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Mechanisms underlying neural cell fate specification in the Drosophila sensory organ precursor lineage

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Mechanisms underlying neural cell fate specification in the *Drosophila* sensory organ precursor lineage

by

Nicholas John Justice

DISSERTATION

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I dedicate this dissertation to my mother.

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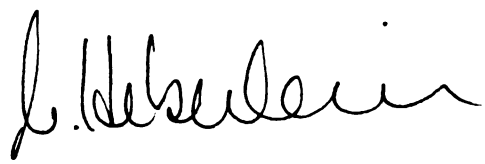
Abstract

Mechanisms underlying neural cell fate specification in the *Drosophila* sensory organ precursor lineage.

By Nicholas John Justice

The sensory organ precursor (SOP) lineage is an excellent model system in which to study the molecular mechanisms that coordinate cell fate decisions in the developing *Drosophila melanogaster* peripheral nervous system. I have used the SOP lineage to search for genes that influence the determination of the hair, socket, neuron, and glial cells that comprise the external sensory (ES) organs in the adult fly. The SOP and all of its progeny cells require Notch signaling to be properly specified. We systematically tested the effects of gene misexpression on Notch mediated cell fate specification in the SOP lineage using a misexpression screen. I characterized two of the genes that exhibit misexpression phenotypes consistent with defects in Notch pathway signaling. *tribbles* misexpression causes cell fate transformations within the lineage, producing extra neurons at the expense of hair and socket cells. *bantam* misexpression results in the tufting of sensory bristles, perhaps by disrupting Notch pathway mediated lateral inhibition during selection of the SOP. Using loss-of-function analysis, we demonstrated that *lethal giant larvae* (*lgl*) is required for the specification of neurons and glia in the SOP lineage. Furthermore, we found that *lgl* acts genetically upstream of *Notch* and downstream of *numb*, suggesting a direct function for Lgl in the Numb-mediated inhibition of Notch signaling activity. Lgl is a tumor suppressor essential for the establishment and maintenance of cell polarity in epithelial cells in both *Drosophila* and mammalian cells. The discovery that Lgl influences Notch pathway signaling suggests

that this might be a general mechanism for the coordination of cell polarity, cell fate, and proliferation.



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Introductory Overview

All of the cells that compose an individual multicellular organism are derived from one cell; in humans this cell is the fertilized egg. Development of a growing organism involves the proliferation, patterning, and determination of cells in a highly organized process that results in the specification of every cell to a distinct fate. The path of differentiation followed by a newborn cell is determined by the intrinsic factors inherited through a cell's lineage and by extrinsic factors present in the extracellular environment. Signaling between cells allows for the coordination of cell fate decisions within a developing tissue. This is important not only for patterning a developing multicellular structure, but also for insuring that related cells of a common origin adopt different fates, such that every cell type needed within the tissue is represented. In this way, the differentiation of cells is orchestrated to construct a tissue, such as the musculature, skeleton, or nervous system, which will function as part of the mature organism. The entire cell lineage that begins with a single-celled oocyte and ends with terminally differentiated and functional cells is far too complex to study as a whole. Therefore, simple lineages within model genetic systems have been used to study the molecular mechanisms that mediate cell fate decisions. Many of the themes of cell fate specification described in these simple lineages recur throughout metazoan development; each mechanism has been adapted to a given phase of cell fate determination to influence how every cell will ultimately differentiate within the organism.

The Sensory Organ Precursor (SOP) lineage in the developing *Drosophila melanogaster* peripheral nervous system is a model system for genetic analysis of the molecular mechanisms that specify neural cell fate during development. The SOP

generates the External Sensory (ES) organs in a well-characterized series of cell divisions, visible as bristles on the adult fly (Figure 1). By creating mutations that cause errors in the development of the ES organs, the involvement a particular gene in the normal process of cell fate specification is revealed. For example, mutations in the genes *achaete* and *scute* result in the failure to specify the SOP, the first cell of the SOP lineage (Cubas et al., 1991). These genes are now known to confer on epithelial cells the potential to become neural precursors. Patches of cells initiate expression of genes in the *achaete – scute Complex (as-C)* to establish neural potential in cells of proneural clusters in the developing *Drosophila* epithelium (Ghysen and Dambly-Chaudiere, 1989). A subset of these equipotent cells will adopt the neural cell fate of the SOP.

Epithelial cells in proneural clusters communicate with each other via the extracellular receptor protein, Notch, and its ligand, Delta. In a process termed lateral inhibition, Notch/Delta signaling activity between cells in contact selects one cell from each cluster to become an SOP (Artavanis-Tsakonas et al., 1999). Notch activity inhibits the acquisition of a neuronal fate, in part by down-regulating the expression of the *as-C*, while creating a feedback loop by inhibiting Delta expression (Heitzler et al., 1996). This negative feedback amplifies differences in Notch activity between cells, resulting in low Notch activity in a single cell within each cluster, which selects that cell as the SOP. Thus, the SOP cell acquires a neuronal fate via activity of the intrinsic transcription factors *achaete* and *scute*, which establish neuronal potential, followed by the extrinsic activity of Notch/Delta signaling between equipotent cells to select one cell as the SOP.

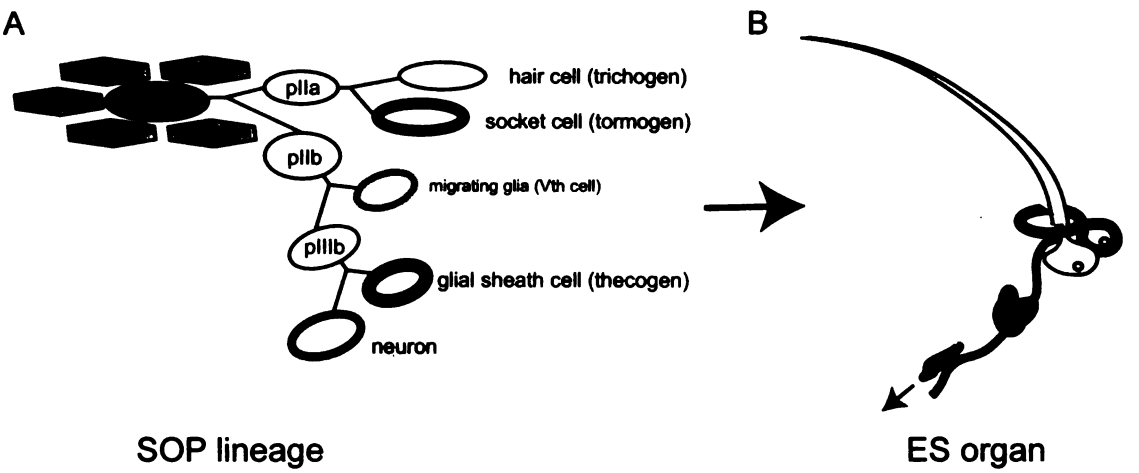
Once the SOP has been specified, this neuronal precursor cell rapidly acquires characteristics that distinguish it from other cells in the epithelium, including changes in

morphology, polarity, and gene expression. As the SOP divides to produce the five cells that will comprise the mature ES organ, proteins are asymmetrically distributed to one pole of the dividing cell such that the cellular factors inherited by each daughter cell differ (Bellaiche et al., 2001a; Gho et al., 1999; Rhyu et al., 1994; Roegiers et al., 2001b). In the first division, which occurs in the plane of the epithelium after cell polarity has been oriented along the anterior-posterior axis, the cell fate determinant Numb is asymmetrically localized to an anterior crescent and inherited by the anterior daughter cell upon division. After cell division, this cell becomes specified as the pIIb and remodels its cytoskeleton to polarize perpendicular to the epithelium, now with its poles oriented along an apical-basal axis (Figure 1A). Numb segregates to the basal daughter cell in each of two successive divisions, which result in the production of two glia and a neuron. Numb inhibits Notch activity through direct interaction with the Notch intracellular domain (Guo et al., 1996), perhaps blocking essential proteolytic cleavage events that occur during the activation of the receptor (Struhl and Adachi, 1998), and thereby biasing the relative levels of Notch activity between the two daughters of a given cell division (Rhyu et al., 1994). This mechanism of Numb mediated inhibition of Notch is used reiteratively in the SOP lineage to specify each of the fates of the progeny cells of the SOP that will form the mature ES organ (Figure 1).

The relatively simple lineage of the SOP, along with many tools developed for its genetic manipulation, provides a system in which we can study how intrinsic and extrinsic molecular mechanisms specify the neuron, glia, hair and socket cell of the mature ES organ (Figure 1B). Screens for enhancer traps have identified genetic elements that selectively express genes only in the SOP and cells of its lineage. Other fly lines

have been engineered that restrict transgenic expression to the pIIb internal branch of the lineage, or to just one cell, such as the socket cell or neuron. The fates of cells can be visualized and altered by selective expression of proteins that are known to influence cell fate decisions within the SOP lineage. Additionally, the MARCM system allows for clonal tissue to be positively marked, making possible the identification of SOP clusters derived from mutant tissue within mitotic clones (Lee and Luo, 1999). Because of the relatively simple nature of the SOP lineage that allows for straightforward analysis of phenotypes, many discoveries are still being made in this model system that continue a long contribution to our understanding of the complex molecular mechanisms that specify cell fate during development.

Figure 1: The SOP lineage produces the cells of the External Sensory Organ



(A) The SOP lineage begins with the division of the SOP in the plane of the epithelium. The pIIb precursor cell remodels its cytoskeleton and divides twice in an apicobasal orientation to produce a migrating glia (light blue), a neuron (lavender) and a glial sheath cell (red). The pIIa precursor cell divides once in the plane of the epthelium to produce the hair and socket cells. (B) All of the cellular components of the mature ES organ are derived from the SOP including the hair and socket cells that are visible externally, and the internal neuron and glia. An extra division in the pIIb branch of the lineage produces an additional glial cell that migrates away from the cluster and undergoes apoptosis (Fichelson and Gho, 2003; Gho et al., 1999).

Chapter 1: Variations on the Notch pathway in neural development

Variations on the Notch pathway in neural development

Nicholas J Justice* and Yuh Nung Jan†

Notch signaling allows cells in contact to adopt different fates. Regulation of the Notch pathway allows for the same signaling mechanism to be used in a wide variety of contexts during development. Intracellular activities of the E3 ubiquitin ligases Sel-10 and Neuralized involve proteasome-dependent degradation in the regulation of Notch pathway activity. Extracellular manipulations of Notch by Fringe and Scabrous regulate the pathway by changing Notch interactions outside the cell. These regulatory mechanisms, along with many others, affect how Notch signaling activity influences cell fate determination.

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Abbreviations

DSL	Delta/Serrate/Lag-1
EGF	epidermal growth factor
Fng	Fringe
Hes	*Hairy/Enhancer of Split*
Jag	Jagged
lufng	*lunatic fringe*
mafng	*manic fringe*
neur	*neuralized*
NICD	Notch intracellular domain
RING	Really Interesting New Genes
Sca	Scabrous
SOPs	sensory organ precursors
VZ	ventricular zone

Introduction

The evolutionarily conserved Notch signaling pathway mediates cell–cell interactions that allow neighboring cells to adopt different fates, providing a mechanism for consistent cell fate determination, differentiation, and patterning of highly organized tissues. In the developing nervous system, Notch signaling functions at many stages to determine cell fate, from the first segregation of neuronal precursors, to the terminal specification of cells as neurons and glia. Notch signaling is also important in differentiating neurons, possibly influencing the elaboration of axons and dendrites as well as the formation and maintenance of synapses. This review discusses recent advances that reveal the intricate regulation of Notch pathway signaling, via both intracellular and extracellular interactions. We then use the Notch-mediated specification of glia as an example to illustrate the possible impact such regulation could have on stem cell specification by influencing the context of Notch pathway signaling.

The Notch signaling pathway

Notch encodes a single pass transmembrane receptor that is proteolytically processed during its maturation into a 200kD extracellular fragment; this fragment is non-covalently linked to a 100 kD fragment containing a transmembrane spanning segment and an intracellular domain which extends into the cytosol [1] (see Table 1 for species orthologues of *Notch* and other pathway components). The Notch receptor is activated by Delta/Serrate/Lag-1 (DSL) ligands presented by neighboring cells. Ligand binding leads to the proteolytic cleavage of Notch, first at an extracellular site by an ADAM/TACE family metalloprotease, then by a presenilin-dependent protease — although this might be presenilin itself [2] — at a site within the transmembrane segment. Once the Notch intracellular domain (NICD) is cleaved and released from the plasma membrane, it enters the nucleus and binds CSL (CBF-1/Suppressor of Hairless/Lag-1) proteins within a complex that modulates the expression of various target genes (reviewed in [3]). Transcriptional repressors encoded by *Hairy/Enhancer of Split* (*Hes*) are transcribed in response to Notch signaling, and mediate many of the primary effects of Notch activation. Although the description of the pathway presented above contains the most consistently present components of Notch signaling in the wide array of systems and organisms in which it is studied, many additional regulators have been found to act on the pathway, in subsets of Notch signaling mediated processes [1].

Tuning the Notch signal inside the cell

The NICD functions in the nucleus to modulate gene expression, and its manipulation by proteins in the cytoplasm can directly impact transcriptional events associated with Notch activity. The identification of E3 ubiquitin ligase homology in many genes that genetically interact with *Notch* has implicated proteasome-dependent proteolysis in the cytoplasmic regulation of Notch signaling [4,5]. The recent discovery that many of these E3 proteins can physically interact with the NICD has suggested that ubiquitin-mediated proteolysis, which is used broadly by cells to direct protein degradation, might play a specific role in Notch signaling, by altering the stability of the NICD.

sel-10 was identified in a screen for mutations that suppress the egg-laying defect seen in certain hypomorphic alleles of *lin-12*, one of the two Notch receptor genes found in *Caenorhabditis elegans* [5]. *sel-10* encodes a protein containing an F-box and seven WD-40 repeats, a structure common to the HECT (homologous to E6 associated protein carboxyl [C] terminus) domain family of E3 proteins that function as constituents of the Skp1/Cdc53/F-box ubiquitin ligase complex in yeast [5]. F-box proteins are thought to confer specificity on the complex by selectively interacting with target proteins, bringing them in proximity to the ubiquitination machinery, which results in covalent attachment of ubiquitin and subsequent proteasome-mediated proteolysis of the target protein [6]. Sel-10 can bind Lin-12/Notch, as well as mammalian Notch4 [5],

suggesting that it might mediate the ubiquitination and degradation of Notch.

The recent identification and analysis of a mouse *sel-10* homologue (*mSel-10*), reported by three independent groups, has demonstrated that a similar interaction occurs between Notch1 and mSel-10 [7•,8•,9•]. Domain analysis shows that the mSel-10 F-box interacts with the region of the NICD that contains the C-terminal PEST protein degradation domain, leading to a reduction of transcriptional activation at target promoters induced by Notch activity [7•,8•,9•]. Moreover, this interaction involves the phosphorylation of the NICD, most likely in the nucleus [8•,9•]. It remains to be shown that ubiquitination leads directly to NICD degradation. However, given the evidence that proteasome inhibitors block NICD degradation, that the NICD is stabilized by truncated mSel-10 containing only the F-box domain, and that the *lin-12* phenotype is suppressed by *sel-10* in *C. elegans* [5], it seems likely that mSel-10 facilitates NICD ubiquitination and degradation [7•,8•,9•].

Notch is known to be ubiquitinated by two other HECT domain E3 proteins. Itch, which is mutant in *itchy* mice that suffer from constant itching of the skin and immune system abnormalities, can cause the ubiquitination of Notch in cell culture assays [10]. Additionally, *suppressor of deltex* negatively interacts with *Notch*, and encodes a HECT domain E3 that is involved in *Drosophila melanogaster* wing development [11]. These two genes, like *sel-10*, are believed to inhibit Notch signaling activity via ubiquitination of the NICD. Perhaps by causing the degradation of actively signaling NICD molecules, these genes increase the temporal resolution of Notch signaling, allowing the pathway to function during multiple signaling events in succession. Alternatively, ubiquitin-mediated proteolysis might not have a regulatory impact on Notch signaling. Instead, it may merely serve to constitutively remove the NICD, a process that occurs during the lifecycle of many proteins within the cell.

Ubiquitin-mediated proteolysis could lead to potentiation of Notch signaling activity rather than inhibition. *neuralized* (*neur*) encodes a putative E3 protein that belongs to the RING (Really Interesting New Genes) domain family of ubiquitin ligases and is required in a subset of Notch pathway-mediated cell fate decisions during development of the *Drosophila* nervous system [4,12,13]. In these cell fate decisions, *neur* loss of function causes similar phenotypes to *Notch* loss of function, suggesting that Neur facilitates Notch signaling. Selective expression of *neur* by the sensory organ precursors (SOPs) during their determination, division, and differentiation into external sensory bristles, suggests the intriguing possibility that Neur adjusts Notch signaling for proper specification of the peripheral nervous system. Another E3 protein in the RING domain family has been shown to activate a membrane spanning transcription factor (reminiscent of Notch) via regulated ubiquitin/proteasome-dependent processing in yeast [14••,15].

Table 1

Notch pathway component orthologues.

	C. elegans	*Drosophila*	Mouse
Notch	Lin-12 (2)	Notch (1)	Notch (4)
DSL	Lag-2 (1)	Delta (2)	Jagged (3)
Presenilin	Sel-12	dPs (2)	Presenilin (2)
CSL	Lag-1	Su (H)	CBF-1/RBP-Jk
Hes	Lin-22 (?)	E (spl)	Hes (7)

In parentheses are the numbers of orthologous genes found in each species, with the named gene being the orthologue most commonly referred to in the text.

Although Notch is known to require proteolysis at an intramembrane site for activation, it is tempting to speculate that Neur causes ubiquitination and proteasome-mediated cleavage of Notch, in an additional proteolytic event during Notch signaling activation. The function of Neur is equally likely to be involved in ubiquitin-mediated degradation of proteins that inhibit Notch pathway activity, yet the identification of Neur as a putative E3 protein supports an important role for ubiquitin-mediated proteolysis in the regulation of Notch signaling.

Tinkering with extracellular Notch influences receptor tone

The regulation of extracellular Notch has the potential to change Notch receptor activation characteristics by changing DSL ligand interactions, thus modulating the dynamics of Notch signaling to effect cell fate determination and differentiation. The extracellular fragment of Notch contains 36 epidermal growth factor (EGF)-like repeats, of which only EGF repeats 11–12 have been shown to be essential for DSL–ligand interaction [16]. The demonstration that Fringe (Fng), a gene known to genetically intereract with the Notch pathways glycosylates Notch, has opened the door to our understanding of the important role that extracellular regulation plays in the proper function of the Notch pathway (reviewed in [17]). More recently, Scabrous (Sca), a secreted protein that is highly expressed by many neural precursors making cell fate decisions in the developing *Drosophila* peripheral nervous system, has also been found to interact with the extracellular domain of Notch, giving rise to a number of new models as to how Sca impacts Notch receptor function [18••].

Fringe influences Notch activation

Identification of distant sequence homology between *fng* and a bacterial glycosyl-transferase led to the hypothesis that Fng glycosylates Notch, resulting in a bias of Notch receptor activation by Delta versus Serrate [19,20]. Glycosylation by Fng adds O-fucose residues, one of the less commonly found forms of glycosylation, to carbohydrate chains at sites between EGF repeats 22 and 36 in the extracellular domain of Notch, as it matures through the trans-Golgi network. Dorsal compartment expression of

Fng during *Drosophila* wing formation results in Notch glycosylation and the selective sensitivity of dorsal cells for Delta over Serrate. This leads to a difference in the levels of Notch signaling activity at the boundary of the dorsal and ventral compartments contributing to formation of the wing margin, which is thought to organize the outgrowth and patterning of the wing [21–23]. In the mouse, *fng* homologs *lunatic fringe* (*lufng*), *manic fringe* (*mafng*), and *radical fringe*, have recently been reported to change the sensitivity of Notch to activation by different DSL ligands. There have been four Notch receptor genes (Notch1–4) and three DSL ligands, Delta, Jagged(Jag)1 (Serrate1) and Jagged2 (Serrate2), identified in the mouse genome. Shimizu *et al.* [24] found that Lufng and Mafng can modify different positions on Notch2, and Hicks *et al.* [25] found that Notch1 is differentially activated by Delta or Jag1 depending on whether it is modified by Lufng [25]. These studies suggest that each Fringe can selectively modify the Notch receptor, specifically altering Notch sensitivity to activation by different DSL ligands.

This idea is supported by phenotypes observed in the inner ear of *jag2* and *lufng* knockout mice, in which Notch pathway function is often examined, due to its very close resemblance to Notch pathway component expression and function in *Drosophila* [26]. *jag2* knockout results in the overproliferation of sensory hair cells of both the inner and outer cellular layers of the mouse cochlea, a phenotype that is, in many ways, similar to the classic neurogenic phenotype of Notch pathway mutants in the fly. Knockout of *lufng* suppresses the overproliferation of inner hair cells in *jag2* knockouts, but fails to suppress the overproliferation of the outer hair cell layer [27••]. So, loss of the Lufng results in selective rescue of one subpopulation of Jag2-dependent hair cells, implying that, in the inner layer, Lufng has the effect of inhibiting Notch receptor activation by ligands other than Jag2. Mutations in *jag1*, present in both the *headturner* and *slalom* mutant mice, cause a reduction in total hair cell number and general patterning defects of the inner ear [28,29]. Thus, in the inner ear, Jag1 promotes the formation of neurons and Jag2 promotes non-neuronal fates, whereas Lufng causes cells of the inner layer to require Jag2 activation of Notch, in order to remain non-neuronal. These observations support the hypothesis, suggested by earlier experiments, that different modification and modulation of Notch by each Fng adds specificity to the Notch signaling pathway, such that closely related cells can use Notch signaling to promote the acquisition of very different cell fates.

Scabrous stabilizes Notch

The recent discovery that Sca physically interacts with the extracellular domain of Notch, has provided another example of Notch receptor regulation by interaction with the portion of the Notch receptor present on the external surface of the cell [18••]. *sca* encodes an 85 kD secreted protein, with C-terminal homology to the blood coagulation factor fibrinogen and causes mispatterning of the ommatidia when mutant in the *Drosophila* eye [30]. *sca* mutants also show mild mispatterning of thoracic bristles, which are derived from the SOPs on the developing pupal notum. SOPs are specified within proneural clusters, by the process of lateral inhibition. In this process, small differences in Notch signaling between equipotent cells are amplified through negative feedback on the pathway, such that by the end of lateral inhibiton, one cell within a cluster has the lowest Notch signaling activity and will be determined as the SOP (reviewed in [31]). Interestingly, *sca* is expressed at the highest levels specifically in the cells with the lowest levels of Notch signaling activity.

