

Lethal Giant Larvae Acts Together with Numb in Notch Inhibition and Cell Fate Specification in the *Drosophila* Adult Sensory Organ Precursor Lineage

Nicholas Justice,¹ Fabrice Roegiers,¹
Lily Yeh Jan, and Yuh Nung Jan*
Departments of Physiology and Biochemistry
Howard Hughes Medical Institute
University of California, San Francisco
533 Parnassus Avenue
San Francisco, California 94143-0725

Summary

The tumor suppressor genes *lethal giant larvae* (*lgl*) and *discs large* (*dlg*) act together to maintain the apical basal polarity of epithelial cells in the *Drosophila* embryo [1]. Neuroblasts that delaminate from the embryonic epithelium require *lgl* to promote formation of a basal Numb and Prospero crescent, which will be asymmetrically segregated to the basal daughter cell upon division to specify cell fate [2, 3]. Sensory organ precursors (SOPs) also segregate Numb asymmetrically at cell division. Numb functions to inhibit Notch signaling and to specify the fates of progenies of the SOP that constitute the cellular components of the adult sensory organ. We report here that, in contrast to the embryonic neuroblast, *lgl* is not required for asymmetric localization of Numb in the dividing SOP. Nevertheless, mosaic analysis reveals that *lgl* is required for cell fate specification within the SOP lineage; SOPs lacking *Lgl* fail to specify internal neurons and glia. Epistasis studies suggest that *Lgl* acts to inhibit Notch signaling by functioning downstream or in parallel with Numb. These findings uncover a previously unknown function of *Lgl* in the inhibition of Notch and reveal different modes of action by which *Lgl* can influence cell fate in the neuroblast and SOP lineages.

Results and Discussion

Lgl functions with *Dlg* to specify the formation of a protein crescent at the basal cortex of asymmetrically dividing neuroblasts in the *Drosophila* embryo [2, 3]. Mutations in *lgl* and *dlg* do not affect the apically localized complex of Bazooka/DaPKC/DmPar-6, which is inherited by neuroblasts that delaminate from the epithelium; however, they disrupt basal Numb and Prospero crescent formation and thereby affect the asymmetric segregation of cell fate determinants upon cell division [2, 3]. Sensory organ precursor (SOP) cells also divide asymmetrically during the development of the *Drosophila* adult peripheral nervous system but differ from neuroblasts in the plane of division and in the role played by *Dlg* [4]. The SOP follows planar polarity cues to divide asymmetrically within the epithelium along the anterior-posterior axis, and, as a result, the anterior daughter *pIIb* differs from the posterior daughter *pIIa* in cell fate

[5, 6]. *Dlg* forms a complex with Pins at the anterior cortex and causes posterior localization of Bazooka and, in turn, anterior localization of Numb [4, 7]. How might *Lgl* be involved in the polarity and asymmetric division of the SOP? Little is known about the function of *Lgl* in the SOP, or whether it acts together with *Dlg*.

To further characterize the role of *Lgl* in the formation of crescents in the SOP, we generated mitotic clones homozygous for either of two protein null *lgl* alleles: *lgl^f*, a small deletion removing the *lgl* coding sequence [8], and *lgl^{fw3}*, a loss-of-function point mutation [1]. Using the MARCM system to restrict UAS transgene expression to clonal tissue [9], we expressed Partner of Numb (Pon)-GFP in SOPs within mutant clones under the control of *neuralized*-Gal4, which allows the visualization of Numb crescent formation as SOPs divide, without affecting cell fate [6, 10, 11]. In SOPs within *lgl* mutant clones, Pon-GFP crescents are seen forming normally at the anterior cortex in all cases (Figure 1B, n = 35), as in control clones. Antibody staining against Numb protein revealed anterior crescents in mitotic SOPs both inside and outside *lgl* mutant clones (Figures 1C and 1D). Given the similarity in cortical and cytoplasmic protein localization of *Lgl* in neuroblasts [2, 3] and SOPs (Figures 1G and 1H), as well as the importance of *Lgl* to the asymmetry of neuroblasts, we were very surprised to find that both null alleles of *lgl* fail to disrupt the formation and segregation of Numb crescents in the dividing SOP. We investigated other aspects of SOP polarity that might be predicted to depend on *Lgl*. Upon staining, however, we saw both DaPKC (Figures 1C and 1D) and Bazooka (not shown) localized to posterior crescents opposite Numb in the dividing SOP within *lgl* clones, and these SOPs were indistinguishable from SOPs within neighboring wild-type tissue. It thus appears that *Dlg* and *Lgl* function independently in the SOP, since the loss of *lgl* does not alter asymmetric localization of posterior crescent components or anterior crescent components, which both depend on *Dlg* [4]. Despite the normal polarity observed in *lgl* mutant SOPs, loss of *Lgl* has a strong influence on cell fate determination of SOP progenies that will form the ES organ in the adult fly.

