Distinct roles of \( \text{G} \alpha \text{i} \) and \( \text{G}\beta13F \) subunits of the heterotrimERIC G protein complex in the mediation of \textit{Drosophila} neuroblast asymmetric divisions

Fengwei Yu,1 Yu Cai,1 Rachna Kaushik,1 Xiaohang Yang,1 and William Chia2

1Institute of Molecular and Cell Biology, Singapore 117609
2MRC Centre for Developmental Neurobiology, London SE1 1UL, UK

The asymmetric division of \textit{Drosophila} neuroblasts involves the basal localization of cell fate determinants and the generation of an asymmetric, apico-basally oriented mitotic spindle that leads to the formation of two daughter cells of unequal size. These features are thought to be controlled by an apically localized protein complex comprising of two signaling pathways: Bazooka/\textit{Drosophila} atypical PKC/Inscuteable/DmPar6 and Partner of inscuteable (Pins)/Gai; in addition, \( \text{G}\beta13F \) is also required. However, the role of Gai and the hierarchical relationship between the G protein subunits and apical components are not well defined. Here we describe the isolation of Gai mutants and show that Gai and \( \text{G}\beta13F \) play distinct roles. Gai is required for Pins to localize to the cortex, and the effects of loss of Gai or pins are highly similar, supporting the idea that Pins/Gai act together to mediate various aspects of neuroblast asymmetric division. In contrast, \( \text{G}\beta13F \) appears to regulate the asymmetric localization/stability of all apical components, and \( \text{G}\beta13F \) loss of function exhibits phenotypes resembling those seen when both apical pathways have been compromised, suggesting that it acts upstream of the apical pathways. Importantly, our results have also revealed a novel aspect of apical complex function, that is, the two apical pathways act redundantly to suppress the formation of basal astral microtubules in neuroblasts.

Introduction

The \textit{Drosophila} embryonic central nervous system is derived largely from neural progenitors called neuroblasts (NBs). NBs divide asymmetrically to generate two unequal size daughter cells: the larger apical daughter remains as a NB and continues to divide asymmetrically, and the smaller basal/lateral daughter (ganglion mother cell) divides terminally to generate two neurons/glial cells (Campos-Ortega, 1995). Three well-characterized features of the NB asymmetric division (Jan and Jan, 2001; Chia and Yang, 2002) are: (a) basal localization and asymmetric segregation of cell fate determinants and their associated proteins such as Numb/Partner of numb (Pon), Prospera (Pros)/Miranda (Mira), and pros RNA/Staufen; (b) reorientation of the mitotic spindle along the apical/basal axis at metaphase; (c) generation of an apically biased asymmetric mitotic spindle (Kaltschmidt et al., 2000) and the displacement of the spindle toward the basal cortex during ana/telophase, which leads to the formation of NB daughter cells that differ in size. An additional feature, which has not been extensively studied, is that late in NB mitosis an extensive astral microtubule network emanates from the apical but not the basal centrosome (Giansanti et al., 2001).

The well-characterized features of the NB asymmetric division are controlled by a complex of proteins that are apically localized in dividing NBs, which include the \textit{Drosophila} homologues of the conserved Par3 (Bazooka [Baz]/Par6 (DmPar6)/aPKC (\textit{Drosophila} atypical [DaPKC]) (Kuchinke et al., 1998; Schober et al., 1999; Wodarz et al., 1999, 2000; Petronzelli and Knoblich, 2001) protein cassette first described in \textit{Caenorhabditis elegans} (Kemphues, 2000; Matsuzaki,
using a relatively mild G
Insc crescent (not depicted) reappears in
fluorescence. Pins crescent (green) and
is also undetectable in GaiP8 (C) and
are antigen minus (arrow). G
locus is deleted partially or fully in all
derived from G
homozygotes of either G
indicates that embryos derived from

Recent findings indicate that the apical proteins are also
involved in daughter cell size determination and can be fur-
ther subdivided into two redundant pathways that control
mitotic spindle geometry and displacement late in NB divi-
sions (Cai et al., 2003). Baz, DaPKC, Insc, and probably
DmPar6 belong to one pathway and Pins and (probably)
Goi belong to the other. Members of each pathway can
asymmetrically localize when members of the other pathway
are mutated, suggesting that localized spindle extension sig-
als derived from either one of these two pathways are suffi-
cient to generate asymmetric spindle geometry and spindle
displacement, resulting in unequal size daughter cells. Si-
multaneous disruption of both pathways destroys the local-
ized spindle extension and displacement signals. Conse-
quent, the two half spindle arms remain identical in length
and mutant NBs produce two daughter cells with equal size.

Heterotrimeric G protein signaling has been shown to be
involved in controlling distinct microtubule-dependent pro-
cesses in C. elegans P0 embryos (Gotta and Ahringer, 2001).
Gβγ is important for correct centrosome migration around
the nucleus and spindle orientation. Gai is required for
asymmetric spindle positioning in the one-cell embryos. In
Drosophila, G protein signaling is also involved in microtu-
bule-dependent processes such as the formation of an asym-
metric spindle. When Gai is overexpressed (Schaefer et al.,
2001) or when GB13F function is abolished (Schaefer et al.,
2001), the ability to generate an asymmetric spindle is dis-
rupted and NBs frequently divide to produce two daughter
cells with equal size (Fuse et al., 2003). However, it has not
been possible to assess the relative roles of GB13F and Gai
in NB asymmetric divisions not only because Gai mutants
are not available but also because in GB13F mutants Gai is
undetectable in all cell types (Schaefer et al., 2001).