It has been proposed that Sca functions to refine the boundaries between cells adopting different fates, by modulating levels of Notch signaling activity. The evidence for this has come primarily from experiments, in which *sca* is misexpressed during development of the eye and wing in *Drosophila*. In both regions, this manipulation results in an antagonism of Notch signaling, causing supernumerary R8 cells in the eye and failure of margin formation in the wing [32,33]. The similarity between these overexpression phenotypes and the phenotype seen in *Notch* mutants, suggests that Sca can disrupt lateral inhibition by antagonizing Notch activity. The recent demonstration that Sca can stabilize Notch protein through physical interaction with the extracellular domain suggests a number of models for how Sca might function to influence Notch signaling activity [18••]. In the case of lateral inhibition, SOPs express high levels of *sca*, which is thought to enhance a difference in Notch activity between the SOP and surrounding cells. Perhaps by stabilizing Notch, Sca blocks the proteolytic events mediated by kuzbanian and presenilin, thereby preventing Notch receptor activation. Alternatively, Sca function may be similar to the proposed action of Fng, changing Notch receptor specificity for Delta or Serrate. Indeed, Delta (but not Serrate) is uniquely required during lateral inhibition, whereas both ligands are equally effective activators of Notch during specification of cell fate within the SOP lineage [34]. A model in which Sca alters the selectivity or stability of the Notch receptor would be consistent with Sca functioning cell-autonomously in the SOP as an antagonist of Notch signaling. However, Sca is a secreted protein, and a model in which *sca* functions to change Notch signaling activity in cells surrounding the proneural SOP remains a possible explanation, consistent with reports that *sca* function is non cell-autonomous in the eye [30]. It will be interesting to see whether further genetic analysis will be able to resolve these issues.

Notch plays a part in glial determination

The recent examination of stem cell lineages that produce both neurons and glia has raised the possibility that Notch signaling can promote glial determination, in addition to inhibiting the determination of neurons (review in [35]). Notch activity often specifies the most undifferentiated state during cell fate decisions and is thought to help maintain the multipotent character of stem cells as they divide,

to generate daughter cells that adopt terminal differentiation states. Notch signaling also specifies many terminal cell fates, after a stem cell finishes dividing. The recent demonstration that Notch activity promotes the determination of glia in the retina [36], neural crest [37], adult hippocampus [38], and cortical ventricular zone [39•], has provided new insights into the role of Notch activity in the determination of glial cell fate.

Notch specifies radial glia

Neuroglial stem cells (neuroglioblasts) in the cortical ventricular zone (VZ) produce neurons and glia at very different time points during their proliferation [40]. In the rat, cortical VZ neuroglioblasts primarily produce neurons until neurogenesis ends at embryonic day E19.5. As the production of neurons declines, gliogenesis is just beginning and generates astrocytes until postnatal day P9 and oligodendrocytes until P21 [41]. A unique glial cell type, generated by VZ neuroglioblasts concomitantly with neurons, are the radial glia, which form a scaffolding perpendicular to the ventricular surface of the brain, used by neurons to migrate from the VZ into the cortical plate (reviewed in [42]; Figure 1). Cells misexpressing NICD1 (simulating high Notch activity) become predominantly radial glia, suggesting that Notch activity is not only restricting neuronal fate, but is also promoting the adoption of a radial glial fate [39•]. However, the radial glial fate is not a terminal differentiation state. After neuronal migration into the cortical plate ends, radial glia regain stem cell characteristics and begin to divide, producing astrocytes for the remainder of development and well into adulthood. Therefore, Notch signaling activity, in this case, can be considered to promote the less restricted fate of the radial glia.

Recently, Chambers *et al.* [43••] found that the effects of Notch activity on glial cell fate determination are highly variable, depending on spatiotemporal context in the VZ. Early examination (E19.5) of embryos, transfected with NICD1 at E14.5, found the accumulation of NICD1 positive cells in the subventricular zone, but later examination, at P21, showed that most NICD1 expressing cells became astrocytes and left the VZ. However, in the olfactory bulb, which is populated by stem cells from the anterior cortical VZ, very few astrocytes expressing NICD1 were found at P21 [43••]. Anterior VZ stem cell lineages are known to produce only neurons [44] and perhaps lack glial competence, resulting in their failure to differentiate in response to high levels of Notch activity. Notch activity can be described as having an anti-neuronal influence on neuroglioblasts in the VZ, but whether that influence results in glial specification depends on the spatial and temporal context of each particular cell fate decision. A large number of cells in the VZ respond to Notch activity with no differentiation, ceasing to proliferate and remaining in the VZ [43••]. At this time, little is known about what makes a proliferating neuroglioblast capable of producing glia in response to Notch signaling, but genes that regulate the Notch pathway are likely candidates.

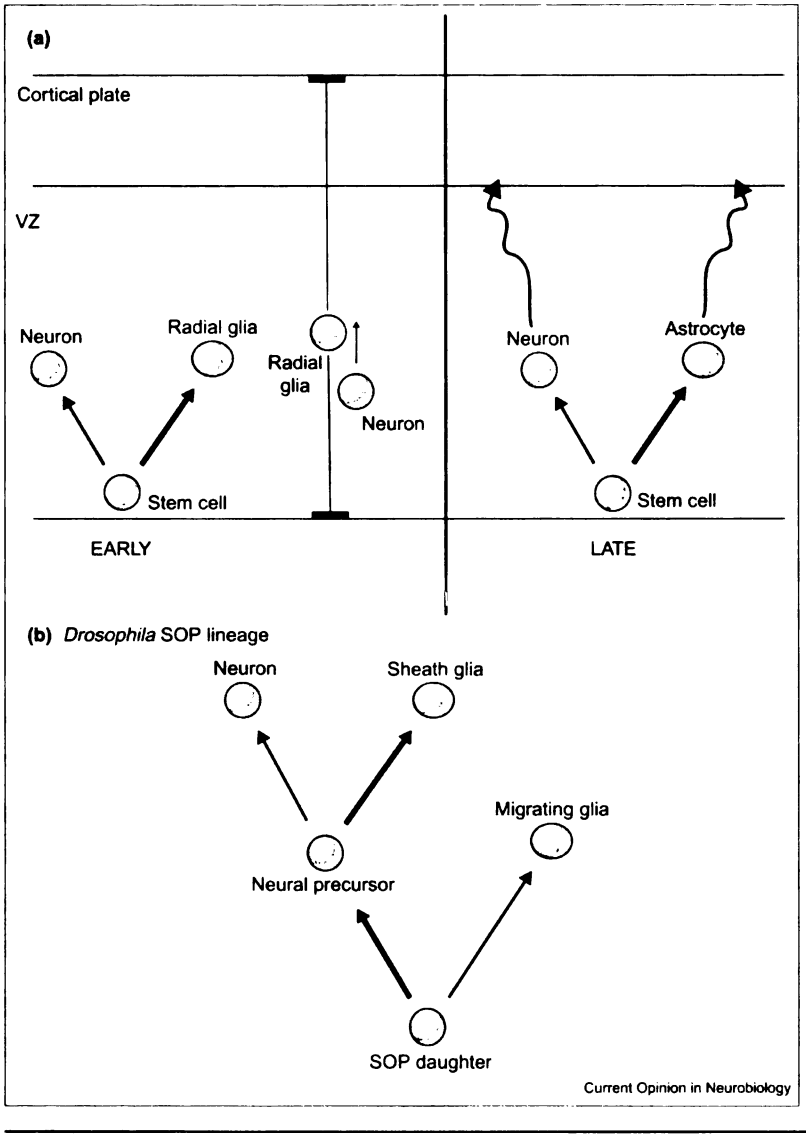
Determination of glia in the sensory organ precursor lineage

In the *Drosophila* peripheral nervous system, the details of neuroglioblast lineages are well characterized. Here, SOPs are specified by lateral inhibition mediated by Notch signaling. Each SOP follows a strict pattern of four cell divisions to produce five daughter cells. Every cell within the lineage adopts a unique fate and together they differentiate to form an external sensory organ — the bristle — in the adult fly. Two of the fates specified by this lineage are glial: the sheath cell serves to support the neuron whereas the other glia migrates away from the organ soon after it is born [45]. Recently, a requirement for Notch activity in the specification of SOPs to neurons and sheath cells, rather than to the migrating glial cell, has been reported [46]. The authors take advantage of a temperature sensitive allele of Notch that can be inactivated at the precise time of each cell fate decision, in experiments similar to early studies of Notch function in the SOP lineage [47]. Inactivation of Notch for the duration of the lineage leads to additional glia, whereas inactivation at later timepoints, after division has finished, results in extra neurons. These experiments show that Notch, in two successive fate decisions made by a neuroglioblast, promotes a neuronal precursor fate in the first fate decision, and a glial fate in the next (Figure 1b). Similar to conclusions made in the developing mouse cortex, the impact of Notch signaling activity is dependent on the context of a given fate decision made between a number of possible fates. A difference in Notch signaling activity is required during fate specification to prevent cells in contact — often the two daughter cells of a division — from choosing the same fate.

An encore for Notch in neuronal differentiation?

The Notch pathway also functions in post-mitotic developmental processes specific to neuronal differentiation. Although the regulation of Notch signaling by proteins, such as Sel-10 and Neur inside the cell and Fng and Sca outside the cell, has an important role in cell fate determination, the modulation of Notch signaling could also alter interactions between neurons during differentiation. Notch has been found to affect the outgrowth of axons and dendrites [48–50]. Furthermore, given that Notch is a transmembrane signaling molecule in many ways similar to cell adhesion molecules [51], it may play a role in establishing and maintaining the specific cell–cell contacts found at neuronal synapses. Additionally, Notch has similar regulation dynamics to those of amyloid precursor protein and appears to be important for the maintenance of neuroglial stem cell lineages in the adult hippocampus [38]. These findings may indicate the involvement of Notch pathway components in the development of Alzheimer's disease and even in aging [52]. Thus, the post-mitotic differentiation of neurons is quickly becoming another area of nervous system development, in which Notch signaling has been found to have a significant impact.

Figure 1



Influence of Notch signaling on cell fate. **(a)** Lineages of neuroglioblasts produce the neurons and glia of the mammalian cortex. Early divisions produce mainly neurons and, in these cells, NICD misexpression promotes radial glial fates. The radial glia serve as a scaffold for the neurons to migrate into the cortical plate. At later timepoints, as neurogenesis ends, misexpression of NICD promotes astroglial fates. **(b)** In the *Drosophila* SOP lineage, Notch activity promotes the neural precursor (IIIb) fate and not the glial fate. Notch then promotes the glial sheath fate after the next division of the lineage. A thick arrow denotes the fate promoted by Notch activity.

Conclusions

Regulation by intracellular mechanisms can change the effects of Notch signaling before modulation of gene expression and potentially influences the temporal resolution of Notch signaling. Extracellular regulation can change the activation of Notch by different DSL ligands, leading to additional specificity in Notch signaling. Regulatory mechanisms contribute to the developmental context of a given neural stem cell, and can alter the production of neurons and glia by altering Notch signaling. Understanding how the history, intrinsic properties, and extrinsic influences converge within a cell to provide the context of Notch signaling and control cell fate determination and differentiation will lead to a better understanding of how Notch signaling functions during the development of the nervous system.

Update

Studies from both *Drosophila* and *Xenopus* have recently suggested Delta as a target of the ubiquitin ligase activity of Neur. Neur interacts with Delta and causes the endocytosis and degradation of Delta protein in *Drosophila* [53•,54•]. A newly discovered *Xenopus* homologue of Neur has also been shown to interact with XDelta1 and cause its ubiquitination *in vitro* [55•]. These observations suggest a model, in which the function of Neur within the Notch pathway is to downregulate Delta, thus altering the relative levels of Notch signaling activity between neighboring cells. Although earlier studies have proposed that the action of *neur* is cell-autonomous, this new evidence indicates a possible non-cell autonomous role for Neur through the manipulation of Delta.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Artavanis-Tsakonas S, Rand MD, Lake RJ: **Notch signaling: cell fate control and signal integration in development.** *Science* 1999, **284**:770-776.

2. Fortini ME: **Notch and presenilin: a proteolytic mechanism emerges.** *Curr Opin Cell Biol* 2001, **13**:627-634.

3. Bray S, Furriols M: **Notch pathway: making sense of *suppressor of hairless*.** *Curr Biol* 2001, **11**:R217-R221.

4. Yeh E, Dermer M, Commisso C, Zhou L, McGlade CJ, Boulianne GL: **Neuralized functions as an E3 ubiquitin ligase during *Drosophila* development.** *Curr Biol* 2001, **11**:1675-1679.

5. Hubbard EJ, Wu G, Kitajewski J, Greenwald I: ***sel-10*, a negative regulator of *lin-12* activity in *Caenorhabditis elegans*, encodes a member of the CDC4 family of proteins.** *Genes Dev* 1997, **11**:3182-3193.

6 Jackson PK, Eldridge AG, Freed E, Furstenthal L, Hsu JY, Kaiser BK, Reimann JD: **The lore of the RINGs: substrate recognition and catalysis by ubiquitin ligases.** *Trends Cell Biol* 2000, **10**:429-439.

7. • Oberg C, Li J, Pauley A, Wolf E, Gurney M, Lendahl U: **The Notch intracellular domain is ubiquitinated and negatively regulated by the mammalian sel-10 homolog.** *J Biol Chem* 2001, **276**:35847-35853.
See annotation to [9•].

8. • Gupta-Rossi N, Le Bail O, Gonen H, Brou C, Logeat F, Six E, Ciechanover A, Israel A: **Functional interaction between Sel-10, an F-box protein, and the nuclear form of activated Notch1 receptor.** *J Biol Chem* 2001, **276**:34371-34378.
See annotation to [9•].

9. • Wu G, Lyapina S, Das I, Li J, Gurney M, Pauley A, Chui I, Deshaies RJ, Kitajewski J: **Sel-10 is an inhibitor of Notch signaling that targets Notch for ubiquitin-mediated protein degradation.** *Mol Cell Biol* 2001, **21**:7403-7415.
The findings reported in [7•,8•,9•] suggest an evolutionarily conserved role for *sel-10* in Notch pathway signaling. All three sets of authors describe mSel-10 dependent Notch ubiquitination in the mouse.

10. Qiu L, Joazeiro C, Fang N, Wang HY, Elly C, Altman Y, Fang D, Hunter T, Liu YC: **Recognition and ubiquitination of Notch by Itch, a HECT-type E3 ubiquitin ligase.** *J Biol Chem* 2000, **275**:35734-35737.

11. Cornell M, Evans DA, Mann R, Fostier M, Flasza M, Monthatong M, Artavanis-Tsakonas S, Baron M: **The *Drosophila melanogaster suppressor of deltex* gene, a regulator of the Notch receptor signaling pathway, is an E3 class ubiquitin ligase.** *Genetics* 1999, **152**:567-576.

12. Lai EC, Rubin GM: **Neuralized functions cell-autonomously to regulate a subset of Notch-dependent processes during adult *Drosophila* development.** *Dev Biol* 2001, **231**:217-233.

13. Yeh E, Zhou L, Rudzik N, Boulianne GL: **Neuralized functions cell autonomously to regulate *Drosophila* sense organ development.** *EMBO J* 2000, **19**:4827-4837.

14. •• Hoppe T, Matuschewski K, Rape M, Schlenker S, Ulrich HD, Jentsch S: **Activation of a membrane-bound transcription factor by regulated ubiquitin/proteasome-dependent processing.** *Cell* 2000, **102**:577-586.
The authors demonstrate the activation of a transcription factor by proteasome-mediated processing. As a result, they suggest that the growing family of RING domain E3 genes might be used in regulatory mechanisms that involve ubiquitination, but not necessarily the degradation and inactivation, of target molecules.

15. Hoppe T, Rape M, Jentsch S: **Membrane-bound transcription factors: regulated release by RIP or RUP.** *Curr Opin Cell Biol* 2001, **13**:344-348.

16. Rebay I, Fleming RJ, Fehon RG, Cherbas L, Cherbas P, Artavanis-Tsakonas S: **Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor.** *Cell* 1991, **67**:687-699.

17. Blair SS: **Notch signaling: Fringe really is a glycosyltransferase.** *Curr Biol* 2000, **10**:R608-R612.

18. •• Powell PA, Wesley C, Spencer S, Cagan RL: **Scabrous complexes with Notch to mediate boundary formation.** *Nature* 2001, **409**:626-630.
In this paper, a physical interaction between Sca and Notch is demonstrated and shown to stabilize extracellular Notch. This finding begins a molecular understanding of a mechanism by which Sca regulates Notch signaling.

19. Bruckner K, Perez L, Clausen H, Cohen S: **Glycosyltransferase activity of Fringe modulates Notch–Delta interactions.** *Nature* 2000, **406**:411-415.

20. Moloney DJ, Panin VM, Johnston SH, Chen J, Shao L, Wilson R, Wang Y, Stanley P, Irvine KD, Haltiwanger RS *et al.*: **Fringe is a glycosyltransferase that modifies Notch.** *Nature* 2000, **406**:369-375.

21. Irvine KD: **Fringe, Notch, and making developmental boundaries.** *Curr Opin Genet Dev* 1999, **9**:434-441.

22. Doherty D, Feger G, Younger-Shepherd S, Jan LY, Jan YN: **Delta is a ventral to dorsal signal complementary to Serrate, another Notch ligand, in *Drosophila* wing formation.** *Genes Dev* 1996, **10**:421-434.

23. Rauskolb C, Correia T, Irvine KD: **Fringe-dependent separation of dorsal and ventral cells in the *Drosophila* wing.** *Nature* 1999, **401**:476-480.

24. Shimizu K, Chiba S, Saito T, Kumano K, Takahashi T, Hirai H: **Manic fringe and lunatic fringe modify different sites of the Notch2 extracellular region, resulting in different signaling modulation.** *J Biol Chem* 2001, **276**:25753-25758.

25. Hicks C, Johnston SH, diSibio G, Collazo A, Vogt TF, Weinmaster G: **Fringe differentially modulates Jagged1 and Delta1 signalling through Notch1 and Notch2.** *Nat Cell Biol* 2000, **2**:515-520.

26. Eddison M, Le Roux I, Lewis J: **Notch signaling in the development of the inner ear: lessons from *Drosophila*.** *Proc Natl Acad Sci USA* 2000, **97**:11692-11699.

27. •• Zhang N, Martin GV, Kelley MW, Gridley T: **A mutation in the *lunatic fringe* gene suppresses the effects of a *Jagged2* mutation on inner hair cell development in the cochlea.** *Curr Biol* 2000, **10**:659-662.
Here, an elegant use of genetics shows the interaction between two Notch pathway components, Lufng and Jag2, in the mammalian system. Suppression of the Jag2 phenotype in a subset of affected cells by LuFng suggests that the Fringe family of proteins may add selectivity to the Notch receptor for a particular DSL ligand.

28. Kiernan AE, Ahituv N, Fuchs H, Balling R, Avraham KB, Steel KP, Hrabe de Angelis M: **The Notch ligand Jagged1 is required for inner ear sensory development.** *Proc Natl Acad Sci USA* 2001, **98**:3873-3878.

29. Tsai H, Hardisty RE, Rhodes C, Kiernan AE, Roby P, Tymowska-Lalanne Z, Mburu P, Rastan S, Hunter AJ, Brown SD *et al.*: **The mouse *slalom* mutant demonstrates a role for Jagged1 in neuroepithelial patterning in the organ of Corti.** *Hum Mol Genet* 2001, **10**:507-512.

30. Baker NE, Mlodzik M, Rubin GM: **Spacing differentiation in the developing *Drosophila* eye: a fibrinogen-related lateral inhibitor encoded by scabrous.** *Science* 1990, **250**:1370-1377.

31. Baker NE: **Notch signaling in the nervous system. Pieces still missing from the puzzle.** *Bioessays* 2000, **22**:264-273.

32. Lee EC, Yu SY, Baker NE: **The Scabrous protein can act as an extracellular antagonist of Notch signaling in the *Drosophila* wing.** *Curr Biol* 2000, **10**:931-934.

33. Lee EC, Yu SY, Hu X, Mlodzik M, Baker NE: **Functional analysis of the fibrinogen-related *scabrous* gene from *Drosophila melanogaster* identifies potential effector and stimulatory protein domains.** *Genetics* 1998, **150**:663-673.

34. Zeng C, Younger-Shepherd S, Jan LY, Jan YN: **Delta and Serrate are redundant Notch ligands required for asymmetric cell divisions within the *Drosophila* sensory organ lineage.** *Genes Dev* 1998, **12**:1086-1091.

35. Wang S, Barres BA: **Up a Notch: instructing gliogenesis.** *Neuron* 2000, **27**:197-200.

36. Furukawa T, Mukherjee S, Bao ZZ, Morrow EM, Cepko CL: ***rax, Hes1, and notch1* promote the formation of Muller glia by postnatal retinal progenitor cells.** *Neuron* 2000, **26**:383-394.

37. Morrison SJ, Perez SE, Qiao Z, Verdi JM, Hicks C, Weinmaster G, Anderson DJ: **Transient Notch activation initiates an irreversible switch from neurogenesis to gliogenesis by neural crest stem cells.** *Cell* 2000, **101**:499-510.

38. Tanigaki K, Nogaki F, Takahashi J, Tashiro K, Kurooka H, Honjo T: **Notch1 and Notch3 instructively restrict bFGF-responsive multipotent neural progenitor cells to an astroglial fate.** *Neuron* 2001, **29**:45-55.

39. • Gaiano N, Nye JS, Fishell G: **Radial glial identity is promoted by Notch1 signaling in the murine forebrain.** *Neuron* 2000, **26**:395-404.
Here, the authors provide an *in vivo* demonstration that radial glia are produced in response to high levels of Notch activity in populations of stem cells that produce mainly neurons. This suggests that Notch promotes a glial fate in the developing cortex.

40. Alvarez-Buylla A, Garcia-Verdugo JM, Tramontin AD: **A unified hypothesis on the lineage of neural stem cells.** *Nat Rev Neurosci* 2001, **2**:287-293.

41. Levison SW, Chuang C, Abramson BJ, Goldman JE: **The migrational patterns and developmental fates of glial precursors in the rat subventricular zone are temporally regulated.** *Development* 1993, **119**:611-622.

42. Parnavelas J, Nadarajah B: **Radial glial cells: are they really glial?** *Neuron* 2001, **31**:881-884.

43. •• Chambers CB, Peng Y, Nguyen H, Gaiano N, Fishell G, Nye JS: **Spatiotemporal selectivity of response to Notch1 signals in mammalian forebrain precursors.** *Development* 2001, **128**:689-702.
This in depth study shows the variety of effects that misexpression of Notch can have on stem cells in the cortical VZ: the spatiotemporal context of stem cells can change the result of this perturbation.

44. Smith CM, Luskin MB: **Cell cycle length of olfactory bulb neuronal progenitors in the rostral migratory stream.** *Dev Dyn* 1998, **213**:220-227.