ES organs are normally visible as two external cells (a hair cell projecting through a single socket), and both cells comprise a single sensory bristle (Figure 2A). Clones of *lgl* on the notum cause large tumors and the disruption of junctions between epithelial cells; this finding is consistent with previous reports of *lgl* mutant phenotypes in imaginal disc tissue [8, 12]. Within these *lgl* mutant clones, ES organs appear malformed, containing additional external cells (Figure 2B, arrows). Very similar phenotypes were found in clones mutant for *lgl^f* or *lgl^{fw3}*, in which no *Lgl* protein was detectable (Figure 2F). Most mutant ES organs consist of three sockets and one hair cell (84%, n = 86, Figure 2B, arrows). Clusters of four sockets are also present at a lower frequency (12%, n = 86, Figure 2B, arrowhead). Within mutant clones stained for markers of neurons (anti-Elav) and glia (anti-Pros) at pupal stages, we find both internal

*Correspondence: ynjan@itsa.ucsf.edu

¹These authors contributed equally to this work.

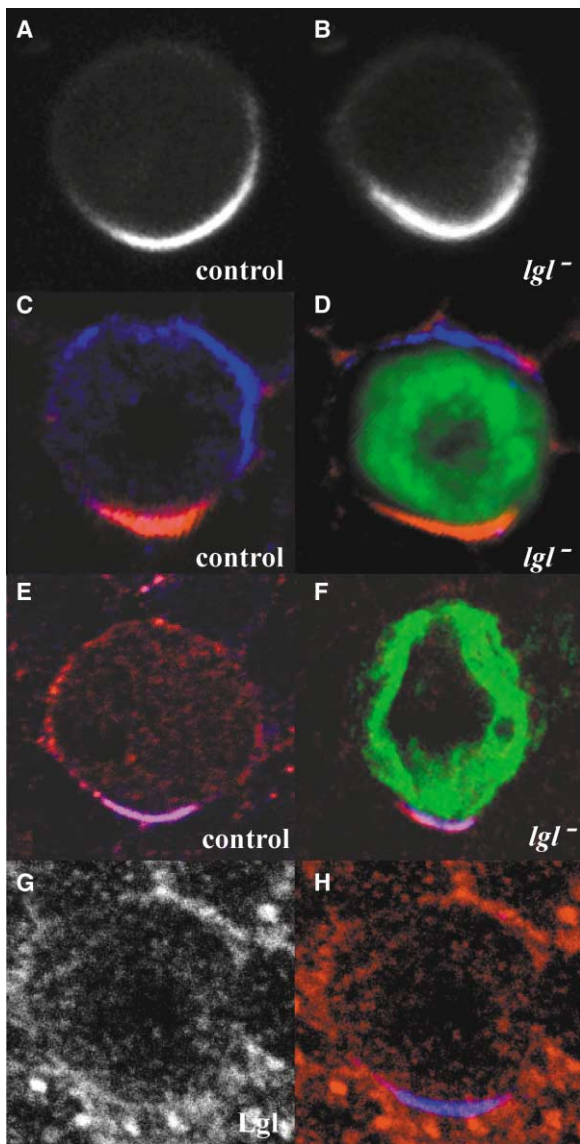


Figure 1. Lgl Is Not Required for Asymmetric Localization of Cell Fate Determinants in the SOP Cell

(A and B) Asymmetric localization of Pon-GFP is not dependent on Lgl. Mitotic SOPs in both (A) control and (B) *lgl*⁻ mutant clones (*lgl*⁻) form crescents of Pon-GFP at the anterior cortex.