In this study, we report the isolation and analysis of loss of
function mutations in Gai and assessing the role of the api-
cal complex components on NB astral microtubules and mi-
totic spindle geometry. Our findings indicate distinct roles
for Gai and GB13F in NB asymmetric divisions. Loss of
Gai releases Pins from the apical cortex into the cytosol and
exhibits a similar array of phenotypes seen in pins mutant
NBs. Mutations in Gai and one of the genes in Baz/ DaPKC/Insc pathway cause NB to generate symmetric spin-
dles and two equal size daughter cells, suggesting that Gai
and Pins act in same pathway with respect to mediating mi-
totic spindle geometry. Formally, GB13F functions up-
stream of both Baz/DaPKC/Par6/Insc and Pins/Gai path-
ways and is required, at least in part, for the asymmetric

Figure 1. Characterization of Gai
deletion alleles. (A) Schematic
representation of three Gai deletion
alleles. The extent of the deletions are
indicated by the parentheses. The Gai
locus is deleted partially or fully in all
three alleles. (B) Western blot analysis
using a Gai COOH-terminal antibody
indicates that embryos derived from
homozygotes of either GaiP8 or GaiP29
are antigen minus (arrow). Gai signal
is also undetectable in GaiP8 (C) and
GaiP29 (not depicted) NBs using immuno-
fluorescence. Pins crescent (green) and
Insc crescent (not depicted) reappears in
GaiP8 NBs (D) with ectopic Gai expres-
sion using a relatively mild sca-gal4 driver.
The mitotic NBs are identified using DNA
staining (cyan, C and D). Apical is up.
Cell boundary is outlined with white
dots. Bar: (C and D) 10 μm.
localization and/or stability of all apical complex members. Mutation in GB13F can disrupt the asymmetric localization of members of both apical pathways in NBs and results in the formation of symmetric spindles and equal size daughter cells. Strikingly, our analyses has also revealed that the two apical pathways act downstream of Gai and GB13F to redundantly suppress the formation of basal astral microtubules during NB divisions.

Results
Generation of antigen-minus alleles of Gai
It has been shown that Gai is apically localized in mitotic NBs and its apical localization requires Pins. Gai interacts directly with the GoLoco motifs (Siderovski et al., 1999) in the COOH-terminal region of Pins, a region required for Pins to target to the NB cortex (Yu et al., 2002). In the absence of pins, Gai is localized uniformly to the cortex of dividing NBs. To ascertain the functions which are specific to Gai during asymmetric NB divisions, we generated Gai mutant alleles by imprecise excision of the P element (KG01907) inserted in the 5’ flanking region of the Gai gene. Three revertants, Gai<sup>p8</sup>, Gai<sup>p29</sup>, and Gai<sup>p20</sup>, associated with flanking deletions were isolated and mapped (Fig. 1). Gai<sup>p20</sup> is an embryonic lethal allele. Deletion in Gai<sup>p20</sup> removes not only the complete coding region of the Gai gene but also the putative gene CG10063. The precise 3’ breakpoint of Gai<sup>p20</sup> has not been determined. Gai<sup>p20</sup> contains a deletion uncovering the first exon that includes the codon for translation initiation, whereas Gai<sup>p8</sup> carries a deletion that removes the first two exons. There is an EST sequence LD18889 with no obvious ORF in the first intron of the Gai gene but also the putative gene iP29. Similar to animals lacking zygotic pins function, homozygous Gai<sup>p8</sup> and Gai<sup>p29</sup> flies lacking zygotic Gai are viable, show locomotion defects, but nevertheless can lay fertilized eggs. The majority of the embryos derived from these homozygous animals lacking both maternal and zygotic components die as larvae. Western blot analysis and immunostaining with an anti-Gai antibody raised against the extreme COOH-terminal region, aa 327–355, of Gai (Schaefer et al., 2001) indicated that these Gai<sup>p8</sup> and Gai<sup>p29</sup> embryos are antigen minus (Fig. 1, B and C). Since these embryos exhibit NB phenotypes which are indistinguishable from germ line clone embryos derived from Gai<sup>p20</sup>.
(a complete deletion of the gene), they are likely to be null alleles. In the following experiments, unless otherwise specified, Ga\textsubscript{i} mutant refers to Ga\textsubscript{i} \textsuperscript{248} embryos lacking both maternal and zygotic Ga\textsubscript{i} function.

**Loss of maternal and zygotic Ga\textsubscript{i} causes Pins to localize to the cytosol and produce phenotypic defects similar to those seen in pins NBs**

Both Pins and Insc, which normally form apical crescents in wt NBs (Fig. 2, A, C, and E), are cytoplasmic in dividing Ga\textsubscript{i} \textsuperscript{248} NBs (Fig. 2, B, D, and F). The apical localization of DaPKC (68%, n = 50) and Baz (unpublished data) remain largely unchanged although the intensity of the staining is reduced, sometimes dramatically (Fig. 2 H). Localization of the basal proteins are also affected. Basal proteins Mira/Pros (Fig. 2, I and J) and Pon/Numb (unpublished data) are often mislocalized in mitotic NBs up to metaphase; however, telophase rescue occurs normally, and basal proteins subsequently segregate primarily to just one daughter during telophase (Fig. 2 L). In Ga\textsubscript{i} mutant NBs, GB13F remains uniformly cortical as in wt NBs (Fig. 2, O and P). The RP2sib to RP2 cell fate change is also observed in Ga\textsubscript{i} embryos (Fig. 2 R), which serves as a good indication of defective ganglion mother cell asymmetric divisions. Anti-Eve staining shows that RP2sib adopts RP2 cell fate in ~10% (n = 248) of mutant hemisegments. In addition, the RP2 missing phenotype is also observed (11%, n = 248). Mitotic spindle reorientation is also affected in Ga\textsubscript{i} mutants. In mitotic domain 9, mitotic spindles fail to undergo 90\textdegree orientation, and these cells divide parallel to the embryonic surface (Fig. 2 N), whereas their wt counterparts reorientate and divide perpendicular to the surface (Fig. 2 M). These defects are similar to those observed for NBs lacking pins function (Yu et al., 2000).

Several observations further support the view that the above described defects are caused by the loss of Ga\textsubscript{i} function. Introduction of the nested gene LD18889 into Ga\textsubscript{i} \textsuperscript{248} does not rescue the defects in asymmetric NB division. Furthermore, the small deletion Ga\textsubscript{i} \textsuperscript{248}, which contains intact LD18889, exhibits the same phenotypes seen in Ga\textsubscript{i} \textsuperscript{248}. Moreover, low level expression of a UAS-Gai using the sca-gal\textsubscript{4} driver in Ga\textsubscript{i} mutant background can partially restore apical localization of Pins (81%, n = 52; Fig. 1 D) and Insc (unpublished data) in mitotic NBs, suggesting that defects in NB divisions are due to loss of Ga\textsubscript{i} function.