45. Gho M, Bellaiche Y, Schweisguth F: **Revisiting the *Drosophila* microchaete lineage: a novel intrinsically asymmetric cell division generates a glial cell.** *Development* 1999, **126**:3573-3584.

46. Van De Bor V, Giangrande A: **Notch signaling represses the glial fate in fly PNS.** *Development* 2001, **128**:1381-1390.

47. Hartenstein V, Posakony JW: **A dual function of the Notch gene in *Drosophila* sensillum development.** *Dev Biol* 1990, **142**:13-30.

48. Berezovska O, McLean P, Knowles R, Frosh M, Lu FM, Lux SE, Hyman BT: **Notch1 inhibits neurite outgrowth in postmitotic primary neurons.** *Neuroscience* 1999, **93**:433-439.

49. Redmond L, Ghosh A: **The role of Notch and Rho GTPase signaling in the control of dendritic development.** *Curr Opin Neurobiol* 2001, **11**:111-117.

50. Sestan N, Artavanis-Tsakonas S, Rakic P: **Contact-dependent inhibition of cortical neurite growth mediated by notch signaling.** *Science* 1999, **286**:741-746.

51. Kamiguchi H, Lemmon V: **IgCAMs: bidirectional signals underlying neurite growth.** *Curr Opin Cell Biol* 2000, **12**:598-605.

52. Berezovska O, Xia MQ, Hyman BT: **Notch is expressed in adult brain, is coexpressed with presenilin-1, and is altered in Alzheimer disease.** *J Neuropathol Exp Neurol* 1998, **57**:738-745.

53. • Pavlopoulos E, Pitsouli C, Klueg KM, Muskavitch MA, Moschonas NK, Delidakis C: ***neuralized* encodes a peripheral membrane protein involved in delta signaling and endocytosis.** *Dev Cell* 2001, **1**:807-816.
This study shows that Neur activity causes the endocytosis of Delta and suggests that the function of Neur within the Notch pathway may be non-autonomous.

54. • Lai EC, Deblandre GA, Kintner C, Rubin GM: ***Drosophila* Neuralized is a ubiquitin ligase that promotes the internalization and degradation of Delta.** *Dev Cell* 2001, **1**:783-794.
In this paper, Neur is shown to function as a ubiquitin ligase. Both the ubiquitin ligase activity and the demonstrated Delta internalization are shown to be RING finger dependent.

55. • Deblandre GA, Lai EC, Kintner C: ***Xenopus* Neuralized is a ubiquitin ligase that interacts with XDelta1 and regulates Notch signaling.** *Dev Cell* 2001, **1**:795-806
These authors describe the cloning of the *Xenopus* homologue of Neur, named Xneur. They provide evidence that Xneur functions as a ubiquitin ligase, and is capable of causing the ubiquitination of Xdelta *in vitro*.

Chapter 2: A gain-of-function screen to isolate genes involved in the specification of neuronal cell fate during the development of the *Drosophila* peripheral nervous system

Introduction

Screens for recessive mutations that cause loss-of-function phenotypes have been a primary source for the discovery of genes involved in the development of the *Drosophila melanogaster* nervous system. Rarely, dominant gain-of-function mutations caused by spontaneous or chemical mutagenesis have led to the identification of novel genes. Recently developed transgenic techniques have made it possible to overexpress genes using the Gal4-UAS system (Brand and Perrimon, 1993). Gal4 activates transcription from UAS promoter elements, which can be inserted into the fly genome by P-element transposition (Spradling and Rubin, 1982). Transcriptional activation at UAS-sequences is restricted to the subset of cells that express Gal4 in a given Gal4 fly line. In this way, UAS-dependent transcription only occurs in specific tissues or cell types, which allows for the precise analysis of particular developmental events. Based on this system, P. Rørth designed the EP screen (Rorth, 1996). She created 2300 lines, each carrying a single P-element containing UAS elements and a minimal promoter, which was randomly inserted into the genome (Rorth, 1996). Theoretically, each EP line will misexpress adjacent downstream genes in the presence of Gal4, which is expressed in cells of a given tissue of interest such as the eye, wing, or nervous system, depending on the Gal4 fly line used.

The invention of the EP element presents a method to systematically screen for gain-of-function phenotypes, offering advantages over standard loss-of-function genetics.

Primarily, the EP lines provided a new genetic manipulation for screening, given the multitude of screens for recessive mutations that have already been performed to near saturation. Recessive screens can fail to identify genes for many reasons. If gene function is required early in development, assaying gene loss-of-function effects on later developmental processes is difficult. For instance, *Notch* null mutations are lethal at early embryonic stages, making analysis of the effects of *Notch* loss-of-function in the adult SOP lineage cumbersome to study. Additionally, *Notch* mutations are pleiotropic, causing phenotypes in almost every tissue of the developing fly that mask its role in the specification of cells derived from the SOP. Pleiotropy is less problematic in gain-of-function screens because genes can be misexpressed selectively in the tissue of interest. Redundancy of gene function is another pitfall of loss-of-function screens that EP screens avoid. Even though the removal of many genes does not produce a visible phenotype, misexpression could reveal an endogenous function.

The EP screen is attractive because of its ease and simplicity. Standard screens for recessive mutations assay phenotypes in F2 progeny, whereas the EP screen looks for phenotypes in the F1 progeny of a single generation cross. Cloning of genes identified in the screen is also relatively straightforward. After identification of EP lines that cause phenotypes when misexpressed, the EP element and surrounding genomic DNA can be recovered by P-element rescue. If the EP element is inserted in or near genes that are misexpressed in cells that express Gal4, those genes are likely candidate genes responsible for an observed phenotype. Additionally, the effects of gene loss-of-function are easily assayed because imprecise P-element excision can be used to create deficiencies in nearby genes. For these reasons, we decided to screen the EP line

collection for phenotypes in the adult peripheral nervous system (Abdelilah-Seyfried et al., 2000), in parallel with other screens examining the development of the eye and wing (Rorth et al., 1998), to assay the effects of gene misexpression.

We used *scabrous*-Gal4 (*sca*-G4), which is expressed selectively in the SOP (Nakao and Campos-Ortega, 1996), to misexpress the EP lines and screened for lines with phenotypes in the ES organ bristles on the adult notum. The well-characterized process of cell fate determination of the SOP and progeny cells of its lineage facilitates the interpretation of bristle phenotypes in terms of cell fate specification (Figure 1, see Introductory Overview). Determination of neurons in the *Drosophila* peripheral nervous system begins with proneural gene expression in a developing epithelium, establishing the potential of clusters of cells to become neuronal precursors (Cubas et al., 1991; Skeath and Carroll, 1991). Acquisition of neuronal potential sets up the next phase of neuronal determination, in which Notch/Delta signaling between equipotent cells in contact coordinates cell fate decisions to select one cell from each cluster as the SOP (Heitzler and Simpson, 1991).

As the SOP divides, terminal cell fates of SOP progeny cells are specified and each cell differentiates as a unique cellular component of the mature External Sensory (ES) Organ (Figure 1C). Transformation of cell fate between branches of the lineage occurs to produce many of the bristle phenotypes observed with loss-of-function mutations in genes that play a role in the specification of cell fate. For instance, loss-of-function mutations in *numb*, which encodes a cytoplasmic inhibitor of Notch, causes ES organs to be composed of four external cells (most often three sockets and one hair cell) that lack internal neuron and sheath cells (Rhyu et al., 1994; Uemura et al., 1989). This

phenotype results from transformation of the pIIb to an ectopic pIIa cell, which divides to produce two ectopic external cells in each mutant ES organ (Rhyu et al., 1994; Uemura et al., 1989). Numb overexpression in the SOP causes loss of ES organs and balding of the notum. Staining for markers of internal cells of the lineage reveals clusters of extra neurons and glia present underneath the cuticle, indicating that the opposite transformation of pIIa to pIIb occurs with Numb gain-of-function (Rhyu et al., 1994). In similar analyses we can categorize the phenotypes produced when genes under the control of an EP insertion are misexpressed in the SOP, and focus on lines that affect specific processes involved in neuronal cell fate specification.

Materials and Methods

Genetic screening

The screen was carried out by crossing female sca-G4 virgins to males from each of the 2300 independent EP element insertion lines generated by P. Rørth, balanced on the X, second, or third chromosomes (Rorth, 1996). Crosses to patched-G4, which expresses Gal4 in cells at the anterior-posterior boundary of the developing wing imaginal disk, and to 109(2)68, which has a similar expression pattern to sca-G4 but is expressed at lower levels in the cells of the SOP lineage, were also included. All crosses were set up in duplicate, allowing for the variation of temperature. One cross was kept at 18°C, while the other cross was kept at 25°C until after embryogenesis (24-48h), then transferred and shifted to 29°C. The transferred cross was kept at 25°C. Adult progeny from each cross were scored for bristle phenotypes or other gross developmental abnormalities. A line was kept for further screening if the genetic combination of Gal4

and EP chromosomes was lethal or if it caused a consistent phenotype. The entire collection of 2300 independent lines yielded approximately 140 positively scored lines, which were further characterized in secondary molecular and genetic analysis.

Molecular analysis – genomic rescue

To determine the genomic location of the EP element lines that generated positive phenotypes, surrounding DNA was recovered by genomic rescue. A brief description of the specific methods we used to rescue EP elements begins with isolation of genomic DNA from adult flies of a given EP line. Genomic DNA was then digested either by *EcoRI* (to rescue 3' sequence) or *SacII* (to rescue 5' sequence). After heat inactivation of the restriction enzyme, the digested DNA is added to a dilute ligation reaction, then transformed into *E. Coli*. Kanamycin resistance was used to select colonies transformed by circularized plasmid containing EP element DNA, which was sequenced using primers facing genomic DNA that lies next to the EP element. Sequences were BLAST queried against the BDGP database to determine the cytological location and identity of nearby genes. The location of most of the positive EP lines was determined using plasmid rescue; the remainder were mapped by inverse PCR, mainly by the BDGP (http://www.fruitfly.org).

Genetic interactions - secondary screening

Secondary genetic screening was carried out by misexpressing a given EP line with sca-G4 in the background of a heterozygous loss-of-function mutation in *Notch* or *Hairless*, looking for either enhancement or suppression of the phenotype, which was

initially observed with misexpression of an EP line alone. For genetic interactions with Notch, N^{55e11}/FM6; sca-G4/CyO females were crossed to males from each EP line. The bristle phenotype of N^{55e11}/+; scaG4/EP was compared with males from the same cross of the genotype FM6/y; scaG4/EP. N^{55e11} is a reported null allele of Notch, and heterozygous flies display a dominant wing notching phenotype, but have little or no visible bristle phenotypes. An EP line was scored as positively interacting with Notch if the EP x scaG4 phenotype was significantly changed when N^{55e11} was heterozygous in the background. In similar crosses, an allele of *Hairless* was included in the background of EP misexpression with sca-G4 by crossing sca-G4/CyO; H^{E21}/TM3 to an EP line and comparing the phenotypes of adult control flies with the genotype EP/scaG4; +/TM3, to sibling flies with the genotype EP/scaG4; H^{E21}/+. H^{E21} is a loss of function allele of *Hairless* that displays a dominant, mild multi-socket bristle phenotype, indicating that *Hairless* activity antagonizes Notch pathway signaling. Therefore, *Notch* or *Hairless* loss-of-function mutations are predicted to interact an EP misexpression phenotype in opposite directions.

Results

Adult progeny of crosses between each of the orginal 2300 EP lines (Rorth, 1996), and *sca*-G4 were screened for bristle phenotypes. 105 EP lines were isolated by the screen group that caused ES organ defects when misexpressed in the SOP (Abdelilah-Seyfried et al., 2000). Rescue of the EP elements from these lines revealed the genomic location of most of the EP insertions. Many EP elements were found inserted near genes that are known to be involved in the specification of SOP lineage cell fates.

Approximately 20% of the EP lines that caused bristle phenotypes were found inserted near genes that were uncharacterized at the time of the screen and had only been reported as sequenced ESTs. Others were found inserted near genes that had been characterized in other systems, but had never been shown to be involved in neuronal cell fate specification. A final class of positive EP lines were found to be inserted in regions of the genome where no clear prediction could be made as to which gene was responsible for the observed misexpression phenotype. EP lines were selected for secondary screening based on the strength and specificity of the misexpression phenotype. Results of the secondary screen provided genetic interaction information that was considered along with information about the molecular nature of nearby gene(s), in order to prioritize each line for further genetic and molecular characterization.

ES organ bristle phenotypes are separable into distinct classes based on the phase of cell fate specification that is most likely disrupted by misexpression. If the initial acquisition of neuronal potential is disrupted as proneural clusters form, the resulting balding of the notum and complete absence of cells derived from the SOP would be the predicted phenotypic outcome (Figure 1A). EP(2)0415 was included in this class of phenotypes, and then shown to be inserted upstream of the gene enocding Extramacrochaete (Emc), a transcriptional inhibitor of the Achaete-Scute Complex (Skeath and Carroll, 1991). EP(2)0415 misexpression with sca-G4 causes complete balding of the notum and the absence of the neural and glial internal cells of ES organ bristles. Inhibition of proneural gene expression by Emc, under the control of EP(2)0415, is a likely cause of the balding phenotype observed, and therefore serves as a good

example of an EP line classified with EP phenotypes that display altered acquisition of neural potential (Table 1).

After a cluster of cells within the epithelium gains the potential to become neuronal, signaling occurs between equipotent cells via the intercellular interaction of Notch and Delta at cell-cell contacts (Figure 1B). Mutations disrupting lateral inhibition often cause the overspecification of SOPs, resulting in tufts of ectopic bristles on the notum (Figure 1B). The gene *big brain* has been proposed to potentiate Notch activity during lateral inhibition, after being isolated as a recessive mutation that causes increased neurogenesis (Rao et al., 1992; Vassin et al., 1985). EP(2)2278 was found to be inserted upstream of *bigbrain* after being isolated in the EP screen due to a tufting phenotype (Abdelilah-Seyfried et al., 2000). This phenotype distinguishes a second class of EP lines that likely disrupt the next phase of neural cell fate specification: the Notch signaling mediated selection of neural precursor cells by lateral inhibition (Table 2).

After the SOP is determined, the function of Notch activity changes from the inhibition of neuronal precursor cell fate to the specification of terminal cell fates within the lineage (Figure 1C). When Notch signaling is disrupted, cell fates are transformed between branches of the SOP lineage, resulting in characteristic bristle phenotypes (Figure 1B). EP lines that give transformation phenotypes when misexpressed in the SOP were included in the class of lines that disrupt a third phase of cell fate determination: the specification of terminal cell fates (Table 3). EP(2)2478 displays balding of the notum and twinned hairs without sockets when crossed to sca-G4, indicating transformations between fates within the lineage (Figure 1C). When rescued and sequenced, this line was found to be inserted upstream of *numb*, which is known to function in the SOP lineage as

a cell fate determinant (Rhyu et al., 1994; Uemura et al., 1989). The asymmetric inheritance of Numb influences the extrinsic signaling between cells via Notch and Delta, coordinating intrinsic and extrinsic mechanisms of cell fate specification to properly achieve the determination of each of five unique cell fates. EP lines that display cell fate transformation phenotypes similar to that of Numb were assigned high priority for further characterization, given that they are good candidates for genes that affect both the intrinsic competence of a cell to be specified, as well as the Notch signaling pathway that will select cell fate.

A final class of EP lines cause defects in bristle development when misexpressed with sca-G4 (Table 4). These lines most likely disrupt cell differentiation, altering the morphology or viability of the cellular components that make up the ES organ. Whether these genes are endogenously involved in differentiation or programmed cell death during the normal course of development remains unclear. Misexpression of genes might be expected to cause broad changes in the ability of a cell to differentiate appropriately, or could lead to the death of a cell due to elevated levels of misexpressed protein. Because of these complexities in interpretation, this class of EP lines was not considered for further investigation.

Characterization of EP lines inserted in novel genes

The first EP lines chosen for further characterization were those that produced consistent and specific cell fate phenotypes, which were not inserted in genes already known to function in cell fate determination. Genetic interaction screens of candidate EP lines with *Notch* and *Hairless* were performed to help predict the activity that was

perturbed by misexpression, and to test whether a putative misexpressed gene might be likely to play an endogenous role in SOP cell fate determination. Of the EP lines classified as having lateral inhibition phenotypes that genetically interact with *Notch* and *Hairless*, I selected EP(3)3622 for characterization. I also selected EP(3)3519 for characterization due to the strength and specificity of the misexpression phenotype, and the proximity of a novel gene as a strong candidate for the gene responsible for the phenotype, from the class of genes that produce cell fate transformation phenotypes. Our findings (Abdelilah-Seyfried et al., 2000), as well as published reports from parallel screens done in other systems (Abdelilah-Seyfried et al., 2000; Brennecke et al., 2003; Grosshans and Wieschaus, 2000; Mata et al., 2000; Rorth et al., 1998), suggest functions for the genes misexpressed by EP(3)3622 and EP(3)3519 that support and contradict the usefulness of misexpression screens for the purposes of discovering novel gene function.

EP(3)3622 misexpression disrupts lateral inhibiton and selection of the SOP

Adult progeny of EP(3)3622 crosses to sca-G4 display tufts of ectopic bristles on the notum (Figure 2A). Extra bristles are composed of the normal complement of one hair and one socket cell externally, along with one glial sheath cell and one neuron. This suggests that later phases of cell fate specification in the SOP lineage are unaffected by EP(3)3622, and that misexpression specifically alters processes that determine the fate of the SOP. Manipulation of Notch signaling results in similar phenotypes due to the role of the Notch pathway in lateral inhibiton. Heterozygous null loss-of-function mutations of *Notch* in the background of a fly misexpressing EP(3)3622 with sca-G4, strongly enhanced the observed tufting phenotype (Figure 2B). Consistent with this genetic

interaction, loss-of-function mutations of *Hairless,* a gene that inhibits Notch pathway activity (Bang et al., 1991), suppress the tufting of bristles that result from EP(3)3622 misexpression in the SOP (Figure 2C). We pursued the question of how the EP(3)3622 insertion causes a tufting phenotype with attempts to identify target genes misexpressed by EP(3)3622, as well as by screening deficiencies of the EP(3)3622 genomic region for bristle phenotypes.

In order to determine which gene is misexpressed by EP(3)3622, we rescued the EP element along with adjacent genomic DNA, and searched the sequence for target gene candidates. Digestion with *EcoRI* rescues sequence 3' of EP insertions (see Materials and Methods). However, to our surprise, we found both 3' and 5' genomic sequence surrounding the EP(3)3622 insertion in two separate rescued plasmids, indicating that two EP elements are inserted at a single site in the genome, oriented back-to-back facing opposite directions (Figure 3; Brennecke et al., 2003; Hipfner et al., 2002). Multiple insertions of EP elements in a single line complicate the search for misexpressed genes because multiple genes may contribute to a phenotype. Nonetheless, we attempted to clone the gene miexpressed by EP(3)3622, using genomic DNA fragments from both 5' and 3' rescued plasmids to screen EST libraries. We cloned a cDNA (GH02109) that lies approximately 2 kb 3' of the EP insertion, which was the best candidate for the misexpression target of EP(3)3622 due to its proximity to the EP insertion(s). In order to test whether misexpression of GH02109 in the SOP causes the same phenotype as EP(3)3622, we made lines carrying a UAS-GH02109 transgene, and crossed them to sca-G4. Misexpression in the SOP, however, failed to recapitulate the tufting phenotype

observed with misexpression of EP(3)3622, indicating that, most likely, GH02109 is not the gene responsible for the phenotype.

In order to establish whether any nearby gene(s), misexpressed by EP(3)3622, might have an endogenous role in the selection of the SOP, we generated imprecise excisions of two lethal P-elements near the EP(3)3622 insertion (l(3)1170 and l(3)05967), then screened for P-element excision events that result in lethality when crossed to a deficiency of the region (Df[emc]). Nine lethal excision lines were established, then intercrossed to see if lethality in each line results from loss of the same gene(s). All of the EP(3)3622 revertants that were isolated as lethal excisions failed to complement Df[emc], but some combinations of alleles yielded adult escapers. Flies with complementing deficiencies, potentially uncovering the same genes nearby EP(3)3622 that represent candidate misexpression targets, failed to display any bristle phenotypes that indicate a requirement for one of these putative genes in SOP determination.

The bristle tufting phenotype observed when EP(3)3622 is misexpressed in the SOP with sca-G4, along with genetic interactions of this phenotype with *Notch* and *Hairless* mutations (Figure 2), suggest that the misexpressed gene disrupts selection of the SOP during lateral inhibition. These experiments, however, do not demonstrate the endogenous function of this gene. Recently, the report of a phenotype associated with a deficiency of the EP(3)3622 locus (Hipfner et al., 2002) and the subsequent cloning of the novel gene *bantam* that is misexpressed by EP(3)3622 (Brennecke et al., 2003), have provided insight into how *bantam* might produce the bristle tufting phenotype observed when it is misexpressed in the SOP.

bantam was found to encode a 21 nucleotide microRNA that is required for the proliferation of cells in the imaginal discs during larval development (Brennecke et al., 2003). *bantam* can affect dividing cells by regulating Hid, a protein that promotes apoptosis. Misexpression of *bantam* with EP(3)3622 causes a decrease in the levels of Hid protein, resulting in decreased apoptosis. How the regulation of apoptosis by *bantam* affects the selection of the SOP remains unclear. Genetic interactions suggest that *bantam* activity affects the process of lateral inhibition, perhaps revealing a role for apoptosis during selection of the SOP. Alternatively, the effects of *bantam* loss-of-function on cell proliferation might explain how misexpression disrupts SOP selection. MicroRNAs bind to the 3' UTR of mRNA transcripts to inhibit mRNA translation (Lee and Ambros, 2001). Many genes that influence cell proliferation contain *bantam* target sequences, including *string*, *lethal giant larvae*, and *discs large* (Brennecke et al., 2003). Misepxression of *bantam* with EP(3)3622 may regulate these genes to disrupt the cell cycle, and directly affect cell fate decisions made during lateral inhibition. The cell cycle may change the ability of a cell to respond to Notch activity, resulting in the overspecification of cells to the SOP fate (Negre et al., 2003). Alternatively, disrupting the cell cycle may cause aberrant division of the SOP after it is specified, leading to the generation of ectopic ES organs, and tufting phenotypes. The role of *bantam* in the endogenous determination of the SOP, however, is still unclear. The lack of phenotypes associated with loss-of-function mutations in *bantam* make discovery of its role in SOP cell fate determination difficult.