(C and D) Polarity of the SOP does not require Lgl. DaPKC (blue) localizes to the posterior cortex opposite the anterior Numb crescent (red) in mitotic SOP cells in both (C) control and (D) *lgl*⁻ mutant clones (marked in green by mCD8-GFP; *lgl*⁻).

(E and F) Lgl is not required for asymmetric accumulation of α -Adaptin in mitotic SOPs. α -Adaptin (red) colocalizes with Numb (blue) in an anterior crescent (magenta) in mitotic SOPs in both (E) control and (F) *lgl*⁻ mutant clones (marked in green by mCD8-GFP; *lgl*⁻).

(G and H) Lgl localization is not polarized in the SOP. (G) Lgl protein was found uniformly distributed along the cell cortex and in puncta throughout the cytoplasm, (H and G) while Numb (blue) forms cortical crescents in a wild-type mitotic SOP.

cells missing in clusters derived from *lgl* mutant SOPs marked with GFP (96%, n = 26 clusters, Figure 2E, arrows). All four cells of these GFP-marked clusters are

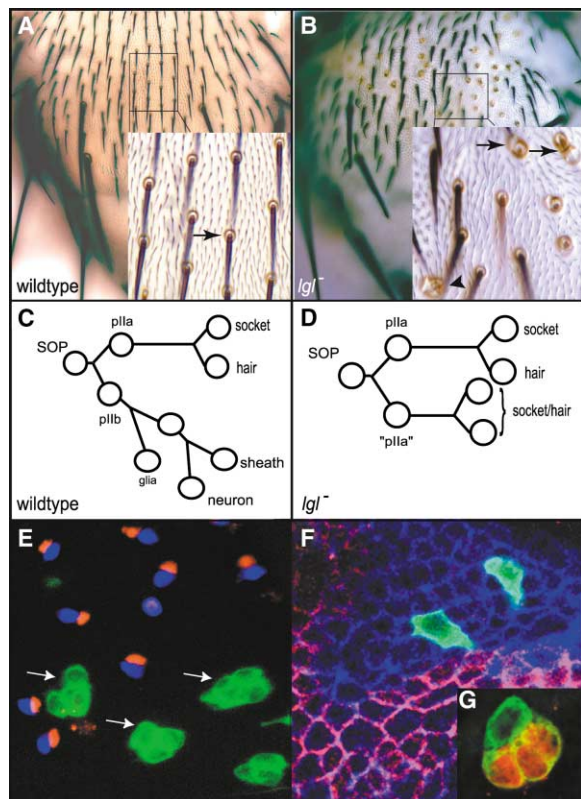


Figure 2. Lgl Is Required for Specification of the Internal Neurons and Glia of the SOP Lineage

(A) The wild-type morphology of the ES organ (arrow), comprised of a single hair and socket, is visible externally on the notum of an adult fly.

(B) An *lgl* mutant clone (marked by yellow bristles) shows the appearance of additional sockets in each ES organ, often apparent as a double socket alongside the normal hair and socket (arrows) or as a four-socket cluster (arrowhead).

(C) Diagram of the wild-type SOP lineage that produces the five cells of each ES organ.

(D) Diagram of the cell fates observed in SOP progeny that lack *lgl*. The *pllb* is transformed into an ectopic *plla* cell, which divides to produce extra sockets and hairs in each ES organ.

(E) Staining of an *lgl* clone marked by expression of mCD8-GFP in SOP progeny cells. Anti-Elav antibodies label neurons in blue, and anti-Pros antibodies label glial sheath cells in red. Internal cells of the SOP lineage are absent in *lgl* mutant clones (the arrows point to examples). The normal neuron and glia are present as a pair of cells in wild-type clusters of SOP progeny outside of the clonal boundary.

(F) An anti-Lgl antibody displays the absence of Lgl protein (red) in an *lgl* mutant clone on the adult notum. Numb protein (blue) is predominantly cortical in cells both within an *lgl* mutant clone and in wild-type tissue. SOPs mutant for *lgl* are marked by mCD8-GFP expression.