**Ga\textsubscript{i} and Pins act in the same pathway to regulate asymmetric spindle geometry and unequal cell size divisions**

Ga\textsubscript{i} has been implicated previously in the generation of spindle asymmetry from overexpression and RNAi experiments (Schaefer et al., 2001; Cai et al., 2003). The availability of Ga\textsubscript{i} loss of function alleles enables us to more definitively assess the role of Ga\textsubscript{i} in NB spindle geometry and the generation of daughters of unequal cell size. In wt NBs, the mitotic spindle is symmetric until metaphase. Starting from anaphase, the differential extension of the apical half spindle arm results in an apically biased asymmetric spindle (Kaltschmidt et al., 2000): the distance from the midspindle to the apical centrosome is larger than that to the basal centrosome. In addition, the spindle is displaced basally: the apical centrosome is located away from the NB apical cortex, whereas the basal centrosome lies close to the basal cortex (Cai et al., 2003). Consequently, the future cleavage plane is located toward the basal side of the NBs. Similar to pins, the majority of Ga\textsubscript{i} mutant NBs generate an asymmetric spindle and produce two daughter cells with different cell sizes; however, similar to pins NBs, 21% (n = 86) of Ga\textsubscript{i} NBs produce a symmetric spindle and give rise to equal size daughters (Fig. 3 B).

To ascertain how Ga\textsubscript{i} acts in the context of our two pathway models for the control of mitotic spindle geometry in NBs, we analyzed spindle geometry and daughter cell size in various combinations of double mutants with Ga\textsubscript{i}. A high frequency of equal size divisions (Ga\textsubscript{i}/insc RNAi, 100%, n = 39 [Fig. 3 C]; Ga\textsubscript{i}/insc, 100%, n = 66 [Fig. 3, D and F]) is observed only when Ga\textsubscript{i} and one of the components of Baz/DaPKC/Insc pathway are simultaneously disrupted. In contrast to wt NBs (Fig. 3 E), in these double mutants, for example, in Ga\textsubscript{i}/insc NBs, the spindle geometry revealed with anticientrosomin (CNN) staining remains symmetric even at telophase with the cleavage plane being equidistant to both

Figure 3. **Ga\textsubscript{i} and pins form part of the same apical pathway for regulating NB mitotic spindle geometry.** Confocal images of triple labeled telophase NBs (BP106, a membrane marker, red; DNA, cyan; Asense, a NB marker, cytosolic green in A–D or CNN, a centrosome marker, green in E–F) showing unequal size divisions in wt (A and E) and equal size divisions in various mutant combinations. 21% of Ga\textsubscript{i} mutant NBs generate two approximately equal size daughter cells (B); further removal of baz function in Ga\textsubscript{i} NBs (C) greatly increases the frequency of equal size divisions; similarly, Ga\textsubscript{i}/insc NBs (D) also show high frequency of equal size divisions (100%, see Results). In wt NBs, the mitotic spindle, deduced from positions of the centrosomes, is asymmetric and displaced toward the basal cortex (E). In equal size NB divisions (e.g., Ga\textsubscript{i}/insc NBs), the mitotic spindle is symmetric and the two centrosomes both lie in close vicinity of the cell cortex (F). Apical is up. Bar, 10 \textmu m.
centrosomes (Fig. 3 F). Furthermore, the spindle is positioned symmetrically with both centrosomes lying in close proximity to the cell cortex (Fig. 3 F). In contrast, the frequency of equal size divisions in the \(G\alpha_i/pins\) double ablation NBs is low, comparable to frequencies seen in \(G\alpha_i\) or \(pins\) single mutants (Cai et al., 2003). These data indicate that \(G\alpha_i\) and Pins belong to the same pathway with respect to regulating asymmetric spindle geometry. Like \(pins\), \(G\alpha_i\) loss of function in combination with mutation in \(baz\), \(DaPKC\), or \(Insc\) will disrupt both pathways which control spindle asymmetry and displacement in mitotic NBs, leading to the formation of a symmetric spindle and equal size daughters.

**Apical functions are necessary to suppress basal astral microtubule formation**

One striking observation seen with anti-\(\alpha\)-tubulin staining of mitotic NBs that had not been noted before is the influence of the apical functions on the asymmetric nature of the astral microtubules associated with the two centrosomes. In wt NBs, astral microtubules are nucleated at the apical centrosome, and the intensity of this staining increases markedly during the later stages of mitosis. Similar astral microtubule cap structures can be seen in dividing epithelial cells (Fig. 4, J–L). In epithelial cells of the epidermis from metaphase onwards, astral microtubules form two cap-like structures; each associates with one of the centrosomes. Overexpression of \(G\alpha_i\) in wt embryos changes the astral microtubule structures in dividing NBs (G–I). In addition to the formation of a symmetric spindle, two astral microtubules cap-like structures (arrows) are formed, associated with each centrosome, similar to that seen in epithelial cells. Similar astral microtubule behavior (arrow) can be observed in NBs in which the Pins/\(G\alpha_i\) and Baz/DaPKC/Par6/Insc pathways are simultaneously compromised: \(G\alpha_i\)/\(insc\) NBs (J–L), \(G\alpha_i\) (M), \(insc/pins\) (N), baz/pins (O), and baz/\(G\alpha_i\) (unpublished data). \(Mira\) is distributed uniformly around the cell cortex in both baz/\(G\alpha_i\) and baz/pins NBs, suggesting the possible involvement of \(Baz\) in “telophase rescue” of basal proteins. For NB panels, apical is up. (D–F) Surface view of epithelial cells. Cell boundary is outlined with white dots. Bar, 10 \(\mu\)m.
to the uniform cortical localization of all apical components, and the loss of GB13F (see next section), also result in the production of prominent astral microtubules over both centrosomes (Fig. 4, G–I). This symmetric astral microtubule association with both centrosomes is similar to the astral microtubule structure seen in dividing epithelial cells (Fig. 4, D–F). These observations suggest that the presence of either of the asymmetric apical pathways is sufficient to suppress the formation of basal astral microtubules in NBs (see Discussion).