EP(3)3519 causes the misexpression of *tribbles* and disrupts Notch mediated cell fate specification in the SOP lineage

Crosses of EP(3)3519 to sca-G4 produce flies that are missing ES organs, and have ES organs with twinned hairs and no sockets (Figure 4). Further internal examination of the nota from these flies reveals additional glia associated with clusters of cells under the cuticle (Figure 5B, inset) suggesting that misexpression causes the transformation of pIIa to pIIb precursor cells, and the transformation of socket cells to hair cells. The appearace of many single hair cells without an accompanying socket suggests that misexpression of EP(3)3519 causes a failure to specify and differentiate socket cells. The loss of socket cells, which are specified by high relative levels of Notch activity, and internal transformation phenotypes very similar to those produced by loss-of-function mutations in *Notch* (Hartenstein and Posakony, 1990), together suggest that the gene misexpressed by EP(3)3519 can inhibit Notch signaling activity.

EP(3)3519 is inserted immediately upstream of a sequenced EST that was novel when the EP lines were first screened, but has since been named *tribbles* (Grosshans and Wieschaus, 2000; Mata et al., 2000; Seher and Leptin, 2000). Flies carrying a UAS-*tribbles* transgene were crossed to sca-G4 and the resulting phenotype was identical to the phenotypes seen with misexpression of EP(3)3519 with sca-G4. *tribbles* encodes a protein homologous to serine/threonine kinases, but lacking the critical "DFG" triplet of amino acid residues that is required for ATP binding. Kinase assays failed to detect any kinase activity (not shown), supporting the hypothesis that Tribbles does not function as a kinase (Grosshans and Wieschaus, 2000; Mata et al., 2000). Because of the specificity of the phenotype produced by EP(3)3519 misexpression in the SOP lineage, and the

identification of *tribbles* as the target of EP(3)3519 that is responsible for the observed transformation phenotype, we asked whether *tribbles* has an endogenous function in the inhibition of Notch during specification of cell fate in the SOP lineage.

tribbles would be predicted to be expressed in the SOP and its progeny cells if it functions as part of the mechanism in which Notch signaling determines cell fate decisions. In order to examine the expression pattern of *tribbles*, we performed *in situ* hybridization on third instar larval imaginal discs using antisense RNA *tribbles* probes. We observed expression of *tribbles* in cells of the wing disc, at the developing anterior wing margin (Figure 5A, B). Third instar larval wing imaginal discs stained with antibodies against the proneural gene product Achaete, display two rows of cells along the anterior wing margin (Figure 5A, inset; Couso et al., 1994). The expression of *tribbles* is like that of *achaete* at the wing margin, suggesting that developing neural precursor cells express *tribbles*. Eye disc expression patterns also suggest that SOPs in the eye express *tribbles* (Figure 5C). *tribbles* is broadly expressed at early embryonic stages, and becomes restricted to the embryonic nervous system at later stages (Figure 5D, E). The evidence that *tribbles* is likely expressed by neuronal precursors of the SOP satisfied one criterion necessary to demonstrate an endogenous role for a gene identified by a gain-of-function phenotype.

The strongest evidence that a gene functions endogenously in a given biological process, such as SOP cell fate determination, is the appearance of a phenotype when the gene is removed from the system. To ask whether *tribbles* loss-of-function causes a bristle phenotype, we mobilized the EP(3)3519 element, and screened for imprecise excisions that result in a viable phenotype or lethality when crossed to a deficiency

covering the genomic region of the EP insertion (Df(3)[rdg], 77C). We recovered three EP(3)3519 revertants that met these criteria. Two lines were lethal and one was semi-viable ($EP3519^{rev20}$) displaying a mild bristle phenotype when crossed to Df(3)[rdg] (Figure 6A). In $EP3519^{rev20}$/Df(3)[rdg] individual bristles were doubled at a low penetrance, however the lethality of the two other revertants led to the hypothesis that $EP3519^{rev20}$ is a hypomorphic mutation in *tribbles*. We therefore pursued these lethal lines with the hope that they were null alleles, and that *tribbles* function is essential for viability. Each putative deficiency was recombined onto an FRT chromosome in order to perform mosaic analysis, looking at clones of cells on the adult notum for associated bristle phenotypes. Clones made up of cells homozygous for either of the lethal revertant lines, however, failed to display any bristle phenotypes (not shown).

Northern analysis revealed that $EP3519^{rev20}$ removes all of the wildtype *tribbles* mRNA, shifting the size of the transcript to over 10kb (Figure 6C). We sequenced genomic DNA from $EP3519^{rev20}$ homozygous flies and found that a 42bp fragment of the P-element remained at the site where EP(3)3519 had been inserted. Meanwhile, parallel studies showed that EP(3)1119, an EP insertion in *tribbles* near EP(3)3519, is a transcript null mutation of *tribbles*. EP(3)1119 is semi-viable, and adult escapers have no associated bristle phenotypes, consistent with the very weak phenotype caused by $EP3519^{rev20}$, and suggesting that loss of *tribbles* has no phenotypic effect on SOP cell fate specification. The strength and specificity of the transformation phenotypes displayed when *tribbles* is misexpressed, however, suggests that *tribbles* can disrupt a cellular process that is important to the specification of cell fate. How might *tribbles* affect the SOP to cause changes in cell fate decisions being mediated by Notch pathway signaling?

Studies on the role of *tribbles* during the development of other *Drosophila* tissues suggest that *tribbles* functions as a checkpoint in the progression of a cell through the cell cycle (Grosshans and Wieschaus, 2000; Mata et al., 2000). When *tribbles* is misexpressed, levels of the cell cycle protein, String (Cdc25), decline, suggesting that *tribbles* can interfere with the cell cycle via modulation of String protein stability (Mata et al., 2000). Progession of the SOP through the cell cycle, which is mediated by String, has been shown to alter the competence of the SOP to make a given cell fate decision (Negre et al., 2003). Thus, regulation of the cell cycle must be coordinated with Notch signaling during cell fate specification. Indeed, *Notch*, *Delta*, and *tribbles*, were discovered together in a screen for enhancers and suppressors of an eye phenotype caused by misexpression of dMyt1, a potent inhibitor of the cell cycle (Price et al., 2002). Additionally, reports that Notch signaling can determine whether follicular epithelial cells in the ovary proceed through mitosis or begin to endocycle, indicate that Notch signaling can have profound effects on dynamics of the cell cycle (Deng et al., 2001). Thus the cell cycle can be influenced by Notch pathway signaling, and can also impact the competence of a cell to respond to Notch signaling activity. This interconnection between the intrinsic process of cell division and extrinsic Notch signaling might help explain why misexpression of *tribbles* results in the mis-specification of cell fates in the SOP lineage.

Within hours of being selected as a neuronal precursor, the SOP divides four times to generate the five cells of the lineage that will terminally differentiate (Gho et al., 1999). During these divisions, the time window in which Notch activity must signal to divert a given cell to another path of differentiation is on the order of minutes (Roegiers

et al., 2001b). While *tribbles* was shown to be able to stall the cell cycle via degradation of String, cells eventually recovered and resumed mitotic cycling (Grosshans and Wieschaus, 2000; Mata et al., 2000). If division of the SOP is stalled, perhaps the competency of this cell to respond to Notch activity, specifically in choosing between pIIa and pIIb cell fates, expires, causing both cells to be specified as pIIb cells, which results in balding. Similarly, in the decision between hair and socket cell fate, disruption of the timing of division of the pIIa, might result in the specification of both progeny as hair cells, resulting in a twinning phenotype (Figure 5A). This demonstration that disrupting the cell cycle, an inherently intrinsic property of a cell, can change the way that a cell responds to an extrinsic signal, exemplifies how intrinsic and extrinsic mechanisms of cell fate specification are coordinated to determine terminal differentiation decisions, as a developing precursor cell proliferates in a lineage.

Discussion

We used the SOP lineage as a model system of neuronal cell fate specification to screen adult flies misexpressing genes at random in the SOP and its progeny. Of the 105 lines isolated with bristle phenotypes, 20 were included in a secondary screen based on certain criteria. First, we evaluated the phase of SOP cell fate determination affected by misexpression. Additionally, secondary screens were conducted to determine whether misexpression phenotypes of the EP lines genetically interact with *Notch* and *Hairless* mutations. Finally, molecular identification of nearby genes likely to be responsible for the phenotype was carefully considered before individual lines were chosen for further characterization. We first tested whether gain-of-function phenotypes observed with

misexpression are relevant to the endogenous function of the misexpressed gene. Most important was the identification of the gene responsible for the phenotype, the determination of the endogenous expression pattern of this gene, and an examination of the loss-of-function phenotypes produced by deficiencies of the gene. These basic characterizations presented obstacles to the analysis of many lines, most often because loss-of-function bristle phenotypes were absent or not interpretable relative to the gain-of-function phenotypes observed in SOP cell fate specification.

Screening for gain-of-function rather than loss-of-function phenotypes was predicted to isolate a different class of genes from previous screens that had assayed recessive-lethal mutations. The limitations of a gain-of-function screen, however, became apparent when numerous labs used the EP lines to look for phenotypes that result from the misexpression of random genes (Abdelilah-Seyfried et al., 2000; Rorth, 1996; Rorth et al., 1998). The EP screen appears attractive due to its simplicity and ease; we isolated 105 lines with specific phenotypes after screening only 2300 first generation crosses. The relatively little effort required to finish the initial screen was not, however, followed by routine cloning and characterization of novel genes. EP lines driving misexpression of known genes were identified due to gain-of-function phenotypes. Suprisingly, of the novel genes discovered by the screen, many failed to display loss-of-function phenotypes consistent with an involvement in the specification of cell fate. This might have been due to redundancies in gene function, a complication of genetics that the EP screen was championed to avoid. However, distinguishing a potentially redundant function from an ectopic or artificial function when studying novel genes can be difficult.

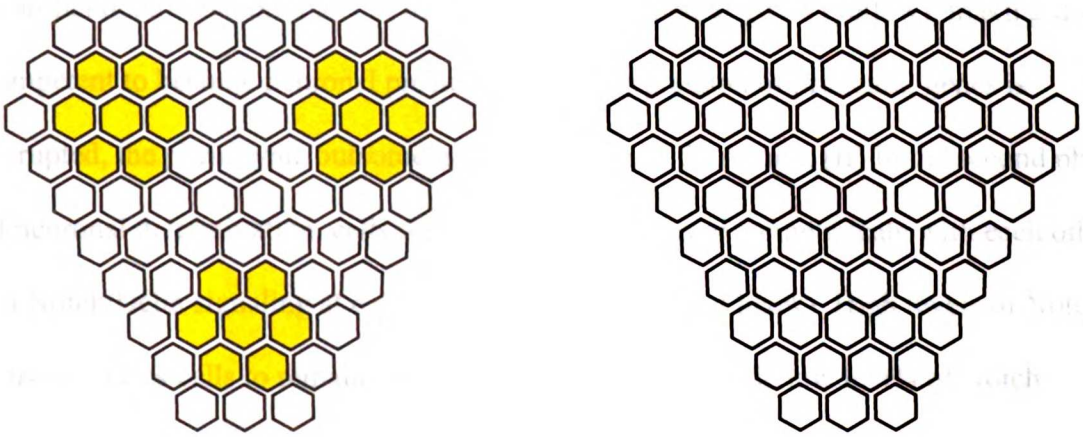
Persistent searching for loss-of-function phenotypes associated with EP lines that misexpress novel or unknown target genes has, in fact, led to the discovery of many important genes that had eluded standard loss-of-function screens. For instance, *tribbles*, a gene that regulates the cell cycle to coordinate morphological events with the cell cycle during development, was first identified in an EP screen (Grosshans and Wieschaus, 2000; Mata et al., 2000). Additionally, the gene *bantam,* which encodes one of the first genetically identified microRNAs in *Drosophila* (Brennecke et al., 2003), was pursued due to a misexpression phenotype caused by the nearby EP element EP(3)3622. Thus, the EP screen has proven to be a successful, but inefficient, tool for gene discovery that was perhaps misunderstood as a shortcut. The EP screen suffered most because standard criteria used to judge the importance of genes discovered by loss-of-function genetics are less effective when applied to genes isolated due to gain-of-function phenotypes.

Traditional genetics relies on the strength of the conclusions that can be derived from loss-of-function evidence that a gene is required for normal execution of a particular biological process. Most genes that have been thoroughly characterized in *Drosophila* are required for viability, and therefore have an essential function during development. However, of the approximately 12,000 genes present in the *Drosophila* genome, loss-of-function in only about one quarter causes lethality, indicating that the majority of genes are not strictly required for viability (Miklos and Rubin, 1996). Importantly, this finding cannot be interpreted to mean that 75% of the genes in the *Drosophila* genome lack essential functions. Non-essential genes might have overlapping functions, such that when one gene is removed, another gene can compensate for its function, which ultimately allows the organism to survive. While loss-of-function genetics has proven to

be an extremely effective means of isolating the genes within the genome that are required for viability, it is not optimal for examining the roles of genes that may be important for specialized functions or for fine-tuning of regulatory systems, but fail to generate phenotypes when removed. Gain-of-function genetics is a more effective tool for the analysis of such genes. Misexpression is well suited for the identification of genes with instructive roles in developmental processes. When these genes are over-expressed they produce strong phenotypes that often reveal a cellular function. For example, we isolated an EP line that misexpresses dLMO (EP(X)1394, Table 1) and causes ectopic outgrowths in the wing when misexpressed by patched-Gal4 (Zeng et al., 1998a). dLMO was shown to interact with and inhibit Apterous to help distinguish dorsal and ventral compartments during patterning of the wing disc (Milan et al., 1998). Mutations of this gene were isolated in the early years of *Drosophila* genetics (*Beadex, heldup-a*), but the lack of an interpretable phenotype, even though wing patterning is disrupted in the loss-of-function mutant, prevented the uncovering of its function. Studies on *tribbles* (EP(3)3519) and *bantam* (EP(3)3622) are additional examples of genes that have specific functions within the cell that were revealed by gain-of-function, misexpression phenotypes (see results). As genetic analysis changes focus from genes that are essential for viability, to genes with subtle loss-of-function phenotypes, these studies may rely more heavily on gain-of-function manipulations rather than standard genetic analysis of recessive lethal mutations.

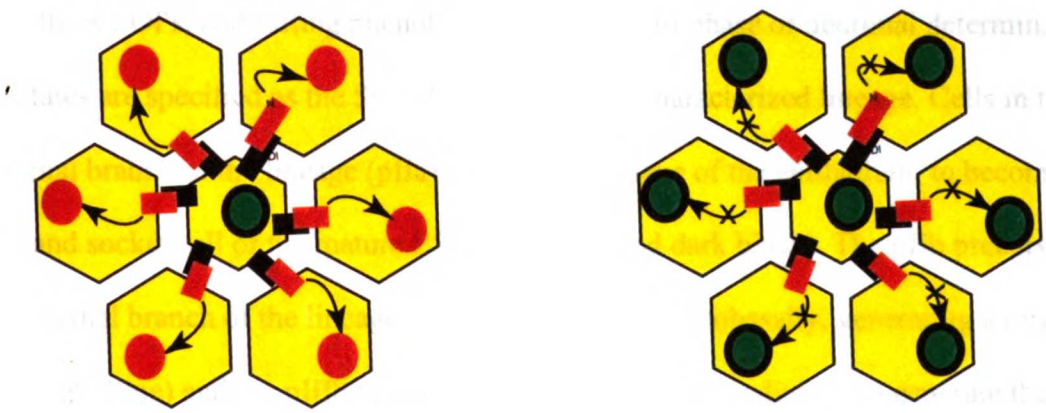
Figure 1: Phases of neuronal cell fate determination

A Phase 1: Proneural gene expression



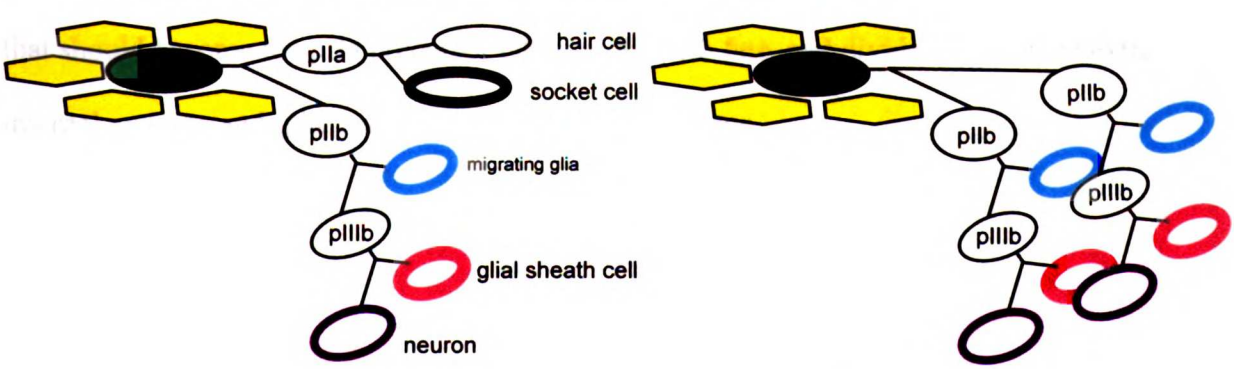
wildtype balding

B Phase 2: Lateral Inhibition



wildtype tufting

C Terminal cell fate specification

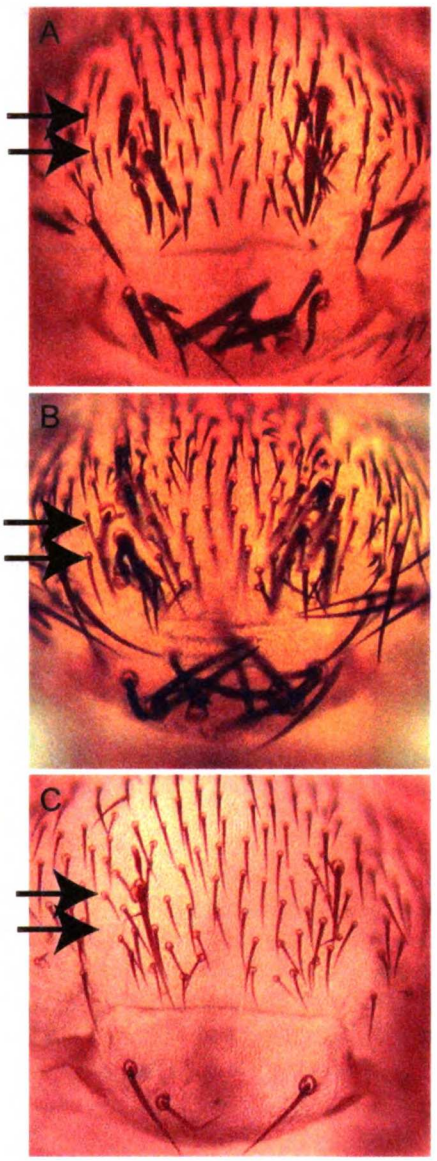


wildtype transformation

Figure 1 Legend

(A) Neuronal potential is acquired in clusters of epithelial cells during the first phase of neuronal determination. Patches of cells express proneural genes (yellow), making them competent to become neuronal precursors. If the expression of proneural genes is disrupted, the phenotypic outcome is often balding of the notum. (B) In the second phase of neuronal determination, cells within a proneural cluster communicate with each other via Notch/Delta signaling at cell-cell contacts (orange and black). High levels of Notch activity causes cells to remain epithelial (orange circle), while low levels of Notch activity selects one cell as the SOP (green circle). When lateral inhibition is disrupted, cells cannot signal to each other via Notch and Delta, resulting in the overcommittment of cells as SOPs, and tufting phenotypes. (C) In a third phase of neuronal determination, cell fates are specified as the SOP divides in a well characterized lineage. Cells in the external branch of the lineage (pIIa) divide in the plane of the epithelium, to become the hair and socket cell of the mature ES organ (light and dark black). The pIIb precursor of the internal branch of the lineage re-orients to divide apicobasally, generating a migrating glia (light blue) and the pIIIb precursor, which subsequently divides to generate the neuron (lavender), and glial sheath cell (red) of the ES organ. When cell fate specification is disrupted, cells transform between fates within the lineage. In this example, the cell that should become the pIIa precursor takes on the pIIb fate, and divides to produce extra internal neurons and glia, resulting in a balding phenotype.

Figure 2: Misexpression of EP(3)3622 results in a tufting phenotype that interacts with *Notch* and *Hairless*



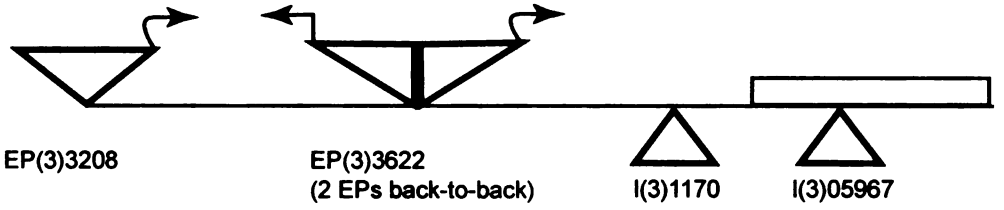
scaG4 x EP(3)3622

Notch / + ;
scaG4 x EP(3)3622

Hairless / + ;
scaG4 x EP(3)3622

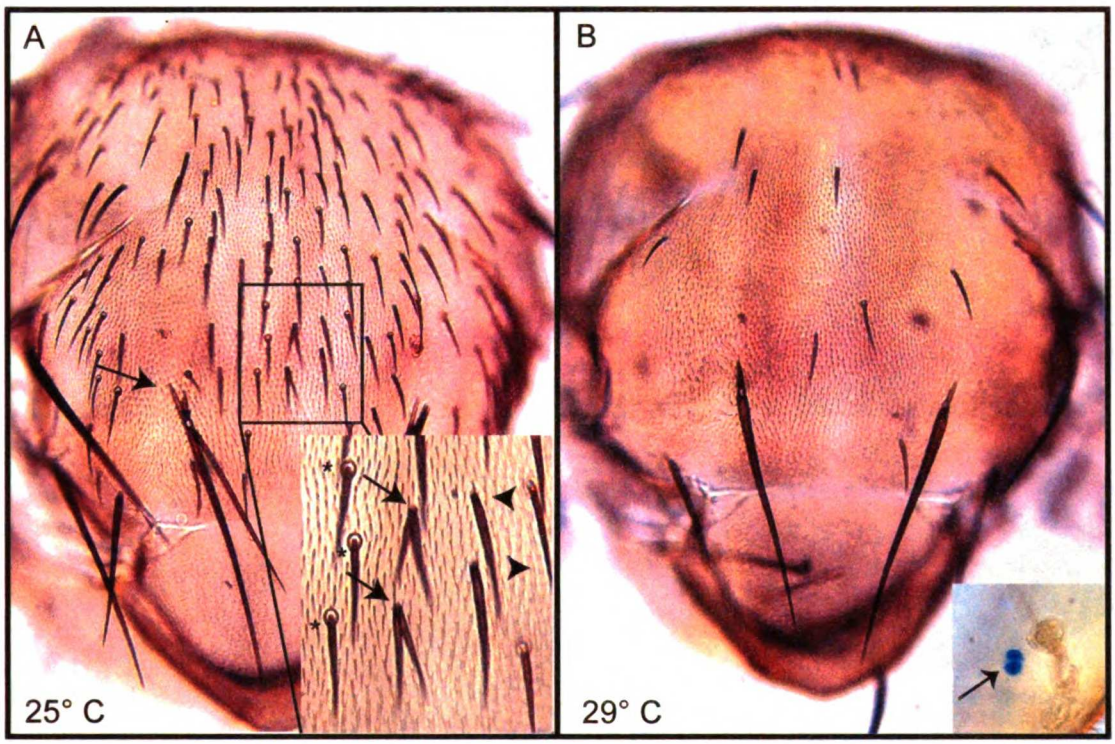
(A) Misexpression of EP(3)3622 with sca-G4 causes bristle tufting. ES organs at dorsal-central and scutellar macrochaete positions are multiplied (arrows), indicating that too many SOPs were specified, and that lateral inhibition was disrupted.
(B) If EP(3)3622 is misexpressed in a heterozygous *Notch* background, the severity of the phenotype is enhanced. Increased numbers of extra ES organs are visible within ectopic tufts of bristles (arrows). (C) If a heterozygous mutation in *Hairless* is included in the background of a fly misexpressing EP(3)3622 in the SOPs, the misexpression bristle tufting phenotype is significantly suppressed (arrows).