(G) An *lgl* mutant ES organ stained for Su(H) (red), which labels socket cells, shows the most common cell fates found (three sockets and one hair) within clusters of externally transformed *lgl* mutant SOP progeny (labeled by mCD8-GFP in green).

typically larger, characteristic of the morphology of external hair and socket cells. When clonal notae are labeled for Suppressor of Hairless (Su(H)), *lgl* mutant clusters most often contain three or four Su(H)-positive cells,

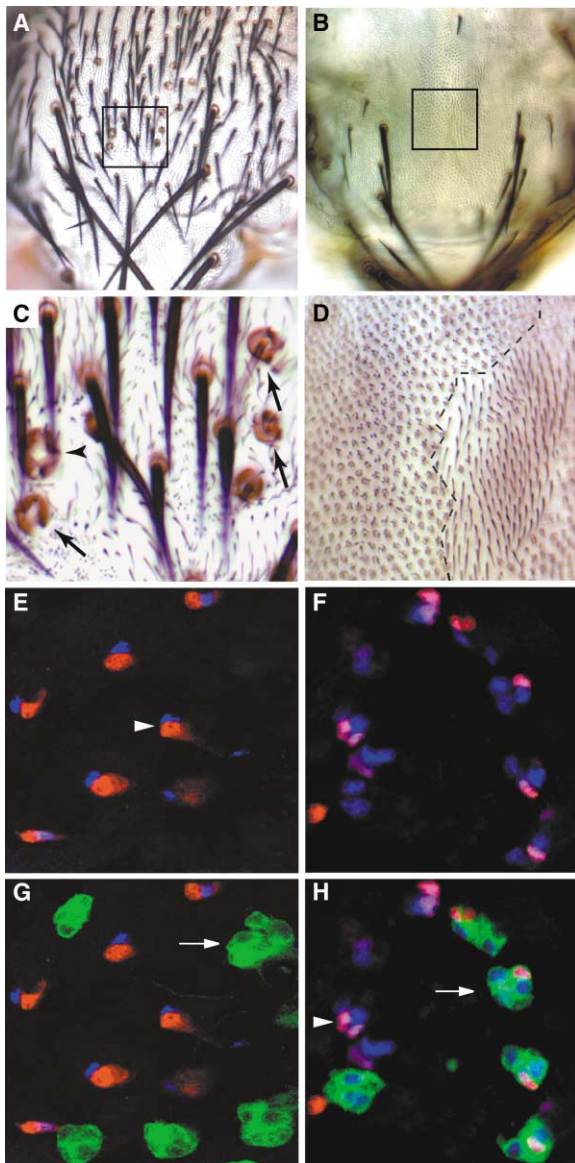


Figure 3. Inactivation of Notch during Cell Fate Specification in the SOP Lineage Reverses the *Igl* Mutant Phenotype

(A–D) (A and C) The adult notum of a female control fly, heterozygous for *Notch^{ts}* and containing *Igl* mutant clones, that has been shifted to the restrictive temperature (29°C) during cell fate specification of the microchaetes (12–20 hr APF). The microchaete exhibit *Igl* loss-of-function multiple socket phenotypes (arrows), including four socket clusters (arrowhead). (B and D) After undergoing the same temperature shift paradigm to 29°C (12–20 hr APF), the adult notum of a male fly, hemizygous for *Notch^{ts}* and containing *Igl* mutant clones, is completely bald. The presence of clones is indicated by *ck*-marked twin spots (dashed line). The near complete loss of bristles in *Igl* mutant and wild-type tissue indicates that the *Notch* phenotype is epistatic to *Igl*. The portions boxed in (A) and (B) are shown in (C) and (D), respectively.