**GB13F function is required for the asymmetric localization of apical components**

To compare and contrast the roles of Goxi and Gb in NB divisions, we analyzed GB13F mutant NBs. In contrast to Goxi, GB13F, which has been shown previously to have a role in NB asymmetric divisions, is evenly distributed to the cortex of mitotic NBs. It has been reported (Schafer et al., 2001) and we have confirmed that in GB13F mutants Goxi is progressively degraded during embryonic development and becomes undetectable at stage 10 with anti-Goxi staining (unpublished data), presumably due to the instability of Goxi in the absence of GB13F. In GB13F mutant NBs, Insc is cytoplasmic (Fig. 5 A) and Pins levels are also strongly reduced and it appears to be distributed throughout the cell cortex and in the cytoplasm of all NBs (100%, n = 21 [Fig. 5 B]). Hence, in all GB13F mutant NBs, both the stability and the asymmetric localization of Pins are drastically affected. In addition, in agreement with the findings of Fuse et al. (2003), we observed that spindle asymmetry is lost in the majority (65%, n = 110 [Fig. 6 B]) of the GB13F NBs, and a similar proportion of NBs divide to produce two equal size daughter cells (Fig. 5 E).

Since we have previously shown that the loss or the uniform cortical localization of both Pins/Gai and Baz/DaPKC pathway members can abolish spindle asymmetry and result in equal size NB divisions, we wondered whether the equal size divisions seen in the GB13F NBs can be rationalized according to our model. If GB13F functions upstream of the apical complex members to regulate their asymmetric localization, stability, or function, we would expect Baz/DaPKC asymmetric localization/function to also be affected in GB13F mutant NBs. Indeed the anti-Baz and anti-DaPKC immunostainings show that Baz (unpublished data) and DaPKC asymmetric localization is lost or undetectable in 71% (n = 45) of GB13F NBs. In the rest of NBs, Baz (unpublished data) and DaPKC (Fig. 5 C) form cortical crescents. Further removal of Baz through RNAi in GB13F germline clones leads to equal size divisions (Fig. 5 F) in 94% (n = 45) (Fig. 6 B), suggesting that the function of the Baz/aPKC pathway is disrupted only in ~71%, whereas the function of the Pins/Gai pathway is compromised in all of the NBs in GB13F embryos. Astral microtubules can be seen associated with both centrosomes in GB13F NBs undergoing equal size divisions (Fig. 5, G–I).

These data suggest that GB13F (presumably in association with Gry) can function upstream of both apical pathways and act to promote the asymmetric localization/stability of the Baz/DaPKC and Pins/Gai pathway members. In the absence of GB13F, the functions of both apical pathways are compromised in the majority of NBs; they fail to generate an asymmetric mitotic spindle and consequently undergo equal size divisions. In the remainder of mutant NBs, although the function of the Pins/Gai pathway is compromised, Baz/DaPKC remain asymmetrically localized and functional; consequently asymmetric spindles and daughter
ectopic expression of Gαi drives higher levels of Gαi (in mata-gal4 embryos) and twofold (in sca-gal4 embryos) higher than that in wt. Immunofluorescence data also show that mata-gal4 drives higher levels of Gαi expression than sca-gal4 in NBs derived from stage 10 embryos. (B) Frequencies of equal size NB divisions induced by ectopic expression of Gαi and Gao and in Gβ13F germ line clone embryos with and without attenuation of baz function. (C) Western blot showing coimmunoprecipitation of Gαo47A with Gβ13F when Gao is overexpressed using a maternal driver. Anti-Gβ antibody was used for immunoprecipitation. PI is a preimmune serum. Bar, 10 μm.

Figure 6. Depletion of free Gβ by overexpression of Gαi or Gao results in equal size NB divisions. (A) Western blot analysis of expression levels of Gαi driven by maternal gal4 driver (mata), sca-gal4 driver, and in wt embryos. Gαi levels based on densitometry are about fivefold (in mata-gal4 embryos) and twofold (in sca-gal4 embryos) higher than that in wt. Immunofluorescence data also show that mata-gal4 drives higher levels of Gαi expression than sca-gal4 in NBs derived from stage 10 embryos. (B) Frequencies of equal size NB divisions induced by ectopic expression of Gαi and Gao and in Gβ13F germ line clone embryos with and without attenuation of baz function. (C) Western blot showing coimmunoprecipitation of Gαo47A with Gβ13F when Gao is overexpressed using a maternal driver. Anti-Gβ antibody was used for immunoprecipitation. PI is a preimmune serum. Bar, 10 μm.

cells of unequal size are produced. These findings support and extend on our earlier two pathway model (Cai et al., 2003) for the generation of an asymmetric mitotic spindle.

**Dosage-dependent effects of Gαi overexpression on equal size NB divisions**

Our previous study (Cai et al., 2003) showed that the equal size NB divisions caused by overexpression of Gαi driven by sca-gal4 was dependent on pins function. Our interpretation of these results was that both proteins need to be present in a complex in order for a signal to be generated. However, recent observations that overexpression of Gαi, but not constitutively activated form of Gαi, in NBs disrupted asymmetric divisions and produced two equal size daughter cells (Schaefer et al., 2001) suggest that it is the depletion of free Gβγ (caused by an excess of GDP-Gαi) that might be the cause for the equal size NB divisions; the equal size NB divisions seen in the Gβ13F embryos provide further support for this view. If this were the case, then one would expect that under conditions in which Gαi was in excess (with respect to all other molecules it can complex with like Pins and Gβγ) free Gβγ should be depleted whether Pins was present or not. How can these seemingly contradictory observations be reconciled?