Figure 3: EP(3)3622 genomic insertion site



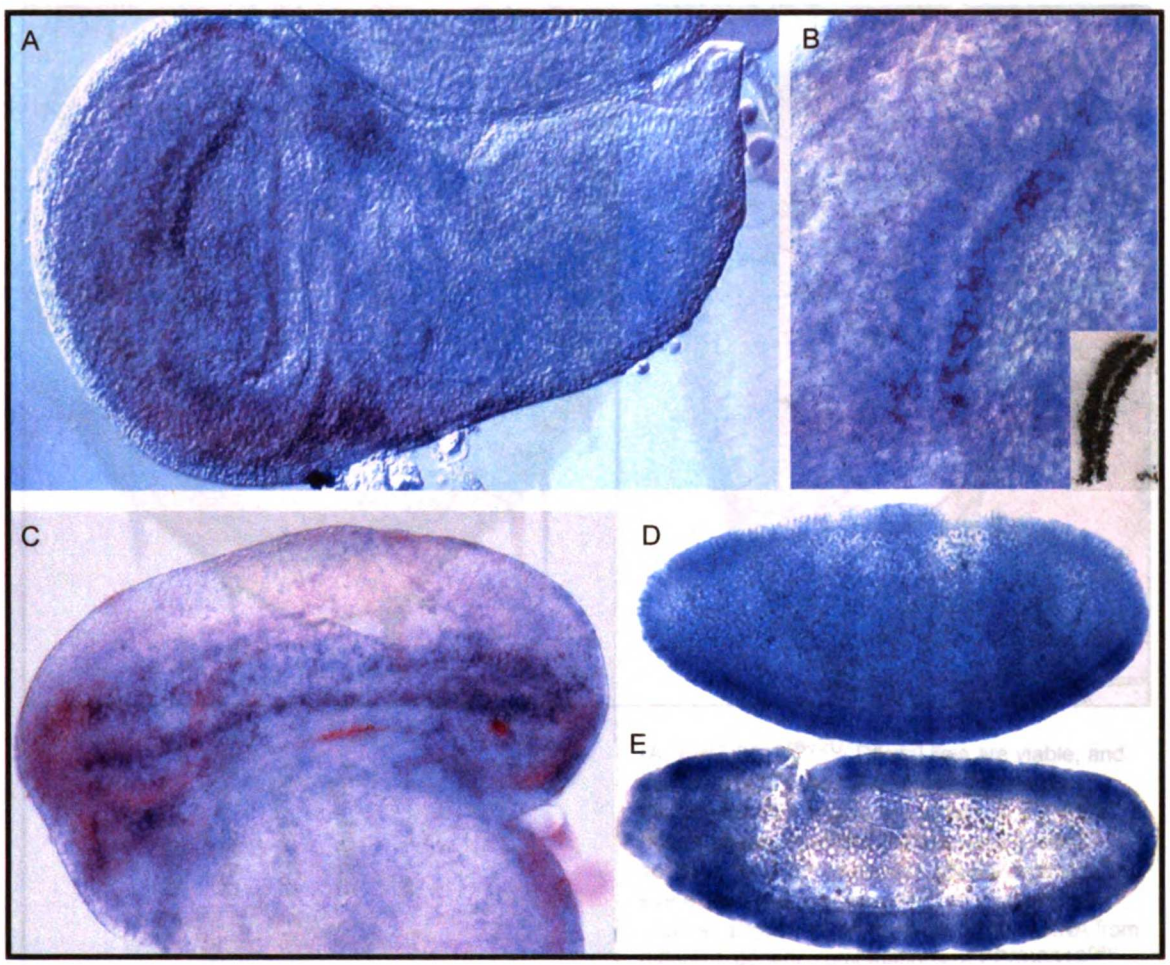
A map of the genomic region where EP(3)3622 is inserted (61C). At the genomic site, 2 EP elements are inserted, back-to-back, facing both 5' and 3'. Approximately 2kb upstream of the EP(3)3622 insertion lies another EP element (EP(3)3208). Downstream (3') of the EP(3)3622 insertion lie 2 lethal P-element insertions (l(3)1170, l(3)05967). *bantam*, which has been shown to be misexpressed by EP(3)3622 near the EP(3)3622 insertion site.

Figure 4: Misexpression of *tribbles* in the SOP causes balding and twinning of ES organ bristles



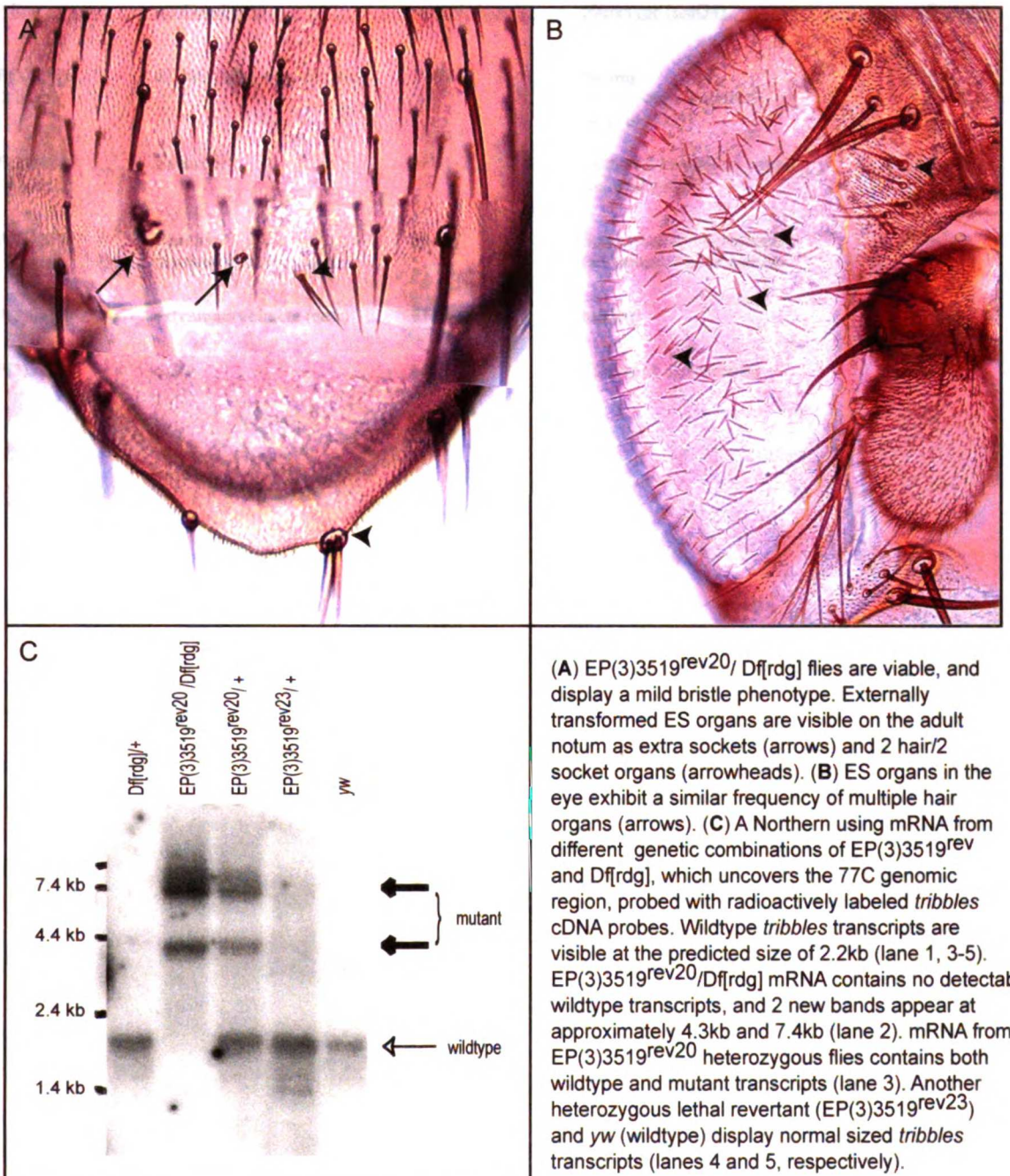
(A) The adult notum of a fly misexpressing *tribbles* with sca-G4 and raised at 25°C. Mild balding, twinning (arrows), and hairs without sockets (arrowheads) are evident across the notum. Normal ES organs are also present (*). (B) Flies of the same genotype raised at 29°C predominantly display a balding phenotype. Internally, double pros-positive cells indicate the duplication of internal glia, suggesting the occurrence of transformations within the SOP lineage (arrow, inset).

Figure 5: *tribbles* is expressed in the embryo and larval imaginal discs



(**A**) *In situ* hybridization of a third instar wing imaginal disc, using antisense *tribbles* RNA probes. Staining is apparent at the wing anterior wing margin, across the ventral and dorsal wing pouch, and on medial regions of the wing disc that will form the adult notum. (**B**) Closeup of the anterior wing margin. Prominent staining is evident at the antieror wing margin, similar to the staining pattern of anti-Achaete antibodies (Couso et al., 1994; inset), and displaying two rows of cells that label positive for tribbles RNA. (**C**) A third instar antennal-eye disc shows *tribbles* expression at the morphogenetic furrow and in a distinct pattern ahead and behing the furrow. (**D**) A stage 5 embryo showing the embryonic pattern of *tribbles* expression. (**E**) A stage 11 embryo showing a segmented pattern of tribbles expression in the embryo.

Figure 6: EP3519^{rev20} has an ES organ phenotype and lacks wildtype *tribbles* mRNA



(**A**) EP(3)3519^{rev20}/ Df[rdg] flies are viable, and display a mild bristle phenotype. Externally transformed ES organs are visible on the adult notum as extra sockets (arrows) and 2 hair/2 socket organs (arrowheads). (**B**) ES organs in the eye exhibit a similar frequency of multiple hair organs (arrows). (**C**) A Northern using mRNA from different genetic combinations of EP(3)3519^{rev} and Df[rdg], which uncovers the 77C genomic region, probed with radioactively labeled *tribbles* cDNA probes. Wildtype *tribbles* transcripts are visible at the predicted size of 2.2kb (lane 1, 3-5). EP(3)3519^{rev20}/Df[rdg] mRNA contains no detectable wildtype transcripts, and 2 new bands appear at approximately 4.3kb and 7.4kb (lane 2). mRNA from EP(3)3519^{rev20} heterozygous flies contains both wildtype and mutant transcripts (lane 3). Another heterozygous lethal revertant (EP(3)3519^{rev23}) and *yw* (wildtype) display normal sized *tribbles* transcripts (lanes 4 and 5, respectively).

Chapter 2 - Table 1: Proneural induction phenotypes

EP line	predicted gene	map position	phenotype (scaG4)	Genetics
EP(X)1306 (+2 lines)	**dLMO** (LIM-only domain)	**17C1-2**	balding ectopic wing outgrowth (with ptc-G4)*	-/-
EP(2)0684 (+ 4 lines)	**Escargot**	**35D1-2**	Complete balding on notum (internal cells?)	-/-
EP(2)2299 (+ 2 lines)	**Beach1** involved in endocytosis	**26A1**	Severe loss of microchaete (abdomen), loss of neurons	no/no
EP(3)0415	**extramacrochaete (emc)**	**61D1-2**	balding	-/yes
EP(3)3168	**dally**	**66E1-2**	balding of macrochaete	-/-

Chapter 2 - Table 2: Lateral inhibition phenotypes

EP line	predicted gene	map position	phenotype (scaG4)	Genetics
EP(X)1216b	CyclinA/cdk2 associated **p19** (RNA pol II associated protein)	**13C7-8**	extra bristles, 2h/2so (small bristle morphology)	no/no
EP(X)1435	**scalloped**	**13F1-2**	extra macrochaete, missing microchaete	-/no
EP(2)0639 (+ 3 lines)	**SD02913**	**53D1-2**	2h/2so Hair/2 socket, abnormal bristle morphology	-/yes
EP(2)2278	**bigbrain**	**30F**	extra bristles	no/-
EP(3)3622	**bantam**	**61C7-8**	tufting, 2hair/2socket	yes/yes

Chapter 2 - Table 3: Cell fate specification phenotypes in the SOP lineage

EP line	predicted gene	map position	phenotype (scaG4)	Genetics
EP(X)1408	Mitochondrial carrier homolog	**3E6-7**	2 hair/2socket	yes/yes
EP(2)0456	**LD26519**	**21A4**	2h/2so, twins	no/no
EP(2)0598	**yan**	**22D1-2**	2h/2so, hair w/o socket hair/2sockets	no/no
EP(2)0647	**lolalike** (BTB transcription factor)	**55B5-10**	2h/2so, hair w/o socket Thick bristle morphology	yes/yes
EP(2)1221 (+ 2 lines)	Genbank ID: AQ025055	**27F3-5**	hair w/o socket, 2hair/2socket	no/no
EP(2)1229	Genbank ID: AQ073484	**52B1-3**	2h/2so, hair/2socket, extra bristles	no/-
EP(2)2010	**Inscuteable**	**57B1-4**	hair w/o socket, 2h/2so, balding	yes/yes
EP(2)2146b	**Ubiquitin conjugating enzyme 2 (UbcD2)**	**32A5**	Loss of external cells, two hair/2 socket, hair w/o socket	no/-
EP(2)2237	**Zinc Finger transcription factor**	**21C4-6**	2 hair/2socket, hair w/o socket socket w/o hair, abnormal bristle morphology	-/-
EP(2)2583	**split ends**	**21B4-6**	hair w/o socket	yes/yes
EP(3)3017	**rab11** (vesicle transport)	**93C1-2**	hair w/o socket, balding	no/-
EP(3)3104	near **klar** and **CG17090** (ser.thr kinase)	**61C3-4**	hair w/o socket, 2so/2h	-/-
EP(3)3121	**eRF-1** (transcription termination factor)	**77B1-9**	2h/2so, Hair w/o socket Thin stumpy bristles	-/-
EP(3)3519	**tribbles**	**77C1-2**	twins, balding internal transformations	yes/yes
EP(3)3673	**Drosophila Zyg homolog (human)** Armadillo repeats, RNI (RNAse inhibitor)-like (centrosome replication)	**62A1-2**	balding, hair w/o socket	-/yes

Chapter 2 - Table 4: Morphology and cell viability phenotypes

EP line	predicted gene	map position	phenotype (scaG4)	Genetics
EP(2)2289	**Kruppel-homolog isoform** Zinc-finger transcription factor	**26B7-9**	severe loss of microchaetae on abdomen, Short and thickened bristle morphology	no/no
EP(3)3463	**taranis** (cell cycle regulation and glial cell migration)	**89B-C**	balding, hair w/o sockets, dead external cells deformed socket morphology	no/no
EP(3)3707	**DNA pol α** – subunit of E2F	**93E8-9**	short and thin shaft morphology	-/-
EP(3)0381	**fat facets** (de-Ubiquitination enzyme) (associated with liquid facets)	**100D1-3**	balding, death of external cells (black dot)	no/-
EP(3)3415	**Pebble** Rho GEF involved in cell cycle	**66A17-18**	balding, extra internal sheath cells	no/no
EP(3)3449	**gliolectin**	**93F6-8**	balding, 2h/2so, abnormal bristle morphology	-/-

Chapter 3: Lethal Giant Larvae Acts Together with Numb in Notch inhibition and cell fate specification in the *Drosophila* adult sensory organ precursor lineage

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

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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^{4}, a small deletion removing the *lgl* coding sequence [8], and lgl^{4w3}, 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^{4} or lgl^{4w3}, 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

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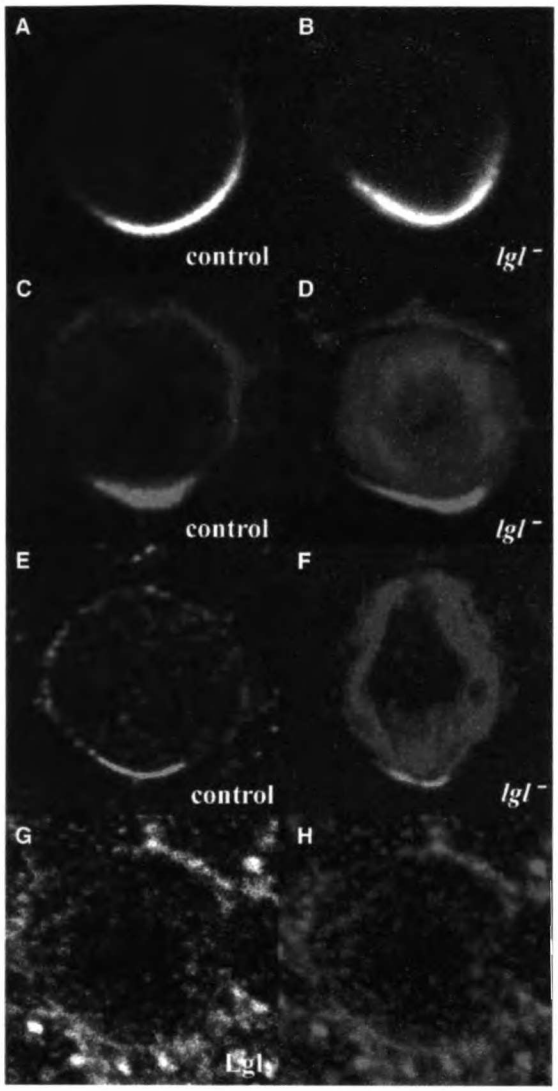


Figure 1. Lgl Is Not Required for Asymmetric Localization of Cell Fate Determinants in the SOP Cell

(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.

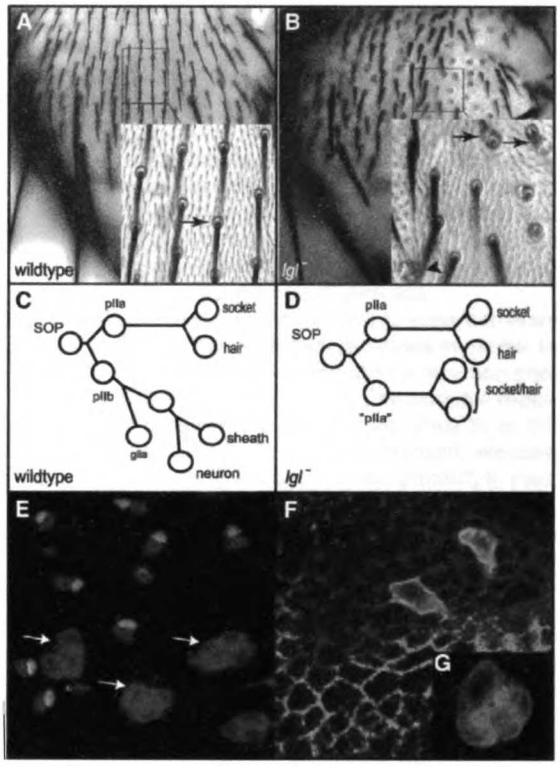


Figure 2. Lgl Is Required for Specification of the Internal Neurons and Glia of the SOP Lineage

(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 pIIb is transformed into an ectopic pIIa 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).

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

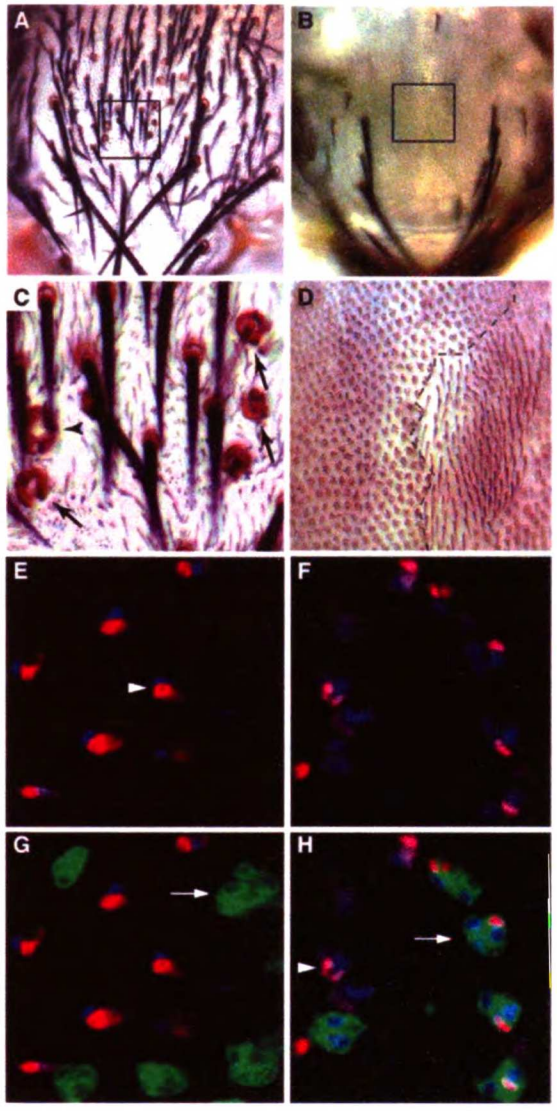


Figure 3. Inactivation of Notch during Cell Fate Specification in the SOP Lineage Reverses the *lgl* Mutant Phenotype

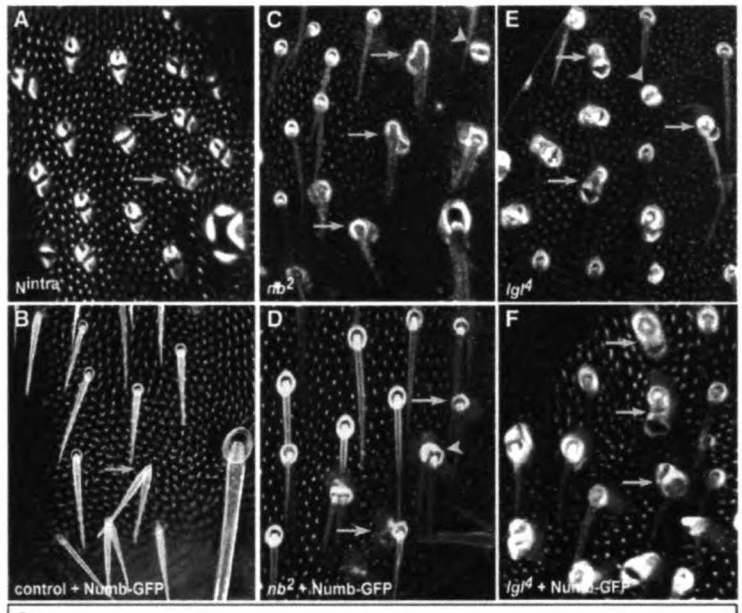
(A–D) (A and C) The adult notum of a female control fly, heterozygous for *Notch*^{ts} and containing *lgl* mutant clones, that has been shifted to the restrictive temperature (29°C) during cell fate specification of the microchaetes (12–20 hr APF). The microchaete exhibit *lgl* loss-of-function multiple 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 *lgl* 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 *lgl* mutant and wild-type tissue indicates that the *Notch* phenotype is epistatic to *lgl*. The portions boxed in (A) and (B) are shown in (C) and (D), respectively.