(E–H) Internal cell clusters in *Igl* mutant clones have increased numbers of neurons and glia in *Notch^{ts}* pupae shifted to the restrictive temperature during cell fate specification. (E and G) A region of the developing notum from a female control fly, heterozygous for *Notch^{ts}* and containing *Igl* mutant clones, that has been shifted to 29°C during cell fate specification of the microchaetes (12–18 hr APF). (E) Groups of one Elav (blue, neuronal marker)-positive cell and one Pros (red, glial marker)-positive cell are present in clusters outside

indicating the presence of extra socket cells within each cluster (94%, $n = 18$ clusters, Figure 2G). In wild-type tissue surrounding mutant clones, internal neuron and glia pairs are present in regularly spaced arrays (Figure 2E). We can interpret the observed cell fate changes seen in *Igl* mutant clones because of the well-characterized lineage of the adult SOP [5]. Loss of *Igl* causes the p11b cell, which normally gives rise to internal cells including a neuron and a glia, to adopt the p11a cell fate and produce two additional external cells at the expense of internal cells of the lineage (Figure 2D).

The transformation of internal cells to supernumerary socket and hair cells in *Igl* mutant clones is similar to *numb* loss-of-function and *Notch* gain-of-function phenotypes in the SOP lineage [13–17]. One possible explanation for this similarity is that Lgl functions to inhibit Notch signaling activity. To test this possibility, we used a temperature-sensitive allele of *Notch* (*Notch^{ts}*) to inactivate Notch in *Igl* mutant clones on the adult notum. *Notch^{ts}* pupae shifted to the restrictive temperature (29°C) during divisions of the SOP (12–24 hr APF) show a loss of external socket and hair cells (balding), accompanied by an increased number of internal cells [18]. Temperature shifts performed on hemizygous *Notch^{ts}* pupae cause loss of external cells (balding), both within and outside *Igl* mutant clones (Figures 3B and 3D), which is in contrast with the external transformation phenotypes that remain in *Igl* mutant clones on *Notch^{ts}* heterozygous control flies (Figures 3A and 3C). When examined internally, GFP-marked *Igl* mutant clusters are composed entirely of internal cells expressing Pros and/or Elav in hemizygous *Notch^{ts}* pupae (Figures 3F and 3H), while, in *Igl* mutant heterozygous controls, clonal clusters lack internal Elav/Pros-positive cells (Figures 3E and 3G). Taken together, these results indicate that the transformation of internal cells to external cells observed in *Igl* mutant ES organs is due to an increase in Notch signaling activity, and that *Igl* functions upstream of *Notch* to inhibit Notch signaling activity and thereby influence cell fate within the SOP lineage.

Numb specifies cell fate, possibly via direct physical interaction with Notch [16], by inhibiting Notch activity in the daughter cell to which it is asymmetrically segregated. *Igl* and *numb* mutants exhibit similar cell fate phenotypes, and both cause transformation of the p11b cell into an ectopic p11a, which divides once to generate two additional external cells in each SOP cluster (Figures 4C and 4E). Our observations that *Notch* is epistatic to *Igl* positions Lgl with *Numb* as an upstream inhibitor of Notch signaling activity and raises the question of whether Lgl functions as part of the same Notch inhibi-

the *Igl* mutant clone (arrowhead). (G) The same region as in (E). The SOP progeny within the *Igl* MARCM mutant clone are positively marked by mCD8-GFP (green); the *Igl* mutant clusters (arrow) do not express Elav or Pros. (F and H) A region of the developing notum of a male fly, hemizygous for *Notch^{ts}* and containing an *Igl* mutant clone, that has undergone the same shift to 29°C (12–20 hr APF). (F) Multiple Elav (blue)- and Pros (red)-positive cells are present in clusters. (H) The same region as in (F). The SOP progeny within the *Igl* MARCM mutant clone are positively marked by mCD8-GFP (green). Clusters of cells expressing Elav (blue) and Pros (red) are present inside (arrow) as well as outside of the clone (arrowhead).