One possible explanation is that under conditions that we used previously (sca-gal4 driving UAS-Gαi) Gαi is not overexpressed to excess. Under these conditions, the phenotypic effects produced are caused by uniform Pins/Gαi signaling from the cortex, and not by the sequestration of Gβγ due to excess Gαi, and therefore are Pins dependent. To test whether the equal size division phenotype is dependent on Pins under circumstances in which Gαi is overexpressed to higher levels, we used a stronger driver (mata-gal4 VP16 V32). This driver increases Gαi levels by about fivefold (compared with wt) compared with a twofold increase by sca-gal4 as judged by Western blot analysis of embryonic extracts (Fig. 6 A). In immunofluorescence experiments using identical conditions, mata-gal4 VP16 V32 also drives a higher level of expression than sca-gal4 in NBs (Fig. 6 A). The increased levels of Gαi overexpression leads to a high frequency of equal size NB divisions (83%, n = 55 [Fig. 6 B]) which is largely independent of Pins, since overexpression in the absence of Pins only marginally reduce the frequency of equal size NB divisions (62%, n = 62 [Fig. 6 B]).

Our interpretation of these observations is that overexpression of Gαi can cause NBs to undergo equal size divisions via two different mechanisms. With the levels of overexpression obtained with sca-gal4, Gαi binds primarily to Pins and recruits Pins uniformly to the NB cortex (Cai et al., 2003). The cortical Pins/Gαi complex, presumably through a signaling function, disrupt the Baz/DaPKC apical localization, resulting in equal size NB divisions. In the absence of Pins, although both endogenous and ectopic Gαi molecules are uniformly cortical, Gαi alone cannot or is less able to interfere with Baz/DaPKC asymmetric localization. With higher levels of ectopic Gαi (mata-gal4 VP16 V32 driver), not only are Pins/Gαi uniformly cortical but the excess Gαi can also bind to and deplete free Gβγ. With limiting levels of free Gβγ, both apical pathways can be disrupted as seen in the Gβ13F mutants. In the presence of higher levels of Gαi,
Figure 7. **Overexpression of Gao mimics Gβ mutant phenotypes.** In mitotic NBs ectopically expressing Gao, double label confocal images (A and B; C and D) show that Gao (red, A) gives a strong uniformly cortical signal whereas Gai (green, B) is weak or undetectable; Pins (green, C) becomes uniformly cortical and Insc (red, D) shows punctuated, delocalized staining. DaPKC is no longer apical as seen in wt (green, E) but weak and uniformly cortical in most of NBs (green, F). Mira basal localization is also disrupted in the presence of ectopic Gao in the majority of the NBs: Mira is delocalized at metaphase (red, G), and the mitotic spindle is asymmetric (red, J; CNN, green, L) like in wt NBs. Apical is up. DNA staining is in cyan. Mira is green in I and J. Cell boundaries are outlined (white dots) or marked by BP106 (K and L). Bars: (A–L) 10 μm.

Pins is not required for the majority of the equal size NB divisions since its absence would not affect the ability of Gai to sequester free Gβy.

**Overexpression of Gao causes equal size NB divisions**

If the depletion of free Gβy can disrupt asymmetric NB divisions, we might expect that other Gα molecules that interact with Gβy may also be able to reproduce the Gαi overexpression phenotypes when ectopically expressed in NBs. One such molecule, Gα47A, which shares high homology with Gai, is able to bind/complex Gβ13F in vivo as indicated by the observation that it coimmunoprecipitates with Gβ13F when it is overexpressed (Fig. 6 C). Anti-Gα47A staining shows a weak cortical localization of the protein in NBs (unpublished data; Schaefer et al., 2001). However, removal of both maternal and zygotic Gα47A does not affect any aspect of NB asymmetric division, indicating that Gα47A is not normally required in wt NBs. When Gα47A is overexpressed, we observe a high frequency of NB equal size divisions (85%, n = 41 [Fig. 7, H, I, and K]), similar to that seen with Gai overexpression (Fig. 4 I). In metaphase NBs overexpressing Gα47A, it shows a strong uniform cortical signal (Fig. 7 A); Gai levels are reduced dramatically (100%, n = 76 [Fig. 7 B]); Pins is cortical (Fig. 7 C); Insc is delocalized (100%, n = 23 [Fig. 7 D]); DaPKC becomes uniformly cortical or undetectable (100%, n = 36 [Fig. 7 F]); and spindle geometry late in mitosis remains symmetric (Fig. 7, I and K), suggesting the disruption of both apical pathways. In addition, Mira is delocalized and can segregate into both daughter cells (75%, n = 40 [Fig. 7, G and H]).

Overexpression of a putative constitutively active Gα0Q205L in NBs does not show any defects in spindle geometry (Fig. 7, J and L), suggesting that it is the GDP-bound Gα which is responsible for the defect in size asymmetry in the overexpression experiments. Our results therefore suggest that depletion of free Gβy either by mutation or by greatly increasing the levels of Gαi subunits can compromise the function of both apical pathways. These data are consistent with the view that Gβ13F (Gβγ) can act genetically upstream of apical complex members to mediate their asymmetric localization.

**Discussion**

Here we report the isolation and analysis of loss of function mutations in Gai and show that the loss of Gai and Gβ13F have distinct effects on NB asymmetric cell divisions. Gai is required for Pins cortical association and asymmetric localization; loss of Gai causes Pins to localize to the cytosol, and mutant NBs exhibit phenotypes which are highly similar to those seen in pins mutants. Analyses of double mutant combinations confirm Gai RNAi results showing that Pins/Gai and Baz/DaPKC/Insc act in an redundant fashion to mediate the formations of an asymmetric mitotic spindle and the generation of NB daughters of unequal size. Importantly, our analyses also revealed a new aspect of apical complex function: that the two apical pathways also act redundantly to suppress the formation of astral microtubules from the basal centrosome of NBs. In contrast, Gβ13F appears to act upstream of the apical components and is required for their asymmetric localization/stability. The defects associated with NBs lacking Gβ13F function are highly similar to those seen when the function of both apical pathways have been compromised. In addition, we show that high level overexpression of two different Gα subunits which can bind/complex to Gβ13F result in similar phenotypes seen in Gβ13F mu-
tant NBs, suggesting that it is the depletion of free Gβ13F, which is responsible for the mutant phenotypes.