(E–H) Internal cell clusters in *lgl* 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 *lgl* 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 the *lgl* mutant clone (arrowhead). (G) The same region as in (E). The SOP progeny within the *lgl* MARCM mutant clone are positively marked by mCD8-GFP (green); the *lgl* 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 *lgl* 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 *lgl* 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).

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 *lgl* mutant clones because of the well-characterized lineage of the adult SOP [5]. Loss of *lgl* causes the pIIb cell, which normally gives rise to internal cells including a neuron and a glia, to adopt the pIIa 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 *lgl* 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 *lgl* 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 *lgl* mutant clones (Figures 3B and 3D), which is in contrast with the external transformation phenotypes that remain in *lgl* mutant clones on *Notch*^{ts} heterozygous control flies (Figures 3A and 3C). When examined internally, GFP-marked *lgl* mutant clusters are composed entirely of internal cells expressing Pros and/or Elav in hemizygous *Notch*^{ts} pupae (Figures 3F and 3H), while, in *lgl* 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 *lgl* mutant ES organs is due to an increase in Notch signaling activity, and that *lgl* 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. *lgl* and *numb* mutants exhibit similar cell fate phenotypes, and both cause transformation of the pIIb cell into an ectopic pIIa, which divides once to generate two additional external cells in each SOP cluster (Figures 4C and 4E). Our observations that *Notch* is epistatic to *lgl* 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-



G

	external transformations		external transformations
nb^2	99% (n=33)	nb^2 + numbGFP	40% (n=43)
lgl^4	99% (n=86)	lgl^4 + numbGFP	89% (n=123)

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

(A) Overexpression of $Notch^{intra}$ 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 *numb²* 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 *numb²* clones (arrows). The appearance of a partially rescued two hair/two socket phenotype is also common (arrowhead).

(E) In an *lgl⁴* clone (marked by GFP expression), the phenotype is similar to that seen in *numb²* 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 *lgl⁴* 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 *numb²* 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^4 and lgl^{4-3} [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 $numb^2$ [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^+ nb^2 *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^4 *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^4 *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-nPKCζ 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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References

1. Bilder, D., Li, M., and Perrimon, N. (2000). Cooperative regulation of cell polarity and growth by *Drosophila* tumor suppressors. Science *289*, 113–116.
2. Ohshiro, T., Yagami, T., Zhang, C., and Matsuzaki, F. (2000). Role of cortical tumour-suppressor proteins in asymmetric division of *Drosophila* neuroblast. Nature *408*, 593–596.
3. Peng, C.Y., Manning, L., Albertson, R., and Doe, C.Q. (2000). The tumour-suppressor genes lgl and dlg regulate basal protein targeting in *Drosophila* neuroblasts. Nature *408*, 596–600.
4. Bellaiche, Y., Radovic, A., Woods, D.F., Hough, C.D., Parmentier, M.L., O'Kane, C.J., Bryant, P.J., and Schweisguth, F. (2001). The Partner of Inscuteable/Discs-large complex is required to establish planar polarity during asymmetric cell division in *Drosophila*. Cell *106*, 355–366.
5. Gho, M., Bellaiche, Y., and Schweisguth, F. (1999). Revisiting the *Drosophila* microchaete lineage: a novel intrinsically asymmetric cell division generates a glial cell. Development *126*, 3573–3584.
6. Roegiers, F., Younger-Shepherd, S., Jan, L.Y., and Jan, Y.N.

(2001). Two types of asymmetric divisions in the *Drosophila* sensory organ precursor cell lineage. Nat. Cell Biol. *3*, 58–67.

7. Roegiers, F., Younger-Shepherd, S., Jan, L.Y., and Jan, Y.N. (2001). Bazooka is required for localization of determinants and controlling proliferation in the sensory organ precursor cell lineage in *Drosophila*. Proc. Natl. Acad. Sci. USA *98*, 14469–14474.
8. Mechler, B.M., McGinnis, W., and Gehring, W.J. (1985). Molecular cloning of lethal(2)giant larvae, a recessive oncogene of *Drosophila melanogaster*. EMBO J. *4*, 1551–1557.
9. Lee, T., and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron *22*, 451–461.
10. Lu, B., Rothenberg, M., Jan, L.Y., and Jan, Y.N. (1998). Partner of Numb colocalizes with Numb during mitosis and directs Numb asymmetric localization in *Drosophila* neural and muscle progenitors. Cell *95*, 225–235.
11. Bellaiche, Y., Gho, M., Kaltschmidt, J.A., Brand, A.H., and Schweisguth, F. (2001). Frizzled regulates localization of cell-fate determinants and mitotic spindle rotation during asymmetric cell division. Nat. Cell Biol. *3*, 50–57.
12. Agrawal, N., Kango, M., Mishra, A., and Sinha, P. (1995). Neoplastic transformation and aberrant cell-cell interactions in genetic mosaics of lethal(2)giant larvae (lgl), a tumor suppressor gene of *Drosophila*. Dev. Biol. *172*, 218–229.
13. Uemura, T., Shepherd, S., Ackerman, L., Jan, L.Y., and Jan, Y.N. (1989). numb, a gene required in determination of cell fate during sensory organ formation in *Drosophila* embryos. Cell *58*, 349–360.
14. Rebay, I., Fehon, R.G., and Artavanis-Tsakonas, S. (1993). Specific truncations of *Drosophila* Notch define dominant activated and dominant negative forms of the receptor. Cell *74*, 319–329.
15. Rhyu, M.S., Jan, L.Y., and Jan, Y.N. (1994). Asymmetric distribution of numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. Cell *76*, 477–491.
16. Guo, M., Jan, L.Y., and Jan, Y.N. (1996). Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. Neuron *17*, 27–41.
17. Frise, E., Knoblich, J.A., Younger-Shepherd, S., Jan, L.Y., and Jan, Y.N. (1996). The *Drosophila* Numb protein inhibits signaling of the Notch receptor during cell-cell interaction in sensory organ lineage. Proc. Natl. Acad. Sci. USA *93*, 11925–11932.
18. Hartenstein, V., and Posakony, J.W. (1990). A dual function of the Notch gene in *Drosophila* sensillum development. Dev. Biol. *142*, 13–30.
19. Strand, D., Unger, S., Corvi, R., Hartenstein, K., Schenkel, H., Kalmes, A., Merdes, G., Neumann, B., Krieg-Schneider, F., Coy, J.F., et al. (1995). A human homologue of the *Drosophila* tumour suppressor gene l(2)gl maps to 17p11.2–12 and codes for a cytoskeletal protein that associates with nonmuscle myosin II heavy chain. Oncogene *11*, 291–301.
20. Koyama, K., Fukushima, Y., Inazawa, J., Tomotsune, D., Takahashi, N., and Nakamura, Y. (1996). The human homologue of the murine Llglh gene (LLGL) maps within the Smith-Magenis syndrome region in 17p11.2. Cytogenet. Cell Genet. *72*, 78–82.
21. Kagami, M., Toh-e, A., and Matsui, Y. (1998). Sro7p, a *Saccharomyces cerevisiae* counterpart of the tumor suppressor l(2)gl protein, is related to myosins in function. Genetics *149*, 1717–1727.
22. Lehman, K., Rossi, G., Adamo, J.E., and Brennwald, P. (1999). Yeast homologues of tomosyn and lethal giant larvae function in exocytosis and are associated with the plasma membrane SNARE, Sec9. J. Cell Biol. *146*, 125–140.
23. Strand, D., Jakobs, R., Merdes, G., Neumann, B., Kalmes, A., Heid, H.W., Husmann, I., and Mechler, B.M. (1994). The *Drosophila* lethal(2)giant larvae tumor suppressor protein forms homo-oligomers and is associated with nonmuscle myosin II heavy chain. J. Cell Biol. *127*, 1361–1373.
24. Arquier, N., Perrin, L., Manfruelli, P., and Semeriva, M. (2001). The *Drosophila* tumor suppressor gene lethal(2)giant larvae is required for the emission of the Decapentaplegic signal. Development *128*, 2209–2220.
25. Petritsch, C., Tavosanis, G., Turck, C.W., Jan, L.Y., and Jan, Y.N. (2003). The *Drosophila* myosin VI Jaguar is required for basal protein targeting and correct spindle orientation in mitotic neuroblasts. Dev. Cell *4*, 273–281.
26. Berdnik, D., Torok, T., Gonzalez-Gaitan, M., and Knoblich, J.A. (2002). The endocytic protein alpha-Adaptin is required for numb-mediated asymmetric cell division in *Drosophila*. Dev. Cell *3*, 221–231.
27. Seto, E.S., Bellen, H.J., and Lloyd, T.E. (2002). When cell biology meets development: endocytic regulation of signaling pathways. Genes Dev. *16*, 1314–1336.
28. Le Borgne, R., Bellaiche, Y., and Schweisguth, F. (2002). *Drosophila* E-cadherin regulates the orientation of asymmetric cell division in the sensory organ lineage. Curr. Biol. *12*, 95–104.

Chapter 4: Lgl function

Summary

The processes of epithelial polarization, asymmetric division, and cell fate specification, share a requirement for *lethal giant larvae* (*lgl*; Betschinger et al., 2003; Bilder et al., 2000; Justice et al., 2003; Ohshiro et al., 2000; Peng et al., 2000). The finding that *lgl* is required for cell fate specification in the SOP lineage led us to predict that *lgl* might affect polarity or asymmetry, and thereby influence cell fate. However, no disruption of asymmetric segregation of proteins is detectable in SOPs lacking *lgl* (Justice et al., 2003). Models for how Lgl might function to influence Notch-mediated cell fate decisions in the SOP will be presented here, using insights from previous studies on the molecular nature of the Lgl protein, and new data from analysis of *lgl* in the developing *Drosophila* adult peripheral nervous system.

Introduction

lgl was originally isolated with *dlg* in screens for mutations that cause increased proliferation in the *Drosophila* imaginal disks (Gateff, 1978). *lgl* encodes a 127 Kd protein containing WD-40 repeats and has no other homologs in the *Drosophila* genome (Mechler et al., 1985). Homologs of *lgl* have been identified in yeast (Kagami et al., 1998; Lehman et al., 1999), mouse (Plant et al., 2003), and human (Strand et al., 1995), all of which share a common domain structure with *Drosophila* Lgl. Biochemical analysis has shown that Lgl can bind myosin II (Zipper in *Drosophila*) and SNARE proteins (Strand et al., 1994b; Strand et al., 1995). The yeast homologs of *lgl*, Sro7/Sro77, are essential for targeted exocytosis, which is consistent with the observed

interaction between Sro7/77 and Sec9p, a yeast t-SNARE involved in vesicular trafficking and exocytosis (Kagami et al., 1998; Lehman et al., 1999). A role for *lgl* in targeted exocytosis has also been observed in *Drosophila*. Loss of function mutations in *lgl* cause defects in the secretion of the morphogenetic signaling factor, Decapentaplegic (Dpp), which disrupts embryo and wing development and results in patterning phenotypes similar to those caused by loss of *dpp* (Arquier et al., 2001). Together, these data from multiple systems support a model in which Lgl functions as a link between vesicles and motor proteins, perhaps localizing proteins to specific domains within the cell by directing vesicular trafficking and targeted exocytosis (Bilder et al., 2000).

A genetic requirement for *lgl* during the apico-basal polarization of epithelial cells has been well-characterized in the follicular, embryonic, and imaginal disc epithelia (Agrawal et al., 1995; Bilder et al., 2000; Tanentzapf et al., 2000). Cells organize into a polarized epithelium due to the coordinated activity of three protein complexes. Bazooka acts with aPKC and Par-6, together termed the Par complex, in an early step that designates the apical pole of the cell. Another protein complex composed of Crumbs, Stardust (Sdt), and Discs Lost (Dlt), responds to apical domain designation by the Par complex, and expands the apical domain basolaterally along the cell cortex (Bilder et al., 2003; Tanentzapf and Tepass, 2003). Meanwhile, Dlg, Scribble (Scr) and Lgl expand the basolateral domain apically. Crumbs/Sdt/Dlt complex activity and coordinated Dlg/Scr, Lgl activity are mutually antagonistic, each promoting apical versus basolateral domain expansion along the cortical cytoskeleton (Bilder et al., 2003; Tanentzapf and Tepass, 2003). The competition reaches a resolution where the adherens junction will form, at the

interface between apical and basal domains (Bilder et al., 2003; Johnson and Wodarz, 2003; Tanentzapf and Tepass, 2003).

Epithelial cells lacking *lgl* fail to form adherens junctions with neighboring cells and round up, disorganizing the developing epithelium and resulting in multilayered epithelial structures due to overproliferation (Bilder et al., 2000). Many of the other genes involved in establishing and maintaining epithelial polarity produce similar loss-of-function phenotypes. Thus, loss of proper polarity in the cells of a developing epithelium leads to a failure to form cell junctions, and the failure of those cells to respond to appropriate controls on proliferation mediated by cell contact (Bilder et al., 2000). However, a mechanism linking cytoskeletal polarity proteins such as Lgl with control of the cell cycle has not been elucidated. The discovery that Lgl is required in neuroblasts for the asymmetric segregation of cell fate determinants during mitosis was one of the first pieces of evidence implicating Lgl in the coordination of cellular polarity with the cell cycle (Ohshiro et al., 2000; Peng et al., 2000). In neuroblasts lacking *lgl*, Miranda, a protein normally segregated to a basal protein crescent during mitosis, is uniformly localized to the cytoplasm and is also found on the mitotic spindle (Ohshiro et al., 2000; Peng et al., 2000). The connection between the onset of mitosis and the formation of asymmetrically localized protein crescents suggests that this process is regulated by the cell cycle machinery (Lu et al., 2000). Lgl may function at the intersection between these two basic cellular processes (see appendix; Betschinger et al., 2003). Our finding that Lgl is required for the inhibition of Notch pathway signaling in the SOP suggests one possible mechanism that might mediate the coordinated regulation of cell polarity, cell-cell contact, and the cell cycle (Justice et al., 2003).

Understanding how Lgl coordinates cellular polarity and cell division to achieve proper segregation of proteins during asymmetric cell division of the embryonic neuroblast does not answer the question of what role Lgl plays to help establish cell polarity. Although a defect in asymmetric segregation is seen in the neuroblast when a mutant Lgl lacking phosphorylation consensus sequences is misexpressed, a corresponding disruption in the polarity of the overlying epithelial cell layer is not observed (Betschinger et al., 2003). This suggests that phosphorylation may not regulate Lgl during cell polarization or in other contexts where *lgl* function is required. One of these contexts is Notch pathway mediated specification of cell fate during the elaboration of the SOP lineage. Our results from studies in the SOP, reveal a role for Lgl in regulating Notch activity, which is required to specify the fates of all of the cells of the lineage (Justice et al., 2003). While it has been demonstrated that the activity of Lgl is required for Numb to appropriately inhibit Notch activity, the precise role of Lgl in this inhibitory mechanism is unclear (Justice et al., 2003). Given previous studies that have implicated Lgl in the regulation of vesicular trafficking, along with new studies suggesting the importance of vesicular trafficking in the regulation of Notch pathway signaling (Berdnik et al., 2002b), and additional preliminary data on the function of Lgl in the SOP, a number of models for how Lgl functions to inhibit Notch activity can be proposed. These models, as well as theoretical and experimental evidence supporting each model, are discussed below.

Results and Discussion

Lgl may inhibit Notch by regulating vesicular trafficking

In one possible scenario, Lgl functions to traffic vesicles and thereby directly participates in the inhibition of Notch activity to specify cell fate. The role of vesicle trafficking in the regulation of Notch pathway signaling has been emerging from many convergent lines of study. First, the discovery in *C.elegans* of a cell non-autonomous requirement for Notch endocytosis during the specification of fates in the vulval lineage, suggests that Notch endocytosis influences the ability of a cell to signal to neighboring cells via Notch and Delta (Shaye and Greenwald, 2002). Second, the recent discovery that Neuralized, an E3 ubiquitin ligase selectively expressed in the SOP (Lai et al., 2001; Yeh et al., 2001), promotes Delta endocytosis to influence cell fate decisions that are mediated by Notch signaling (Deblandre et al., 2001; Lai et al., 2001), suggests that endocytosis is a critical aspect in Notch pathway regulation. Studies in other Notch signaling contexts have indicated that Delta may have a cis-inhibitory (intracellular) impact on Notch signaling activation (Sakamoto et al., 2002), perhaps explaining how Neuralized positively regulates Notch pathway activity (Vassin et al., 1985). Finally, a requirement for α-Adaptin, a protein central to the process of endocytosis, in the Numb mediated inhibition of Notch activity during specification of cell fates in the SOP lineage has been revealed (Berdnik et al., 2002a). Lgl might function in one or more of these contexts, perhaps regulating the movement of Delta to the plasma membrane, or in moving Notch to the lysosome where it can be degraded, to inhibit Notch signaling activity.

Evidence for a direct role for Lgl in Notch inhibition

In support of a model in which Lgl participates in a step of Notch regulation that requires vesicular trafficking, we observed Lgl in vesicular-like structures when we labeled wildtype SOPs for Lgl protein (F. Roegiers, pers comm., Justice et al., 2003). In the SOP, which is specified by low levels of Notch activity during the process of lateral inhibition, we see strong Lgl staining in what appear to be vesicles within cells surrounding the SOP (Figure 1). Neuralized has been recently found to mono-ubiquitinate Delta, thereby inducing endocytosis and removal of Delta from the cell surface (Deblandre et al., 2001; Lai et al., 2001; Yeh et al., 2001). Perhaps during specification of cell fates in the SOP lineage, cis-interactions between Notch and Delta block Notch activation, and Delta functions to inhibit Notch signaling activity. The endocytosis of putative Notch/Delta complexes that cannot signal may be resolved by transport of Notch to the lysosome, and recycling of Delta to the plasma membrane where it can again exert an inhibitory influence on cis-oriented (intracellular) Notch receptors. Alternatively, when Delta interacts with Notch in a trans-orientation (intercellular), this endocytic step would potentiate signaling between cells, and could explain why Notch endocytosis is required non-autonomously in the *C.elegans* vulval lineage (Shaye and Greenwald, 2002). Additionally, this vesicular model of Notch pathway regulation could explain why Neuralized functions as a potentiator of Notch signaling via the endocytosis of Delta (Justice and Jan, 2002; Lai, 2002).

Lgl has been shown to be required for exocytosis in yeast and to regulate the secretion of Dpp during embryonic and larval development in *Drosophila* (Arquier et al., 2001; Kagami et al., 1998; Lehman et al., 1999). This evidence implicating Lgl in the

exocytosis of vesicles and secretion of proteins would lead one to predict that Lgl is not also part of an endocytic mechanism because these two cellular mechanisms rarely share protein machinery (Gundelfinger et al., 2003; Richmond and Broadie, 2002). This issue may be resolved, however, if Lgl functions in a stage of vesicular trafficking that is shared between the endocytic and exocytotic pathways, perhaps during transcytosis. For instance, if Numb-mediated inhibition of Notch by endocytosis involves the movement of an endocytic vesicle, containing Notch, from an endosome to an inhibitory compartment (Seto et al. 2002), this step may require Lgl.

A model for Lgl function with Numb in an endocytic mechanism.

Our finding that *lgl* functions genetically downstream of *numb* (Justice et al., 2003), which has been proposed to function in an inhibitory mechanism involving the endocytosis of Notch (Berdnik et al., 2002b), is hard reconcile with an *lgl* function in the exocytosis of an inhibitor of Notch. These two cellular mechanisms have not been reported to share common protein elements (Richmond and Broadie, 2002). One possibility is that Lgl is responsible for the movement of proteins involved in an endocytic inhibitory mechanism to the plasma membrane, placing *lgl* function genetically downstream of *numb.* In this scenario, *lgl* function would be permissive. Lack of Lgl would render Numb ineffective, due to the loss of an intact endocytic machinery at the plasma membrane, which is needed to inhibit Notch. Components of the endocytic machinery, such as α-Adaptin, have been recently found to be required for the Numb mediated inhibition of Notch activity to appropriately specify cell fate in the SOP lineage (Berdnik et al., 2002a). α-Adaptin binds Numb and is asymmetrically segregated during

division of the SOP. This asymmetric localization of α-Adaptin is dependent on the presence of Numb protein, consistent with the demonstration that Numb and α-Adaptin proteins physically interact both *in vitro* and *in vivo* (Berdnik et al., 2002a). We tested whether the localization of α-Adaptin is disrupted by loss of *lgl*, and found no change in α-Adaptin localization in *lgl* mutant SOPs (Justice et al., 2003). However, a potential explanation for the loss-of-function phenotype observed in *lgl* mutants still remains in a direct function for Lgl in the endocytic mechanism in which Numb inhibits Notch (Justice et al., 2003). This function would place *lgl* in a similar position as *α-adaptin* in a genetic pathway including *numb* and *Notch* (Berdnik et al., 2002a). Additionally, if Lgl functions during endocytosis, it might be predicted that Lgl physically interacts with Numb and/or α-Adaptin. In order to test whether a complex of Numb and α-Adaptin might also include Lgl, we assayed the ability of Numb and Lgl to interact *in vitro*.