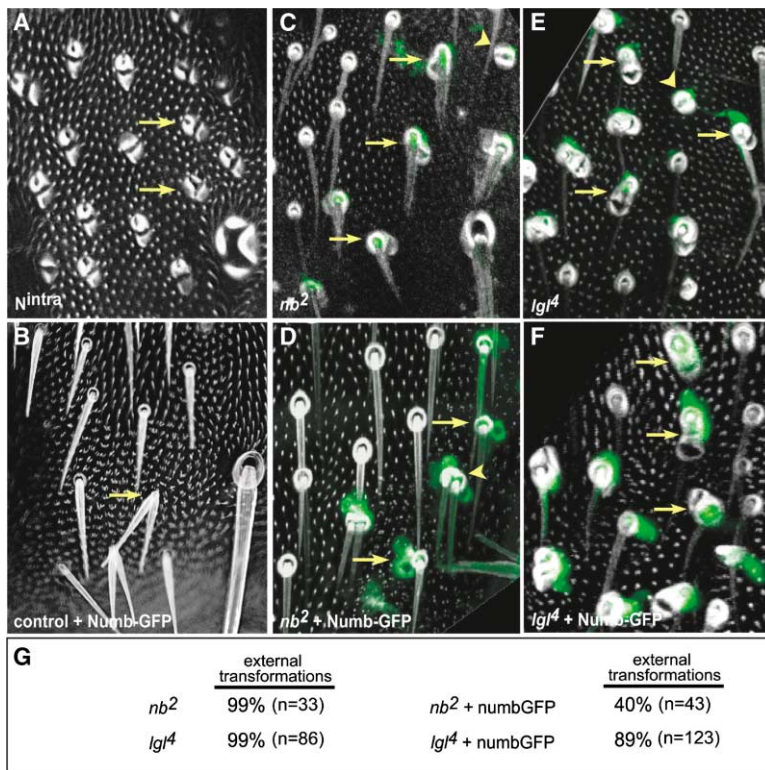


Figure 4. *Lgl* Is Required for Numb-GFP to Inhibit Notch

(A) Overexpression of Notch^{tra} in the SOP lineage causes a multiple socket phenotype (arrows).

(B) Expression of the Numb-GFP causes transformation of external to internal cells within the SOP lineage (balding) and transformation of socket to hair (twinning, arrow).

(C) In a *numb*² mutant clone (marked by GFP expression), multiple sockets are present throughout the clone. The most common phenotype is three sockets and one hair cell (arrows). Double sockets are also present (arrowhead).

(D) Expression of Numb-GFP rescues 60% of ES organs to wild-type morphologies within *numb*² clones (arrows). The appearance of a partially rescued two hair/two socket phenotype is also common (arrowhead).

(E) In an *lgl*⁴ clone (marked by GFP expression), the phenotype is similar to that seen in *numb*² clones, and the clone exhibits external transformations such as three sockets and one hair (arrows) and ectopic socket clusters (arrowhead).

(F) Expression of Numb-GFP fails to rescue the external transformation phenotype in an *lgl*⁴ clone (arrows).

(G) Quantification of the bristle phenotypes from genotypes shown in (C)–(F). An ES organ was counted as externally transformed if it contained more than one hair and one socket;

thus, all three external phenotypes observed, four socket, three socket/one hair, and two hair/two socket, were considered external transformations. The n values are the total number of clonal bristles counted for each genotype.

tory mechanism as Numb to specify cell fate. In order to test this possibility, we examined the epistatic relationship of *numb* and *Lgl* during cell fate specification in the SOP lineage. Expression of Numb-GFP in SOPs on the notum with *neuralized*-Gal4 results in balding and twinned hairs without sockets, due to the transformation of *pIIa* to *pIIb* and transformation of socket to hair (Figure 4B); thus gain-of-function phenotypes opposite to *numb* loss-of-function phenotypes are produced [6]. We further verified that Numb-GFP could replace endogenous Numb function by expressing Numb-GFP in SOPs within *numb*² clones that lack endogenous Numb protein. Numb-GFP rescued the *numb* loss-of-function multiple socket phenotype (Figure 4C) in 60% (Figure 4G) of mutant ES organs (Figure 4D, arrows). Given that Numb-GFP can restore Numb function and rescue cell fate transformations caused by loss of *numb*, we tested whether Numb-GFP could rescue cell fate transformations caused by loss of *Lgl* function. Misexpression of Numb-GFP in SOPs mutant for *Lgl* failed to alter external cell fate transformation phenotypes. The majority (89%) of *Lgl* mutant SOPs (Figure 4G) expressing Numb-GFP produced multiple socket ES organs identical to bristle phenotypes seen with *Lgl* mutations alone (Figures 4E and 4F). The only detectable difference between experimental and control *Lgl* mutant SOPs was the presence of Numb-GFP (Figure 4F), which was asymmetrically segregated to the anterior cell upon division (not shown). The failure of rescue by Numb-GFP suggests that *Lgl* functions downstream or in parallel with *numb* to inhibit Notch and influence cell fate.

The discovery that *Lgl* function is required to specify cell fate within the SOP lineage, but does not affect asymmetric segregation of Numb, suggests that *Lgl* function is distinct from *Dlg* function in the SOP. *Lgl* function is most likely required after polarization of the SOP and somehow contributes to the selective inhibition of Notch activity that specifies the fate of the *pIIb* cell. How might *Lgl* fulfill this function? *Lgl* is a WD repeat-containing protein conserved in eukaryotes ranging from yeast to man [8, 19–22]. Similar to many other WD repeat-containing proteins, *Lgl* likely interacts with multiple partners in a dynamic manner. It binds type II myosins and t-SNAREs on the plasma membrane and is known to be involved in exocytosis in yeast and *Drosophila* by presumably targeting vesicles to the plasma membrane and thereby inserting membrane proteins at specific zones along the apical-basal axis of epithelial cells and releasing extracellular signaling molecules such as DPP [1, 21–24]. The requirement for *Lgl* function, however, is not restricted to membrane proteins and secreted proteins that require vesicular transport. For example, formation of the basal crescent in neuroblasts involves cytoplasmic and cortical movements of globular proteins, such as Numb, Pon, Prospero, and Miranda, that attach to the cytoplasmic side of the membrane via lipid modifications or association with membrane proteins [2, 3, 10]. One plausible scenario for the role of *Lgl* in mediating basal Numb crescent formation in neuroblasts is that *Lgl* and motor proteins form a complex that mediates basal transport of determinants [2, 3, 25]. Such *Lgl*-containing adaptor complexes in the

SOP must differ from those in embryonic neuroblasts under this scenario, given that anterior Numb crescent formation in the SOP is independent of Lgl.

Recently, the AP2 complex-protein α -Adaptin has been shown to asymmetrically localize to the anterior crescent in mitotic SOPs in a Numb-dependent manner [26]. α -*adaptin* mutations result in cell fate phenotypes strikingly similar to both *numb* and *lgl* [26]. A requirement for Lgl to appropriately localize proteins essential for Numb-mediated inhibition of Notch, such as α -Adaptin, could account for the cell fate transformations that result from *lgl* loss of function. Given the proposed function of Lgl in vesicle targeting, we imagined that Lgl might be required in the SOP to deliver proteins that function in a Numb-mediated mechanism to promote Notch inhibition. We tested this idea by staining *lgl* mutant clones with antibodies against α -Adaptin and looking for differences in its localization in dividing SOPs. We found no effect of *lgl* mutations on the asymmetric localization of α -Adaptin to anterior crescents in dividing SOPs (Figures 1E and 1F). While the asymmetric localization of α -Adaptin is not dependent on *lgl*, a scenario in which Lgl is required to deliver components of the machinery required for Numb-mediated inhibition of Notch cannot be excluded. Alternatively, Lgl could directly participate in such a mechanism and could perhaps target endocytic vesicles containing Numb and Notch to the lysosome for degradation. A direct role for Lgl in the Notch pathway is supported by recent studies suggesting that vesicle trafficking of Notch and Delta plays a critical role during Notch pathway signaling [27]. Lgl might bring Notch inhibitors to the plasma membrane or traffic endocytic vesicles in an inhibitory mechanism with Numb and α -Adaptin that specifies cell fates in the SOP lineage.

Experimental Procedures

Fly Stocks and Genetics

Mosaic clones of *lgl* were made by using *FRT40A*-recombined alleles of *lgl^f* and *lgl^{nb2}* [1] in a background containing either *yw Ubx-flp* (kindly provided by J. Knoblich), which generates large clones [28] in wing imaginal discs, or *yw heat-shock flp*, crossed to either *y⁺ ck FRT40A/CyO* to generate externally marked clones or to *p(tub)-gal80 FRT40A*; *neuralized-Gal4*, UAS-mCD8::GFP, or UAS-Pon::GFP/*TM6y⁺* to generate MARCM clones [9], positively marked by expression of GFP in the SOP lineage (see [6, 11]). First instar larvae were heat shocked for 30 min at 37°C to generate large clones with *hs-flp*. Similar crosses were performed to generate clones of *numb* by using the previously described *FRT40A* recombinant of *numb²* [15]. In Numb overexpression experiments, UAS-Numb-GFP was present on the third chromosome and was selectively expressed in SOPs and their lineage by *neuralized-Gal4* (now in the absence of UAS-mCD8::GFP) within clonal tissue. Clones in which Numb was overexpressed in a background wild-type for *lgl* and *numb* were generated by crossing *yw Ubx-flp*; *y⁺ ck FRT40A* to *p(tub)gal80 FRT40A*; *neuralized-Gal4/TM6y⁺*. Numb rescue clones were generated by crossing *yw Ubx-flp*; *y⁺ nb² ck FRT40A/CyO*; UAS-Numb-GFP to *p(tub)gal80 FRT40A*; *neuralized-Gal4/TM6y⁺*. Clones that overexpressed Numb in an *lgl* mutant background were generated in similar crosses, but *yw Ubx-flp*; *lgl^f FRT40A*; UAS-Numb-GFP was used instead. For *Notch^{ts}* epistasis experiments, *Notch^{ts}* was recombined with *yw Ubx-flp* to generate *Notch^{ts} Ubx-flp*, which was then crossed to *lgl^f FRT40A/CyO*, from which males were obtained for use in MARCM crosses as described above. White pupae were collected and aged for 12 hr at 25°C, then shifted to 29°C for 8 hr to inactivate Notch activity specifically during cell fate specification in the SOP lineage [18]. Overexpression of *Notch^{intra}*

was achieved by crossing a UAS-Notch-dB2A2 (kindly provided by E. Giniger) to 109(68)-Gal4 [16].

Live Imaging and Immunohistochemistry

All live cells and immunohistochemical-labeled cells were visualized on a Leica PS2 confocal microscope, with one exception stated below. For staining and live imaging, pupae were selected at pupariation and were aged at 25°C for 15–18 hr to visualize the divisions of the SOP lineage, for 24–28 hr to analyze cell fate, or to the pharate adult stage (approximately 80–100 hr) to analyze external morphology. Adult nota were dissected and placed in 80% isopropanol and were mounted in Hoyer's medium. Images of adult nota were taken on a Nikon E800 microscope equipped with a Spot digital camera and Software (Diagnostic Instruments). Live imaging of control and *lgl* mutant clones was performed essentially as described in [6, 7]. However, for the purposes of quantification, external phenotypes were visualized by using reflective confocal microscopy; the nota of live pharate adults were imaged by using 488/568 excitation laser lines, the reflection images were collected by using the Cy3/Rhodamine filter set (585nm LP), and the GFP images were collected by using the Cy2/GFP filter set (522 nm DF) on a Biorad MRC600 confocal microscope. Mutant ES organs were identified due to their expression of GFP, and they were then scored for the number of hairs and sockets present. Control and mutant clone pupae were fixed and stained by using standard protocols. The antibodies used were rabbit anti-Prospero (1/1000), rat anti-Elav 7E8A10 (1/100; Developmental Studies Hybridoma Bank, University of Iowa), rat anti-mCD8 (1/100; Caltag), rabbit anti-Bazooka (1/1500; kindly provided by A. Wodarz), rabbit anti-nPKC ζ C-20 (1/1000; Santa Cruz Biotechnology), rabbit anti- α -Adaptin (1/100; kindly provided by M. Gonzalez-Gaitan), guinea pig anti-Numb (1/1000), rat anti-Su(H) (1/1500; kindly provided by F. Schweisguth), rabbit anti-Lgl (1/1000; kindly provided by F. Matsuzaki), and rat anti-Lgl (1/100; kindly provided by C.Q. Doe).

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