**Gαi is required to target Pins to the NB cortex**

Our results indicate that Pins and Gαi apical localization are mutually dependent. In pins NBs, Gαi is evenly distributed to the NB cortex, and in Gαi mutant NBs, Pins localizes to the cytosol. We have provided evidence previously that Pins asymmetric localization to the apical cortex of the NBs is a two-step process (Yu et al., 2002): Pins need to be targeted to the cortex first, which requires the COOH-terminal Goloco motifs that can bind Gαi before it can be recruited to the apical cortex in a process which requires its NH2-terminal TPR that can interact with Insc. Our current results therefore suggest that Pins cortical targeting is most likely mediated by Gαi, which cannot only bind Pins but is also able to localize to the plasma membrane through lipid modifications (Casey, 1994).

However, in Gβ13F mutant NBs, although the levels of Pins are drastically reduced, the residual Pins is localized both to the cytosol and to the cell cortex. This poses a problem since in the Gβ13F mutant NBs not only is Gβ13F absent but Gαi is undetectable with an anti-Gαi antibody. One possible explanation is that although Gαi is undetectable, there is still some Gαi remaining in the Gβ13F NBs which may account for the low level residual uniform cortical distribution of Pins. Alternatively, we cannot formally rule out the possibility that the cortical Pins in Gβ13F NBs is due to some unknown molecule that can recruit Pins to cortex in the absence of both Gαi and Gβ13F.

**Gβ13F acts upstream of the apical components to mediate their asymmetric localization**

The analysis of Gβ13F function is complicated by the fact that in the Gβ13F mutant NBs, Gαi levels are also downregulated presumably due to the instability of the protein in the absence of Gβ13F. Although loss of either Gαi or Gβ13F causes aberrations in localization of the basal components and orientation of the mitotic spindle, it is clear that at least some of the defects associated with the loss of Gβ13F cannot be attributable solely to the depletion of Gαi. In the great majority of Gαi mutant NBs, DaPKC and Baz still localize asymmetrically to a subset of the cell cortex. And consistent with our proposal that spindle geometry and the size asymmetry of the NB daughters are mediated by two redundant apical pathways, Pins/Gαi and Baz/DaPKC, the great majority (79%) of the Gαi mutant NBs generate an asymmetric mitotic spindle and divide to produce unequal size daughters. In contrast, in Gβ13F NBs not only do Pins/Gαi always fail to become asymmetrically localized but the majority of mutant NBs (71%) also fail to asymmetrically localize Baz/DaPKC; consequently ~65% of NBs fail to generate an asymmetric mitotic spindle and divide to produce equal size daughters. Therefore, at least formally, Gβ13F acts upstream of the two apical pathways (Fig. 8 A).

We believe that the major reason for the phenotypes associated with loss of Gβ13F function is due to the disruption of Gβγ signaling. We show, as previously reported (Schafer et al., 2001; Cai et al., 2003), that overexpression of Gαi will cause a high frequency of equal size divisions. In addition, we show here that the overexpression of Gαo, a Gα subunit that interacts with Gβ13F but is not itself required for asymmetric divisions in wt NBs, will also mimic the Gβ13F loss of function phenotype. For both overexpression of Gαi and Gαo, the frequency of equal size divisions is significantly higher than that seen in Gβ13F loss of function (~80 versus 65%). This difference may be due to the existence of other Gβ subunits which might also function in NB asymmetric divisions. Three Gβ genes have been identified by the Drosophila genome project, and although one of these genes, concertina, appears not to be involved in the process (Schafer et al., 2001), it is possible that overexpression of Gα subunits may deplete not only Gβ13F but also Gβ7C. This possibility could be addressed by the analysis of double mutants of Gβ genes. Nevertheless, these observations are consistent with the view that the depletion of free Gβγ, and not Gαi, is the major cause for the symmetric divisions seen in Gβ13F mutant NBs (Fuse et al., 2003).
Hence, although previous analysis of GB13F loss of function did not report any effects on NB daughter size, our data are in agreement with those of Fuse et al. (2003) and consistent with the notion that GB13F plays a major role in mediating the distinct size of NB daughter cells.

Apical pathways act redundantly to prevent basal astral microtubule formation

The apical centrosome associates with prominent astral microtubules, whereas the basal centrosome connects to few if any astral microtubules in wt NBs and in mutants in which one of the two apical pathways is compromised. In contrast, in NBs that lack both apical pathways a symmetric mitotic apparatus is established that features extensive arrays of astral microtubules at both centrosomes. Therefore, either of the two apical pathways appears sufficient to prevent formation of basal astral microtubules. It is not clear how this might be accomplished at a mechanistic level. However, one might speculate that there exists an asymmetrically localized molecule, which can act to promote the formation of astral microtubules. When either of the apical pathways is functional, this molecule is asymmetrically localized and promotes the formation of astral microtubules over the centrosome it overlies. However, when both apical pathways are mutated, or when GB13F is mutated or when all apical components become uniformly cortical, e.g., when Gai is overexpressed, then the hypothetical molecule becomes uniformly cortical and can promote the formation of astral microtubules over both centrosomes (Fig. 8 B). This type of model can readily explain why either loss or uniform cortical localization of both apical pathways leads to symmetric astral microtubule formation over both centrosomes.

In summary, our results demonstrate that for NB asymmetric division Gai and GB13F play distinct roles. Gai and Pins are members of one of the two apical pathways and Baz/DaPKC/InsC forms the other. Loss of Gai function results in defects in NB asymmetry that are essentially indistinguishable from those seen in insc mutants. GB13F (Gβγ) functions upstream of both Pins/Gai and Baz/DaPKC/InsC pathways to mediate their stability and/or asymmetric localization (and function). Without GB13F, the function of both apical pathways are attenuated; Gai levels are dramatically reduced and Pins/Gai pathway is defective; in addition, the asymmetric localization of members of the Baz/DaPKC/InsC pathway is often defective. Consequently, loss of GB13F function yields phenotypes which are similar to those seen when both apical pathways are disrupted by mutations. A schematic summary depicting the hierarchical relationship between GB13F and the apical pathways and our speculative model of how the apical pathways might act to “suppress” the formation of basal astral microtubules are depicted in Fig. 8.