Lgl binds Numb *in vitro*

To gain insight into whether the function of Lgl in Numb mediated Notch endocytosis might be direct or indirect, we performed *in vitro* binding assays with Lgl and Glutathione-S-Transferase (GST) fused to full length Numb, N- or C-terminal fragments of Numb, or the intracellular domain of Notch. GST fusion proteins containing either the full length or N-terminal portion of Numb, which includes a phosphotyrosine binding domain, were able to interact with *in vitro* translated Lgl (Fig 3). GST alone or GST fused to the C-terminal portion of Numb, however, exhibited no significant binding to Lgl (Fig 3, lanes 2, 5). Because Numb has been shown to bind Notch (Fig 3, lane 7; Guo et al. 1996), we tested whether Lgl could also bind the intracellular domain of

Notch. No significant interaction was detected between GST-Notch^{intra} and Lgl (Fig 3, lane 6). Our finding of a direct interaction between Lgl and Numb suggests that Lgl might be a member of a complex that mediates Notch endocytosis.

A role for Lgl in directing vesicles from locations in the cell where signaling is activated, to an inhibitory environment, could involve either endocytosis or transcytosis of Notch containing vesicles to endosomes destined to reach the lysosome and be degraded (Seto et al., 2002). This model of Numb-mediated Notch inhibition predicts the removal of Notch receptors before they are activated, given our current understanding of the proteolytic nature of Notch signaling activation (Chan and Jan, 1998). Notch is activated by proteolytic cleavage and release of the intracellular domain from the membrane to the nucleus (Artavanis-Tsakonas et al., 1999; Struhl and Adachi, 1998), where it modulates transcription, and would no longer be subject to downregulation via endocytosis. Perhaps the asymmetric segregation of Numb to one of the two daughter cells of a cell division, leads to the immediate endocytosis of Notch in the cell that receives Numb, and subsequent movement of those enodcytic vesicles to a degratory compartment. This temporally defined decrease in Notch activity would then specify a difference in cell fate.

Indirect models for Notch inhibition by Lgl

Many alternative, mutually non-exclusive models, in which loss of Lgl function indirectly leads to increases in Notch signaling activity and the mis-specification of cell fate, cannot be ruled out. For instance, Lgl may be necessary to specify the basolateral domain of the pIIb cell, which remodels to polarize and divide in an apico-basal

orientation. The inability to specify this domain might cause the anterior daughter to divide in the incorrect orientation within the plane, similar to the orientation in which the posterior daughter cell (pIIa) divides, indirectly resulting in the specification of the anterior daughter as a pIIa. Perhaps the remodeling of the cytoskeleton plays a necessary part in the inhibition of Notch activity. Misexpression of Numb causes transformation to a pIIb cell fate, and subsequent re-modeling of the cytoskeleton to achieve an apico-basal polarity. Thus, it seems that Numb mediated Notch inhibition, which depends on Lgl, is instructive to the acquisition of the pIIb cell fate. In contrast, Lgl is permissive; misexpression of Lgl in the SOP does not cause a phenotype (data not shown). The demonstration of a dependence of the Notch pathway on the formation of intracellular domains, however, would represent a new feature of Notch regulation, and perhaps suggests another possible indirect model for Lgl function.

A scenario in which loss of *lgl* might have an indirect effect on Notch signaling is suggested by its importance in the establishment and maintenance of cell-cell contacts in epithelial cells. Perhaps Notch signaling inhibition requires proper cell contacts to be established between sibling daughter cells of the SOP. In a similar role for Lgl in the SOP as that in epithelial cells, Lgl might be required to establish and maintain points of intercellular contact, where Notch signaling between cells occurs. We have observed that Armadillo, an important component of cell junctions, is delocalized in SOPs that lack Lgl (Figure 5; F. Roegiers, pers. comm.). This suggests that cell-cell contacts, where Cadherin, Frizzled, and Notch signaling occurs in the SOP, may be disrupted in the absence of Lgl. When Lgl is missing, a lack of contact between cells may be responsible for the observed increase in Notch signaling, which results in cell fate transformation

phenotypes observed in *lgl* mutant ES organs (Figure 6). Similar to cells in an epithelial layer where it has been suggested that cell-cell contacts provide inhibition of the cell cycle to suppress tumorigenesis (Bilder et al., 2000), cells in the SOP lineage may require cell contacts to inhibit Notch signaling. It is tempting to speculate that increased Notch signaling activity is also responsible for the overproliferation of epithelial cells that lack Lgl, however, there is little experimental evidence in support of this hypothesis. The connection between cell polarity, cell contact, and the cell cycle is exemplified in this scenario, in which all of these aspects of cell biology converge to influence cell fate decisions being made during development.

Investigating a role for Lgl in regulating Notch pathway activity

An imaging approach

In order to distinguish between potential scenarios for the role of Lgl in Notch inhibition and cell fate specification, it will be important to gain a better understanding of how Notch pathway activity is regulated by the vesicular distribution of Notch and Delta protein. Recently, many lines of evidence have indicated that the dynamics of Notch and Delta protein localization is important for regulating Notch activity in the SOP lineage, as well in other contexts of Notch mediated cell fate specification (Berdnik et al., 2002b; Lai et al., 2001; Seto et al., 2002; Shaye and Greenwald, 2002). However, the details of this mechanism are still relatively unclear. In order to determine the role of Lgl in this mechanism, the subcellular localization of Delta and Notch protein should be analyzed in wildtype and *lgl* mutant SOPs. Labeling wildtype and *lgl* mutant SOPs with antibodies against the extracellular and intracellular

domains of Notch and Delta might uncover which aspect of the vesicular distribution of these proteins is disrupted by loss of Lgl. For example, if *lgl* loss-of-function mutations cause a reduction in the number of Notch containing vesicles in the SOP, this might suggest that Lgl is required in an initial endocytic step, and support a model in which Lgl functions during endocytosis in cooperation with Numb and α-Adaptin (Figure 4). If removal of Lgl causes the accumulation of vesicles containing Delta protein, this would support a model in which Lgl is required to bring Delta to the plasma membrane, where it might function to inhibit the activation of Notch (Figure 2). Loss of Lgl might cause Delta and Notch to change localization relative to each other, as has been suggested by preliminary experiments on the effect of *numb* mutations on the vesicular localization of Notch and Delta (R LeBorgne, F Schweisguth pers comm.). A change in how Delta interacts with Notch might be predicted to occur at the transition between lateral inhibition and cell fate specification phases of SOP determination, given the difference in the effects of Notch activity during the selection of the SOP compared to its role during terminal cell fate decisions made by progeny cells of the lineage (Hartenstein and Posakony, 1990; Zeng et al., 1998b). This change in activation might correlate with a change from transcytosis, which has been proposed as a mechanism of Notch activation (Klueg and Muskavitch, 1999), to endocytosis, which is likely inhibitory (Berdnik et al., 2002).

Additional approaches that would help uncover how vesicular dynamics, potentially involving Lgl, function to regulate Notch pathway signaling, include the construction of epitope or GFP tagged Notch or Delta extracellular and intracellular fragments for immunohistochemistry and live imaging. Additionally, membrane

associated dyes such as FM-143, which has been used extensively for imaging the details of synaptic vesicle movements (Ryan et al., 1997), might also help uncover the step in which Lgl participates during Notch signaling. Due to the dynamic nature of a vesicle mediated regulatory mechanism, a combination of antibody labeling and live imaging techniques will be most successful.

A genetic approach

If Delta does, in fact, exert an inhibitory influence on Notch activity during cell fate specification in the SOP lineage, the predicted phenotypic outcome of misexpression of Delta in the SOP lineage with neuralized-Gal4 would be balding due to internal transformations. Previous overexpression studies using sca-G4, however, have not been reported to produce consistent internal transformations in the lineage, perhaps due to an effect on lateral inhibition as well as cell fate specification (Zeng et al., 1998b). Other Gal4 drivers that are expressed by one branch, or by single cells of the lineage (pros-G4, Moore et al., 2002; ase-G4, Barolo et al., 2000), may produce consistent Delta overexpression phenotypes that would make clear whether Delta ligands activate or inhibit Notch. A function of Lgl to regulate Delta localization, as has been proposed to be the role of Neuralized in Notch pathway signaling, could then be tested.

lgl loss-of-function causes external transformations, which is predicted by the exocytotic model to result from a failure to move Delta to the plasma membrane (Figure 2). In this model, Delta exerts a cis-inhibitory influence on Notch, which explains why *lgl* mutations cause phenotypes similar to Notch gain-of-function. Using the same genetic setup used to test the epistatic relationship of *numb* and *lgl* (see Chapter 3), it is possible

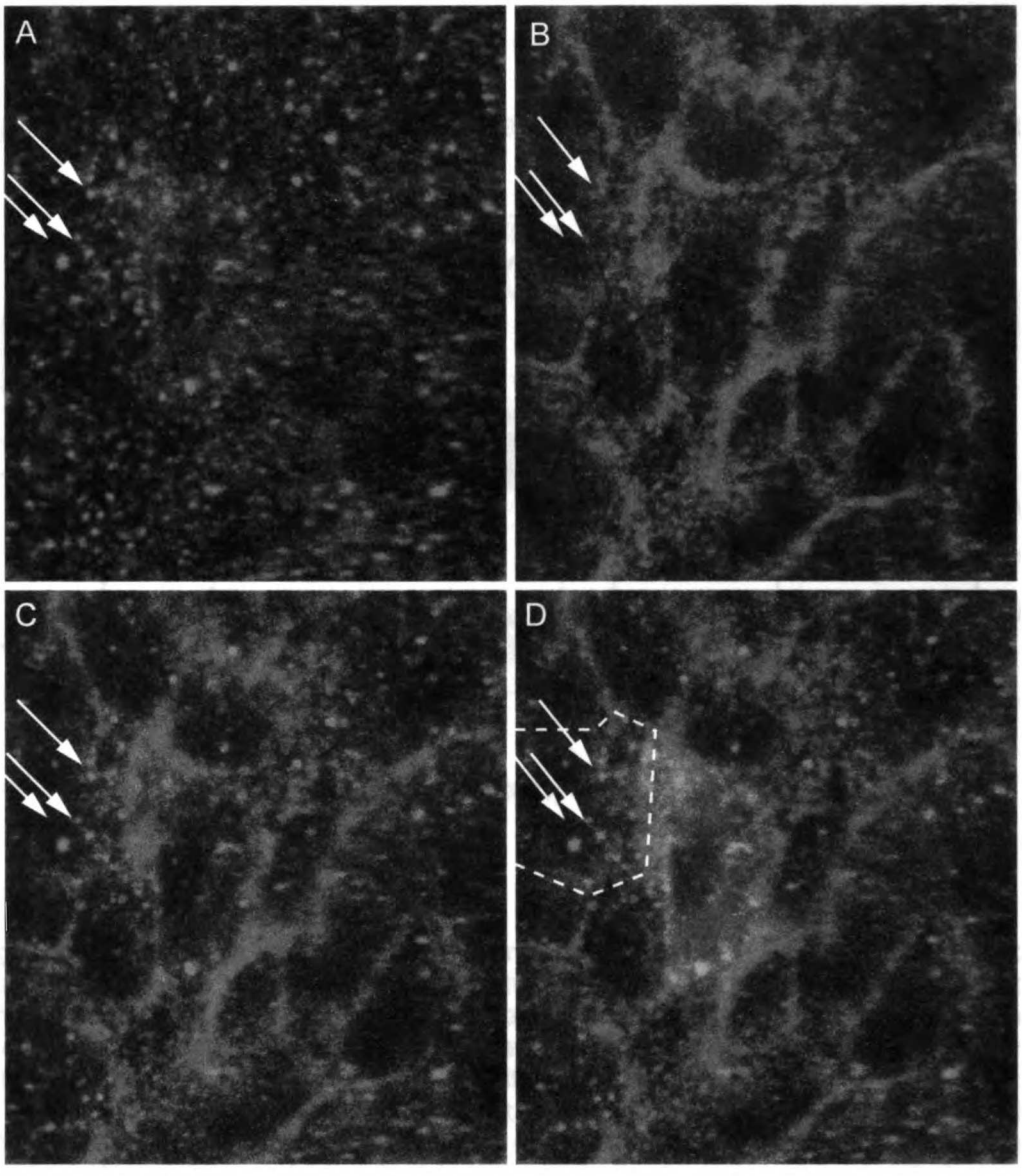
to over-express Delta in the SOP lineage while removing *lgl*, and thereby test whether Delta requires Lgl in order to effect cell fate decisions made by Notch pathway signaling. If Delta misexpression alone causes phenotypes consistent with a Notch inhibitory function, and this inihibition requires the activity of Lgl for localization to the plasma membrane, then removing Lgl should suppress or block the effects of Delta over-expression.

A biochemical approach

Recent studies suggest that Lgl complexes with Par-6 and aPKC to influence cell polarity and asymmetric cell division (see appendix; Betschinger et al., 2003; Plant et al., 2003; Yamanaka et al., 2003). aPKC was found to phosphorylate Lgl and thereby impact the subcellular localization of Lgl by altering its interaction with the cytoskeleton (Betschinger et al., 2003). Preliminary experiments in the SOP using flies carrying a transgene containing a mutant Lgl lacking aPKC phosphorylation sites and over-expressed with Neuralized-Gal4, display a lack of asymmetric Numb and Pon crescents (J Knoblich pers comm.). The cell fate phenotype that results, however, is relatively mild when compared with the *lgl* loss-of-function cell fate phenotype observed in mitotic clones of *lgl* null mutations (J Knoblich pers comm.; Justice et al., 2003), suggesting that phosphorylation does not regulate the activity of Lgl that is responsible for its inhibition of Notch activity. This raises the question of which protein associations are important for the function of Lgl in Notch mediated cell fate specification. If new interactions can be detected between Lgl and proteins important for Numb mediated Notch inhibition in the SOP lineage, such as α-Adaptin, this would suggest that the function of Lgl with Par-6

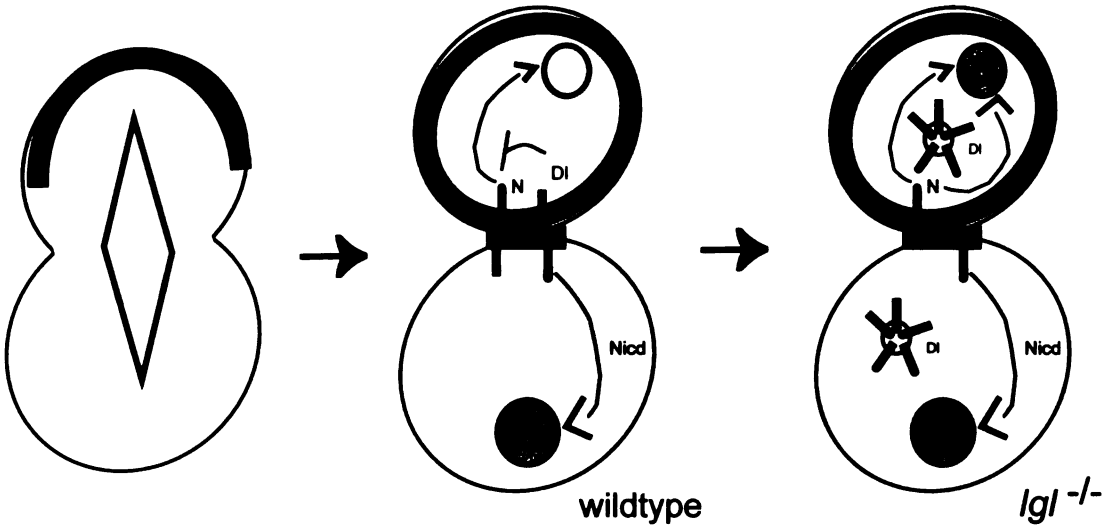
and aPKC in cell polarity is independent of its function in Notch inhibition. For example, Lgl may be a member of an endocytic complex containing both Numb and α-Adaptin, that is asymmetrically localized at cell division and functions in the endocytosis and selective inhibition of Notch through direct interaction with the Notch intracellular domain (Figure 4). Alternatively, Lgl may interact with proteins involved in the vesicular movements of Delta, such as Neuralized, to regulate Notch activity (Figure 2). Lgl may interact with junctional proteins, such as Armadillo, which is delocalized in *lgl* mutant epithelial cells (Figure 5), which would suggest an indirect role for Lgl in Notch pathway regulation due to a function in establishing and maintaining cell contacts (Figure 6). Because Lgl interacts with many proteins in many different contexts, a biochemical approach that isolated the SOP lineage would be most effective at finding a specific role for Lgl in Notch pathway signaling. This might be possible if an epitope-tagged Lgl transgene was overexpressed in the SOP lineage with Neuralized-Gal4, and Lgl interacting proteins were isolated by immunoprecipitations using antibodies against the epitope. Alternatively, immunoprecipitations could be performed using antibodies against candidate proteins, such as Numb, α-Adaptin or Armadillo, then probed to see if Lgl is a member of a larger complex that is known to function in the SOP.

Figure 1: Lgl is localized to vesicles



(A) Flourescent micrograph showing Lgl immunostaining of the pupal notum. Lgl immunoflourescence is found in puncta (arrows). (B) The same field of cells immunostained for Numb protein. Numb is predominantly localized to the plasma membrane but is also found in puncta. (C) A merged image of both Lgl and Numb stainings shown in panels A and B. Positively labeled puncta overlap in a subset of puncta in each population (arrows). (D) The same merged image as in C, now merged with a image showing an SOP positive for mCD8-GFP, which is selectively expressed in the SOP by Neuralized-Gal4. Numerous Lgl positive puncta appear to accumulate to higher levels in only one cell adjacent to the SOP (dashed line).

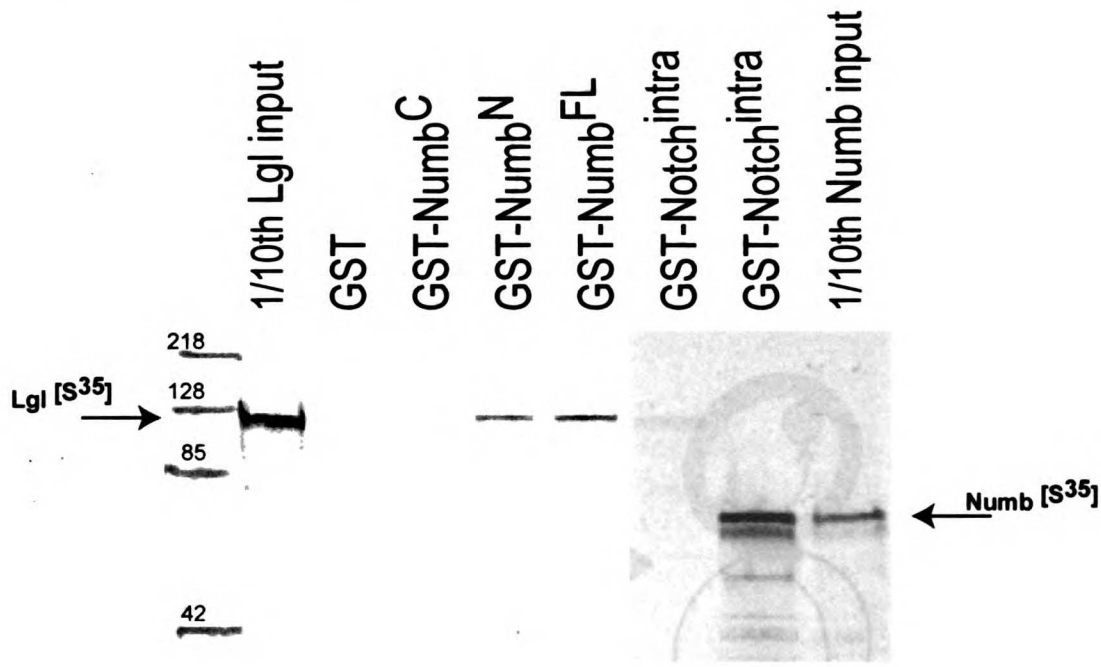
Figure 2: Exocytosis Model - Lgl is required for the exocytosis of a Notch inhibitor



Delta (or another protein) is "cis-inhibitory"

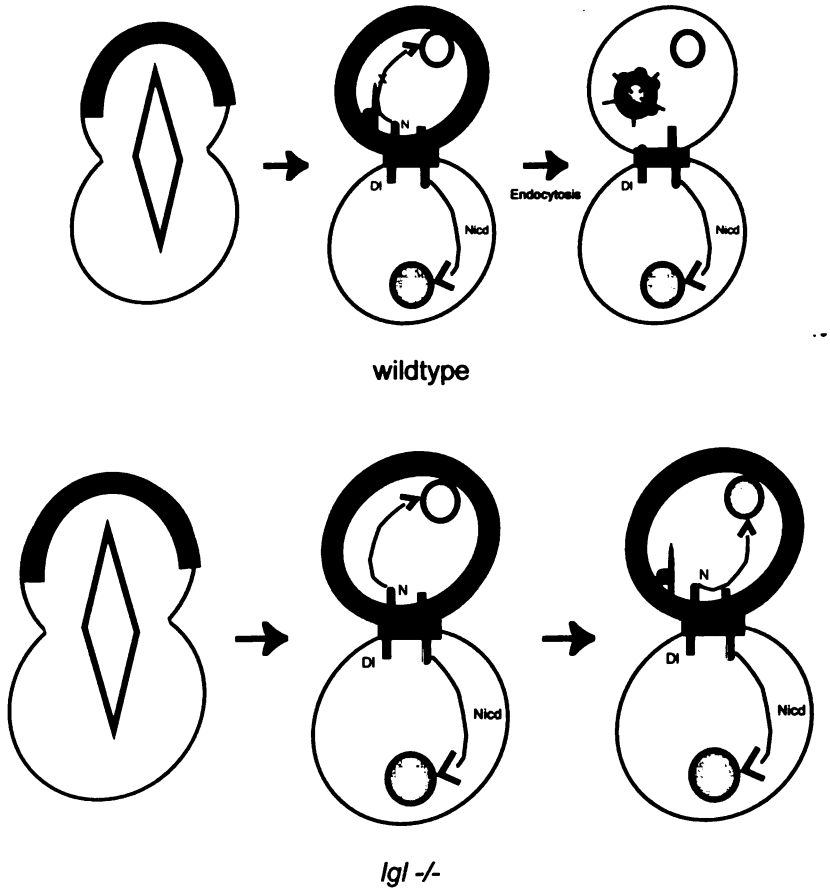
If Lgl inhibits Notch via a role in exocytosis, it may be required to move an inhibitor of Notch to the plasma membrane. In the wildtype, after cell division, the anterior daughter must inhibit Notch signaling activity to be appropriately specified as the pIIb precursor (open blue circle), which is accomplished through the asymmetric segregation of Numb (orange) to the anterior daughter cell. Delta has been proposed to function as an inhibitor of Notch activation during some contexts of cell fate specification, and has been shown to be endocytosed in response to mono-ubiquitination by Neuralized. If Delta must be recycled to the plasma membrane in order to manintain appropriate inhibition of Notch, Lgl may be required during exocytosis of vesicles containing Delta (or another Notch inhibitor).

Figure 3: Lgl binds Numb *in vitro*



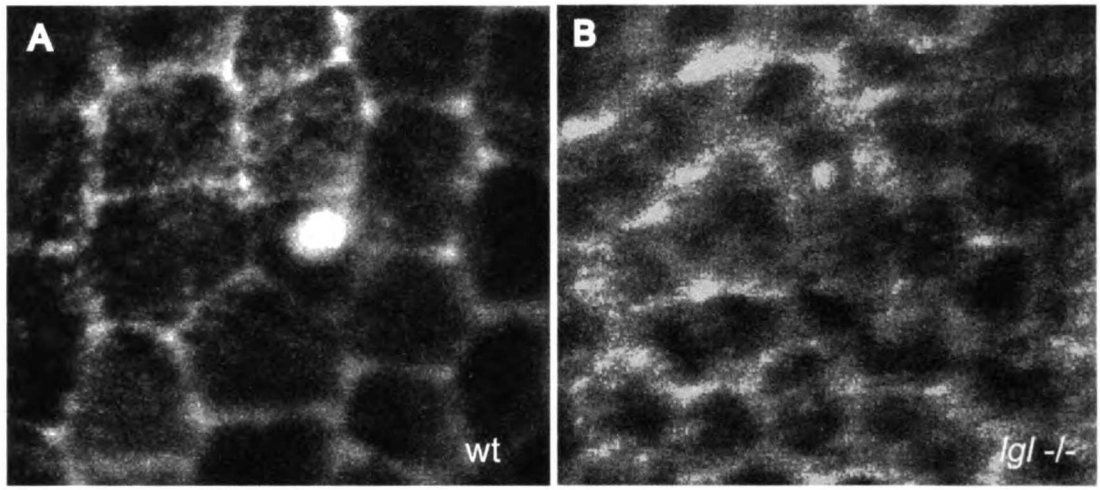
The results of a GST pulldown assay using [S^{35}]-radioactivity labeled, in vitro translated (IVT), Lgl protein, and GST-fused Numb or Notch fragments. Lgl does not pulldown with GST alone or with C-terminal fusions of Numb (GST-Numb^{C}; lanes 2-3). Lgl does pulldown with an N-terminal Numb fragment (GST-Numb^{N}), as well as with full-length Numb (GST-Numb^{FL}) GST fusions (lanes 4-5). GST fused to the intracellular domain of Notch (GST-Notch^{intra}) does not interact with IVT, [S^{35}]-labeled Lgl (lane 6), but does interact with IVT [S^{35}]-labeled Numb (lane 7). Input lanes show 1/10th (lanes 1,8) of the radioactively labeled input protein that was used in each of the experimental lanes (lanes 2-7). The ladder on the left is measured in kilodaltons.

Figure 4: An Endocytic model of Notch inhibtion



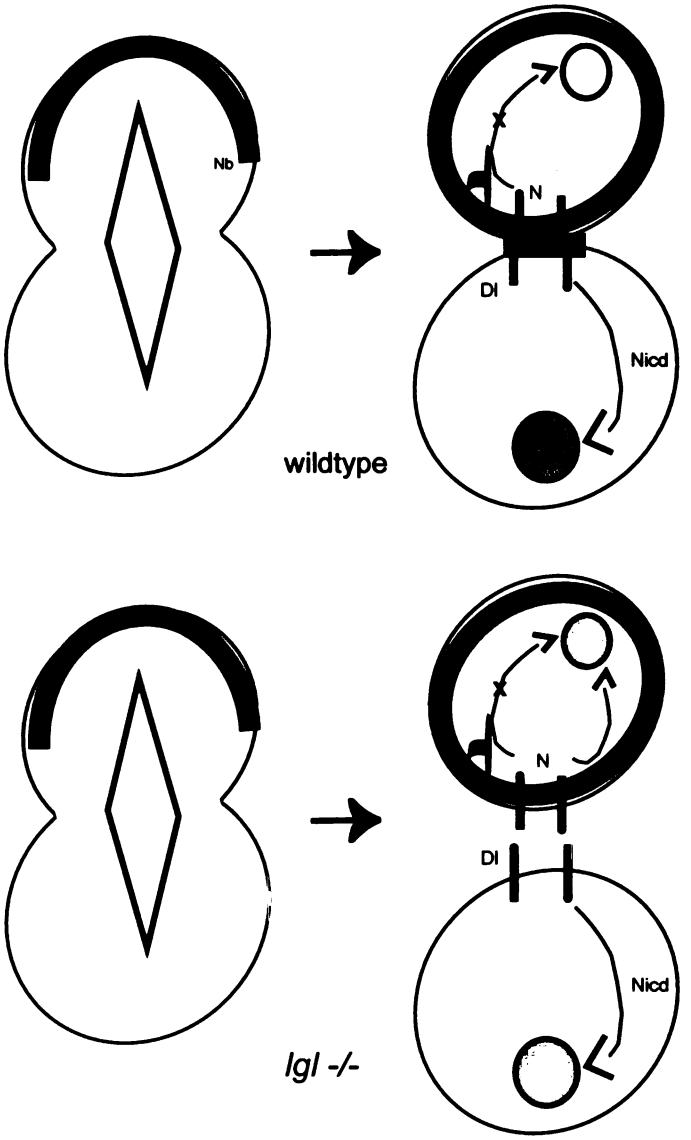
In this model, Lgl participates directly in the endocytosis of Notch, in a Numb-mediated inhibitory mechanism. Numb is asymmetrically segregated to the anterior daughter cell at cell division (orange), where it functions in the endocytosis of Notch receptors. This results in lower relative levels of Notch activity in the anterior daughter, which specifies this cell as the pIIb neuronal precursor (open blue circle). In the absence of Lgl this endocytic mechanism might be disrupted, leading to high levels of Notch activity in both daughters of the SOP division, which results in the specification of two pIIa precursors (filled blue circle), and external transformation phenotypes.

Figure 5: Armadillo localization is disrupted in *lgl* mutants



(**A**) A wildtype SOP (bright spot) surrounded by epithelial cells, and stained with antibodies against Armadillo, shows a restricted localization of the protein to the plasma membrane. (**B**) In the absence of Lgl, SOPs (along with surrounding epithelial cells) display increased cytoplasmic localization of Armadillo, and less staining at the interface between cells, suggesting that cell contacts may be disrupted by loss of Lgl.

Figure 6: Indirect model for Lgl function - loss of cell-cell contact



One indirect model that might explain the cell fate transformations seen in *lgl* loss-of-function mutants postulates that a properly developed cell-cell junction is disrupted in the absence of Lgl, which is required to inhibit Notch signaling activity. In the absense of proper cell contact, Notch activity is high in both cells, leading to the specification two pIIa precursors (filled blue circles), and loss of the internal pIIb lineage that generates the neuron and glia of the mature ES organ.

Appendix: A lethal giant kinase in cell polarity

A lethal giant kinase in cell polarity

focus on
cell polarity

Nicholas J. Justice and Yuh Nung Jan

The coordinated action of several conserved multiprotein complexes establishes polarity in an asymmetrically dividing cell. How apically localized cues affect the basal distribution of proteins has remained unclear. However, new studies provides a direct link between the two poles, showing that the apical Par6–aPKC complex directly regulates Lethal giant larvae (Lgl).

Cellular diversity arises when two daughters of a dividing cell become different from one another. Asymmetric cell division is one such mechanism that creates differences between daughter cells and is used in development to select neural precursors, to specify terminal cell fates and to maintain multipotent stem cell populations. During asymmetric cell division, a polarity cue in the mother cell is used to direct the asymmetric localization of cell-fate determinants. Asymmetrically dividing cells often rely on the earlier establishment of polarity in an epithelial cell layer for such a polarizing cue. For example, in the *Drosophila melanogaster* embryo, neural precursor cells termed 'neuroblasts' delaminate from a polarized epithelium, inheriting polarity cues at the apical cortex that will direct basal formation of a crescent containing the cell-fate determinants Numb and Prospero (Fig. 1). These proteins, together with their adaptors Partner-of-Numb and Miranda (Mir), are then asymmetrically segregated to the basal daughter cell during cell division. The apical Par complex directs their basal localization and consists of three factors: Bazooka (ASIP or mPar-3 in mammals, Par-3 in *Caenorhabditis elegans*), dPar-6 (mPar-6 in mammals, Par-6 in *C. elegans*) and aPKC (PKCλ/PKCζ in mammals, PKC-3 in *C. elegans*). Importantly, mutations in any one of these genes delocalizes the Par complex, resulting in a failure of basal crescent formation and subsequent changes in the cell fates produced by the division.

So how do apically localized proteins affect basal crescent formation at the basal pole? The first clue came from the discovery that Lgl, a WD protein originally identified in *Drosophila* as an epithelial tumour suppressor[1], is required specifically for basal crescent formation[2,3]. In *lgl* mutants, the Par complex forms normally at the apical cortex; however, the basal crescent fails to form. This requirement for Lgl suggests that it functions in a mechanism that couples the apical Par complex to basal crescent formation. Now, the connection between Lgl and the Par complex in this mechanism has become clear. Betschiger *et al.* in *Nature*[4] and Plant *et al.* on page 301 of this issue of *Nature Cell Biology*[5] independently report that Par-6, aPKC and Lgl physically interact in a complex, and that Lgl is in fact a substrate of aPKC. The direct interaction between apical proteins and those required for basal crescent formation reveals a functional link between the polarity complexes that is essential for cell polarization and asymmetric cell division.

Betschiger *et al.* set out to find what factors might mediate *Drosophila* Par complex activity by using antibodies against Par-6 to immunoprecipitate proteins that interact with the Par complex. This approach identified Lgl in a complex with Par-6 and aPKC. Independently, Plant *et al.* isolated an analogous complex from mammalian neural tissue. Kinase assays performed in both systems show that Lgl is phosphorylated at conserved PKC consensus sites by aPKC, raising the possibility that the apical Par complex regulates Lgl through phosphorylation to direct basal crescent formation. Indeed, when Betschiger *et al.* expressed an Lgl mutant that cannot be phosphorylated, Mir — a protein that is normally restricted to the basal crescent — became uniformly localized around the cell cortex in dividing neuroblasts. Moreover, expression of an aPKC mutant that lacks the Par-6-binding domain resulted in uniform cortical localization of aPKC and a redistribution of Miranda to the cytoplasm, similar to the altered localization of Miranda observed in *lgl* mutants. Thus, it seems that localized phosphorylation of Lgl at the apical cortex is necessary for proper basal crescent formation. The fact that phosphorylation of aPKC causes Lgl to dissociate from membranes and the cytoskeleton indicates that phosphorylation inhibits the cortical localization of Lgl. Taken together, these observations suggest a parsimonious model to explain how the apical Par complex directs basal formation of a crescent in the dividing neuroblast. Apically localized Par-6–aPKC activity results in phosphorylation of Lgl on the apical side of the cell, causing Lgl to disassociate from the cytoskeleton selectively at the apical cortex. Basally localized Lgl remains non-phosphorylated and therefore maintains an association with the cortical cytoskeleton, where it targets and/or retains anchors for cell fate determinants and their adaptor proteins. These proteins form a basal crescent that will be segregated to the basal daughter cell at cell division (Fig. 1).

This model, although attractive in its simplicity, may need some additional elements to explain observations that have been made in asymmetrically dividing neuroblasts. For instance, if the basal crescent forms as a result of Lgl dissociating from the apical cortex, why is it that the basal Miranda crescent does not abut the apical Par complex, but instead seems to be separated from the apical crescent by a gap? One explanation may be that the apical and basal crescents initially meet, but then, after Lgl is inactivated apically, proceed to separate along the lateral cortex through the action of myosins, such as Zipper or Jaguar[6]. Another possible explanation for this gap is the spreading of Par-6–aPKC activity beyond the apical crescent. As Par-6–aPKC releases apical Par-3/Baz and binds Lgl, consistent with observations that Par-6–aPKC–Lgl complexes do not contain Par-3/Baz, the complex might dissociate from the apical cortex. In the absence of an apical tether, aPKC activity could spread beyond the detectable apical crescent, phosphorylating cortical Lgl localized to the lateral cortex and creating a gap between apical and basal crescents.

Studies of mammalian homologues of the Par complex have identified many proteins that physically interact with Par-6–aPKC to influence polarity[7]. For example, mPar6 has been shown to interact with Par-3 (refs 8,9), Pals/Sdt[10] and Cdc42 (refs 8, 9). Furthermore, interaction of Par-6 with activated Cdc42 at the leading edge of astrocytes polarizes their migration in response to integrin signalling[11], as shown in the scratch wound assay. The recent report that Par-6 interacts with GSK3β[12] to influence cell migration (reviewed in a News and Views piece on page 275 of this issue), further supports a central role for Par-6 in organizing cell polarity. But is the interaction of Par-6, aPKC and Lgl important in establishing mammalian cell polarity? Plant *et al.* show that endogenous mLgl localizes to post-Golgi complexes, as well as to the leading edge of polarizing astroctyes. aPKC activity at the leading edge might be predicted to locally phosphorylate Lgl, thereby influencing polarization. Indeed, when

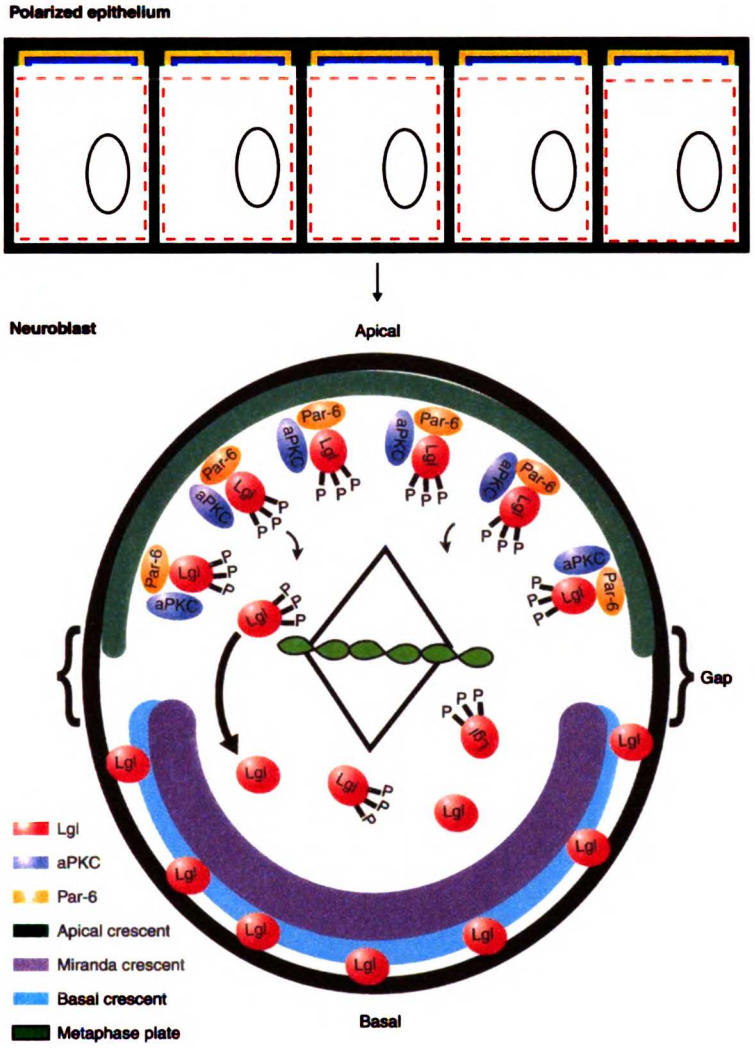


Figure 1 **A model for directing asymmetry: Lgl is apically phosphorylated by aPKC. Neuroblasts delaminate from a polarized epithelium, inheriting Par complex proteins at the apical cortex (green). Apically localized Par-6 (yellow) and aPKC (blue) bind to Lgl (red) at the apical cortex. As the neuroblast divides, aPKC phosphorylates Lgl, resulting in local dissociation of Lgl from the cortical cytoskeleton. At the opposite pole, basally localized Lgl is not phosphorylated, and therefore maintains cytoskeletal association, where it functions to target and retain proteins selectively at the basal cortex (light blue). Basally localized anchors recruit the adaptor proteins Miranda and Pon for cell fate determinants Pros and Numb that together form a basal crescent (purple). Basally localized Numb and Pros will be asymmetrically segregated to the basal daughter during cell division, where they will function to specify cell fate. A gap is often observed between apical and basal crescents along the lateral cortex, suggesting that additional components may function to further restrict the apical and basal crescents as the cell progresses through the cell cycle (adapted from ref. 4).**

Plant *et al.* microinjected astrocytes with constructs encoding a mutant of *Lgl* that lacks aPKC phosphorylation sites, they observed a significant decrease (19%) in the number of cells that could polarize in response to scratch wounding. This finding suggests that phosphorylation of Lgl is an important step, mediated by association with Par-6 and activated aPKC, that influences the acquisition of mammalian cellular polarity.

How might Lgl activity establish polarity? Lgl has been proposed to function in epithelial polarization by regulating the delivery of vesicles to specific domains through its interaction with vesicle/membrane-associated SNARE proteins and non-muscle myosin II (called Zipper in *Drosophila*)[13,14]. Consistent with this role for Lgl, yeast Lgl homologues have been shown to be required for vesicular transport and secretion[15]. How this function for Lgl correlates with the phosphorylation-dependent association of Lgl with the cell cortex is unclear. Previous studies of Lgl suggest that Lgl phosphorylation inhibits its interaction with myosins, which might result in dissociation of Lgl from cytoskeletal contacts[16]. In this way, a specific domain could be targeted for vesicle/protein delivery by spatially restricting the cytoskeletal localization of Lgl by phosphorylation, as seems to be the case during crescent formation in the asymmetrically dividing neuroblast.

The mechanism of cell polarization and migration in mammalian cells and the interactions critical for asymmetric segregation of fate determinants in the *Drosophila* neuroblast reveal the importance of Lgl phosphorylation in both cases. However, the extent to which phosphorylation by aPKC regulates Lgl is not yet known. Betschiger *et al.* note that although expression of a phosphorylation-defective Lgl mutant disrupts asymmetry in the neuroblast, it has little or no effect on the polarity of adjacent epithelial cells, which also require Lgl function to polarize appropriately. This suggests that Lgl may be regulated by alternative mechanisms in epithelial cells. Parallel studies in these systems, as well as in additional contexts where Lgl and aPKC are known to function, will be able to address how general the regulation of Lgl by phosphorylation is in coordinating cell polarity. □

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1. Gateff, E. *Science* **200**, 1448–1459 (1978).
2. Ohshiro, T., Yagami, T., Zhang, C. & Matsuzaki, F. *Nature* **408**, 593–596 (2000).
3. Peng, C. Y., Manning, L., Albertson, R. & Doe, C. Q. *Nature* **408**, 596–600 (2000).
4. Betschinger, J., Mechtler, K. & Knoblich, J. A. 422, 326–330 *Nature* (2003).
5. Plant, P. J. *et al. Nature Cell Biol.* **5**, 301–308 (2003).
6. Petritsch, C., Tavosanis, G., Turck, C. W., Jan, L. Y. & Jan, Y. N. *Dev. Cell* **4**, 273–281 (2003).
7. Ohno, S. *Curr. Opin. Cell Biol.* **13**, 641–648 (2001).
8. Lin, D. *et al. Nature Cell Biol.* **2**, 540–547 (2000).
9. Joberty, G., Petersen, C., Gao, L. & Macara, I. G. *Nature Cell Biol.* **2**, 531–539 (2000).
10. Hurd, T. W., Gao, L., Roh, M. H., Macara, I. G. & Margolis, B. *Nature Cell Biol.* **5**, 137–142 (2003).
11. Etienne-Manneville, S. & Hall, A. *Cell* **106**, 489–498 (2001).
12. Etienne-Manneville, S. & Hall, A. *Nature* **421**, 753–756 (2003).
13. Strand, D. *et al. J. Cell Biol.* **127**, 1361–1373 (1994).
14. Bilder, D., Li, M. & Perrimon, N. *Science* **289**, 113–116 (2000).
15. Lehman, K., Rossi, G., Adamo, J. E. & Brennwald, P. *J. Cell Biol.* **146**, 125–140 (1999).
16. Kalmes, A., Merdes, G., Neumann, B., Strand, D. & Mechler, B. M. *J. Cell Sci.* **109**, 1359–1368 (1996).

References Cited

Abdelilah-Seyfried, S., Chan, Y. M., Zeng, C., Justice, N. J., Younger-Shepherd, S., Sharp, L. E., Barbel, S., Meadows, S. A., Jan, L. Y., and Jan, Y. N. (2000). A gain-of-function screen for genes that affect the development of the Drosophila adult external sensory organ. Genetics *155*, 733-752.

Agrawal, N., Kango, M., Mishra, A., and Sinha, P. (1995). Neoplastic transformation and aberrant cell-cell interactions in genetic mosaics of lethal(2)giant larvae (lgl), a tumor suppressor gene of Drosophila. Dev Biol *172*, 218-229.

Alvarez-Buylla, A., Garcia-Verdugo, J. M., and Tramontin, A. D. (2001). A unified hypothesis on the lineage of neural stem cells. Nat Rev Neurosci *2*, 287-293.

Arquier, N., Perrin, L., Manfruelli, P., and Semeriva, M. (2001). The Drosophila tumor suppressor gene lethal(2)giant larvae is required for the emission of the Decapentaplegic signal. Development *128*, 2209-2220.

Artavanis-Tsakonas, S., Rand, M. D., and Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. Science *284*, 770-776.

Baker, N. E. (2000). Notch signaling in the nervous system. Pieces still missing from the puzzle. Bioessays *22*, 264-273.

Baker, N. E., Mlodzik, M., and Rubin, G. M. (1990). Spacing differentiation in the developing Drosophila eye: a fibrinogen-related lateral inhibitor encoded by scabrous. Science *250*, 1370-1377.

Bang, A. G., Hartenstein, V., and Posakony, J. W. (1991). Hairless is required for the development of adult sensory organ precursor cells in Drosophila. Development *111*, 89-104.

Barolo, S., Walker, R. G., Polyanovsky, A. D., Freschi, G., Keil, T., and Posakony, J. W. (2000). A notch-independent activity of suppressor of hairless is required for normal mechanoreceptor physiology. Cell *103*, 957-969.

Bellaiche, Y., Gho, M., Kaltschmidt, J. A., Brand, A. H., and Schweisguth, F. (2001a). Frizzled regulates localization of cell-fate determinants and mitotic spindle rotation during asymmetric cell division. Nat Cell Biol *3*, 50-57.

Bellaiche, