically localize and maintain residual levels of asymmetry despite the loss of free Gβγ, suggesting that some aspects of NB asymmetry and PAR polarity cues act in parallel or upstream of heterotrimeric G proteins (Fuse et al. 2003; Yu et al. 2003; Izumi et al. 2004). This study provides evidence that in *Drosophila* NBs, both Loco and Pins contribute toward the generation of free Gβγ and the asymmetric localization of Pins/Loco/Gαi depends not only on Gβγ but also the right balance of GDP-Gαi and GTP-Gαi. It remains to be seen whether in NBs Gβγ mediates the formation of an asymmetric spindle by regulating Gα subunits.

Materials and methods

Isolation of new loco *alleles*

EY04589 was mobilized using *P(ry Δ2–3)(99B)* as a transposase source, and 500 independent w^- revertant lines were established and analyzed. Three small deletions, $loco^{P237}$, $loco^{P283}$, and $loco^{P452}$, that remove part or all of the *loco-c1*-coding region were subjected to PCR mapping and DNA sequencing to determine their precise breakpoints. The recessive lethal allele $loco^{P237}$ removes the entire *loco-c1* and *loco-c2* transcripts as well as the flanking gene *mRpL45*. The allele $loco^{P283}$ removes the region from nucleotide –310 to +2195 of the *loco-c1* transcript, while $loco^{P452}$ removes the region from nucleotide –310 to +1277 of the transcript (the start point of *loco-c1* transcription is +1). The region that is removed in the $loco^{P283}$ allele includes the RGS domain, two RBD domains, and the GoLoco motif, while $loco^{P452}$ deletes only up to and including the region encoding the RGS domain.

In $loco^{P283}$ mutant neuroblasts (lacking both maternal and zygotic components) overexpressing Loco-C1 (uas-*loco-C1* driven with *mata-Gal4 VP16 V32*), Gαi apical crescents can be restored in 89% of metaphase NBs ($n = 74$), and Pins crescents can been observed in 70% of metaphase NBs ($n = 60$), indicating that these defects in *loco* mutant NBs are due to loss of *loco* function. When we attempted to rescue using the same procedure with a truncated form of Loco-C1 lacking the GoLoco motif but including the RGS and RBD domains (Loco-C1ΔGoLoco, containing amino acids 1–640), Gαi apical crescents could be restored in 64% of mitotic neuroblasts ($n = 33$), and Pins apical crescents could be seen in 85% of neuroblasts ($n = 20$). However, in the rescue experiments with a truncated form of Loco-C1 lacking the RGS domain (Loco-C1ΔRGS, containing amino acids 232–830), the majority of NBs exhibit uniform cortical distribution of Pins (81%, $n = 26$) and Gαi (95%, $n = 23$). Together with the biochemical experiments, these rescue results indicate that the RGS domain of Loco, and its associated GAP activity for Gαi, is important for NB asymmetric divisions.

Plasmid constructs, fusion proteins, and anti-Loco antibodies

MBP-Gαi was constructed by introducing the coding region of *Gαi* into pMAL-c2x (NEB). Various GST fusion proteins of Loco-C1 (amino acids 61–298, 337–502, 357–636, and 564–731) were generated using pGEX 4T-1 (Amersham). GST-C-Pins was generated according to Yu et al. (2002). Anti-Loco antibodies were generated in guinea pigs and affinity-purified as described in Yu et al. (2003). An anti-Gαi antibody was raised against the full-length Gαi fused to MBP in mice and guinea pigs. No Gαi signal could be detected in Gαi mutant embryos by Western blotting and immunofluorescent staining (data not shown), indicating that this anti-Gαi antibody can recognize Gαi specifically.

Yeast two-hybrid, protein binding assays, and GDI and GAP assays

Yeast two-hybrid assays were carried out as described in Yu et al. (2000). The fragments encoding amino acids 564–829 of Loco-C1 or amino acids 378–658 of Pins were inserted into pAS2-1. The full-length *Gαi* and the mutant version *GαiQ205L* were inserted into pACT2. Their corresponding binding activities were tested based on the ability of colonies to turn blue in an X-gal filter lift assay: +, 60 min; –, no significant staining.

Full-length *Gαi* and the mutant version, *GαiQ205L*, were inserted into pET15b (Novagene). ^{35}S-labeled Gαi and GαiQ205L proteins were produced by using TNT in vitro transcription and translation kit (Promega). The GST pull-down assays were conducted as described in Yu et al. (2000). To test for the nucleotide-dependent interaction between Gαi and the RGS domain of Loco, 10 μL of ^{35}S-labeled Gαi was incubated for 30 min at room temperature by adding 90 μL of buffer A (50 mM Tris-HCl at pH 8.0, 0.1 M NaCl, 1 mM $MgSO_4$, 20 mM imidazole, 10 mM

mercaptoethanol, 10% glycerol) supplemented with GTPγS (10 μM), GDP (10 μM) or GDP and AlF_4^- (10 and 30 μM), respectively. GST-RGS (1 μg) or control GST (3 μg), bound to agarose beads, was separately incubated with the Gαi mixture for 30 min at 4°C. The agarose beads were washed four times with buffer containing the respective nucleotides and/or AlF_4^-. To test whether GST-RGS can pull down endogenous Gαi, 200 μg of GST-RGS or GST alone was incubated with embryo extracts, followed by three washes in the lysis buffer. Bound proteins were Western-blotted with anti-Gαi antibody.

[^{35}S]GTPγS binding experiments were essentially performed as described in Natochin et al. (2000). Reaction mixtures containing 1 μM MBP-Gαi-GDP, 1 μM GST-GoLoco (amino acids 564–731), GST-C-Pins (amino acids 378–658), or control GST were mixed with 2 μM [^{35}S]GTPγS (1000 Ci/mmol) and incubated at 30°C for different time periods. The reactions were terminated and measured for scintillation counts.

GTPase activity assays were performed according to the manufacturer's instructions (Enzcheck Phosphate Assay Kit; Molecular Probes). In brief, 15 μL of 1 nmol of MBP-Gαi fusion protein was mixed with 10 μL of 0.2 mM GTP, 0.2 mL of 2-amino-6-mercapto-7-methylpurine ribonucleoside, 1 unit of purine nucleotide phosphorylase, and 0.78 mL of HEPES buffer (pH 7.5) and measured for the absorbance at 360 nm. Five microliters of 1 M $MgCl_2$ solution containing either GST or GST-RGS (amino acids 61–298) fusion protein was added to initiate the single turnover reaction, and the absorbance at 360 nm was recorded every 5 sec.

Flies, germline transformation, and RNAi experiments

Insc^{22}, *pins*^{P89}, *pins*^{P62}, *baz*^{Xi106} FRT$^{9-2}$, *scabrous-gal4* (*sca-gal4*), *mata-gal4 VP16 V32*, and *UAS-Gαi* were described earlier in Yu et al. (2000) and Yu et al. (2003). *Gβ13(Ff261(FRT*$^{9-2}$ and *Gγ1(N159(FRT*$^{2R\text{-}G13}$ were kindly provided by F. Matsuzaki (Center for Developmental Biology, RIKEN, Lobe, Japan). *UAS-GaiG204A* was obtained by introducing the mutant *GαiG204A* cDNA in which Gly 204 had been replaced with alanine into pUAST (Brand and Perrimon 1993). Overexpression of GαiQ205L and GαiG204A in either wild-type or *Gαi* mutant embryos was driven by *mata-gal4 VP16 V32* at 26°C. Full-length *loco-c1* (GH08607 from BDGP), *loco-c1ΔGoLoco* (encoding the region amino acids 1–630 of the Loco-C1 protein), and *loco-c1ΔRGS* (encoding the region amino acids 232–830) were inserted into pUAST. The coding region of *loco-c2* fused to two tandem Flag epitopes was also cloned into pUAST and hs-Casper vectors and was used for germline transformation. The RNAi experiments were performed essentially as previously described in Yu et al. (2003).

Immunocytochemistry and confocal microscopy

Embryos were collected and fixed according to Yu et al. (2003). Rabbit anti-Asense (Y.N. Jan, University of California, San Francisco, Howard Hughes Medical Institute, CA), rabbit anti-Baz (F. Matsuzaki), rabbit anti-Insc, rabbit and mouse anti-Pins, rabbit anti-Gαi (amino acids 327–355; J.A. Knoblich, Institute of Molecular Biotechnology, Vienna, Austria), guinea pig anti-Gαi (this study), rabbit anti-PKCξ C20 (Santa Cruz Biotechnology), rabbit anti-Gβ13F (F. Matsuzaki), rabbit anti-Miranda (F. Matsuzaki), rabbit anti-Pon (Y.N. Jan), rabbit anti-Numb (Y.N. Jan), mouse anti-α-tubulin (Sigma; DM1A), rabbit anti-CNN (T.C. Kaufman, Indiana University, Howard Hughes Medical Institute, IN), anti-Pros MR1A (C.Q. Doe, University of Oregon, Howard Hughes Medical Institute, Eugene, OR), mouse anti-β-gal (Promega), Rabbit anti-β-gal (Cappel), and anti-Nrt BP106 (DSHB) were used in this study. Cy3- or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were from Jackson Laboratories. Stained embryos were incubated with ToPro-3 (Molecular Probes) to visualize DNA, and embryos were mounted in Vectashield (Vector Labs). Immunostainings were analyzed with laser scanning confocal microscope (Zeiss Meta LSM510).

CoIP and Western blot

Embryos collected from transgenic flies carrying *hs-loco-c2* were heat-shocked at 34°C for 10 min. Embryo extraction and CoIPs were performed as described in Yu et al. (2003). Anti-Gαi or anti-Flag (m2) was used for immunoprecipitation. Bound proteins were analyzed with anti-Flag, anti-Pins, and anti-Gαi by Western blots (Yu et al. 2000).

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