Materials and methods

Flies

insc (insc), pins (pins), scabrous-gal4 (sca-gal4), and UAS-Gai were described earlier (Yu et al., 2000; Cai et al., 2003). KG01907 was a gift from H. Bellin ( Baylor College of Medicine, Houston, TX). UAS-Geo and bkh, an allele of Geo, were a gift from M. Semeriva (LGPD, Centre National de la Recherche Scientifique, Marseille, France). FRT101-GB13F was provided by J.A. Knoblich (Research Institute of Molecular Pathology [IMP], Vienna, Austria).

Mobilization of P element

KG01907 carrying a P element derivative that contains the white gene is inserted near the 5’ end of the Gai transcription unit at cytological location 65D6. The P element in this stock was mobilized using Ppy Dr2/3 (99B) as a transposase source. 300 independent white revertant lines were established. These were analyzed on Southern blots using various portions of the Geo cDNA as hybridization probes. Several small deletion events which resulted in deletions that removed some or all of the Gai coding region were recovered.

Germline transformation, overexpression studies, and RNAi experiments

Transgenes were expressed in NBs using either the maternal GAL4 driver V32 (obtained from D. St. Johnston, Welcome/CRC Institute, Cambridge, UK) or scabrous-gal4 (Brand and Perrimon, 1993). UAS-Gao and UAS-Gao were created by cloning the full-length Gao cDNA (Flemion et al., 1999) or a mutant version in which glutamine 205 had been replaced with leucine into pUAST (Brand and Perrimon, 1993). Rescue experiments were performed by driving the expression of the UAS-Gai transgene with a scabrous-gal4 driver in Gai mutant background.

A 0.8-kb PstI fragment of baz cDNA (from Andreas Wodarz, University of Dusseldorf, Dusseldorf, Germany) was used as a template for RNAi experiments and subcloned into a modified pBluescript vector (pKS-ds-T7) (Cai et al., 2001) for double strand RNA synthesis.

Immunocytochemistry and confocal microscopy

Embryos were collected and fixed according to Yu et al. (2000) for α-tubulin and β-tubulin stainings, embryos were fixed with 38% formaldehyde for exactly 1 min. Rabbit anti-Axense (provided by Y.-N. Jan, University of California, San Francisco, San Francisco, CA), rabbit anti-Baz (provided by F. Matsuzaki, Center for Developmental Biology, RIKEN, Kobe, Japan), mouse anti-Eve (Kai Zinn, Caltech, Pasadena, CA), rabbit anti-InsC, rabbit and rat anti-Pins, rabbit anti-Gai (aa 327–355; provided by J.A. Knoblich, IMP), guinea pig anti-Gao (provided by M. Forte, Oregon Health Sciences University, Portland, OR), rabbit anti-PKCe C20 (Santa Cruz Biotechnology, Inc.), rabbit anti-Gβ13F (provided by J.A. Knoblich), rabbit anti-Mira (provided by F. Matsuzaki), rabbit anti-Pon (provided by Y.-N. Jan), rabbit anti-Numb (provided by Y.-N. Jan), mouse anti-α tubulin (DM1A; Sigma-Aldrich), rabbit anti-γ-tubulin (provided by D. Glover, University of Cambridge, Cambridge, UK), rabbit anti-CNN (provided by T.C. Kaufman, Indiana University, Bloomington, IN), anti-ProxMR1A (provided by C.Q. Doe, University of Oregon, Eugene, OR), mouse anti-β gal (Chemicon), anti-β-tubulin E7 (Developmental Studies Hybridoma Bank [DSHB]) and anti-Nrt BP106 (DSHB) were used in this study. Cy3- or FITC-conjugated secondary antibodies were obtained from Jackson Laboratories. Stained embryos were incubated with ToPro3 (Molecular Probes) for chromosome visualization and mounted in Vectashield (Vector Laboratories). Embryos were analyzed with laser scanning confocal microscopy (Bio-Rad Laboratories MRC 1024 and Zeiss LSM510 [Carl Zeiss Microimaging, Inc.]). Images were processed with Adobe Photoshop.

Comunoprecipitation and Western blot

Embryos overexpressing Geo using the maternal GAL4 driver V32 were ground in liquid nitrogen and mixed with five times volume of the lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, and protease inhibitor cocktail from Roche) for 30 min at 4°C. The embryo lysate was centrifuged at maximum speed in a microcentrifuge for 20 min. The supernatant (embryo extract) was used to immunoprecipitate with anti-Gβ13F antibody and the protein A/G beads (Amer sham Biosciences). Beads were washed three times (10 min each) in lysis buffer. Bound proteins were analyzed by Western blots with anti-Geo and anti-Gβ13F.

We thank our colleagues referred to in the Materials and methods section, DSHB (University of Iowa), and the Bloomington stock center for generously providing antibodies and fly stocks. We are grateful to F. Matsuzaki and N. Fuse (Center for Developmental Biology, RIKEN) for generously providing conditions for anti-α-tubulin staining and exchanging and discussing data prior to publication. F. Yu would like to thank S. Ohterukeno for helpful discussion.

X. Yang is an adjunct staff, Department of Anatomy, National University
of Singapore. W. Chia is a Wellcome Trust Principal Research fellow. This work was supported by A*STAR Singapore and the Wellcome Trust.

Submitted: 26 March 2003
Accepted: 7 July 2003

References

Brand, A.H., and N. Perrimon. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development. 118:491–415.

Cai, Y., W. Chia, and X. Yang. 2001. A family of snail-related zinc finger proteins regulates two distinct and parallel mechanisms that mediate Drosophila neuroblast asymmetric divisions. EMBO J. 20:1704–1714.

Cai, Y., F. Yu, S. Lin, W. Chia, and X. Yang. 2003. Apical complex genes control mitotic spindle geometry and relative size of daughter cells in Drosophila neuroblast and pluripotent asymmetric divisions. Curr. Opin. Genet. Dev. 13:51–62.

Campos-Ortega, J.A. 1995. Genetic mechanisms of early neurogenesis in Drosophila melanogaster. Mol. Neurobiol. 10:75–89.

Casey, P.J. 1994. Lipid modifications of G proteins. Curr. Opin. Cell Biol. 6:219–225.

Chia, W., and X. Yang. 2002. Asymmetric division of Drosophila neural progenitors. Curr. Opin. Genet. Dev. 12:459–464.

Doe, C.Q., and B. Bowerman. 2001. Asymmetric cell division: fly neuroblast meets worm zygote. Curr. Opin. Cell Biol. 13:68–75.

Fremion, F., M. Astier, S. Zaffran, A. Guillen, V. Homburger, and M. Semeriva. 1999. The heterotrimeric protein Go is required for the formation of heart epithelium in Drosophila. J. Cell Biol. 145:1063–1076.

Fuse, N., K. Hisata, L.A. Katzen, and F. Matsuzaki. 2003. Heterotrimeric G proteins regulate daughter cell size asymmetry in Drosophila neuroblast division. Curr. Biol. 13:947–954.

Giansanti, M.G., M. Gatti, and S. Bonaccorsi. 2001. The role of centrosomes and astral microtubules during asymmetric division of Drosophila neuroblasts. Development. 128:1137–1145.

Gotta, M., and J. Ahrlinger. 2001. Distinct roles for Galpha and Gbetagamma in regulating spindle position and orientation in Caenorhabditis elegans embryos. Nat. Cell Biol. 3:297–300.

Jan, Y.N., and L.Y. Jan. 2001. Asymmetric cell division in the Drosophila nervous system. Nat. Rev. Neurosci. 2:772–779.

Kaltenbronn, S.A., C.M. Davidson, N.H. Brown, and A.H. Brand. 2000. Rotation and asymmetry of the mitotic spindle direct asymmetric cell division in the developing central nervous system. Nat. Cell Biol. 2:7–12.

Kemphues, K. 2000. PARsing embryonic polarity. Curr. Opin. Dev. Biol. 10:345–348.

Knoeblich, J.A. 2001. Asymmetric cell division during animal development. Nat. Rev. Mol. Cell Biol. 2:11–20.

Kraut, R., and J.A. Campos-Ortega. 1996. incsucutable, a neural precursor gene of Drosophila, encodes a candidate for a cytoskeleton adaptor protein. Dev. Biol. 174:65–81.

Kraut, R., W. Chia, L.Y. Jan, Y.N. Jan, and J.A. Knoblich. 1996. Role of inscutable in orienting asymmetric cell divisions in Drosophila. Nature. 383:50–55.

Kuchinke, U., F. Grawe, and E. Knust. 1998. Control of spindle orientation in Drosophila by the Par-3-related PDZ-domain protein Bazooka. Curr. Biol. 8:1357–1365.

Matsuoka, F. 2000. Asymmetric division of Drosophila neural stem cells: a basis for neural diversity. Curr. Opin. Neurobiol. 10:38–44.

Ohshiro, T., T. Yamashita, C. Zhang, and F. Matsuoka. 2000. Role of cortical tumour-suppressor proteins in asymmetric division of Drosophila neuroblast. Nature. 408:593–596.

Pammentier, M.L., D. Woods, S. Greig, P.G. Phan, A. Radovic, P. Bryant, and C.J. O’Kane. 2000. Rapynoid/partner of incsucutable controls asymmetric division of larval neuroblasts in Drosophila. J. Neurosci. 20:RC84.

Peng, C.Y., L. Manning, R. Albertson, and C.Q. Doe. 2000. The tumour-suppressor genes lgl and dlg regulate basal protein targeting in Drosophila neuroblasts. Nature. 408:596–600.

Petronczki, M., and J.A. Knoblich. 2001. DmPAR-6 directs epithelial polarity and asymmetric cell division of neuroblasts in Drosophila. Nat. Cell Biol. 3:43–49.

Schaefer, M., A. Shevchenko, and J.A. Knoblich. 2000. A protein complex containing Incsucutable and the Galpha-binding protein Pins orients asymmetric cell divisions in Drosophila. Curr. Biol. 10:353–362.

Schaefer, M., M. Petronczki, D. Dorner, M. Forte, and J.A. Knoblich. 2001. Heterotrimeric G proteins direct two modes of asymmetric cell division in the Drosophila nervous system. Cell. 107:183–194.

Schober, M., M. Schaefer, and J.A. Knoblich. 1999. Bazooka recruits Incsucutable to orient asymmetric cell divisions in Drosophila neuroblasts, Nature. 402:548–551.

Siderovski, D.P., M. Diverse-Pierluissi, and L. De Vries. 1999. The GoLoco motif: a Galpha/e binding motif and potential guanine-nucleotide exchange factor. Trends Biochem. Sci. 24:340–341.

Wodarz, A. 2002. Establishing cell polarity in development. Nat. Cell Biol. 4:E39–E44.

Wodarz, A., A. Ramrath, U. Kuchinke, and E. Knust. 1999. Bazooka provides an apical cue for Incsucutable localization in Drosophila neuroblasts. Nature. 402:544–547.

Wodarz, A., A. Ramrath, A. Grimm, and E. Knust. 2000. Drosophila stryphal protein kinase C associates with Bazooka and controls polarity of epithelia and neuroblasts. J. Cell Biol. 150:1361–1374.

Yu, F., X. Morin, Y. Cai, X. Yang, and W. Chia. 2000. Analysis of partner of incsucutable, a novel player of Drosophila asymmetric divisions, reveals two distinct steps in incsucutable apical localization. Cell. 100:399–409.

Yu, F., C.T. Ong, W. Chia, and X. Yang. 2002. Membrane targeting and asymmetric localization of Drosophila partner of incsucutable are discrete steps controlled by distinct regions of the protein. Mol. Cell. Biol. 22:4230–4240.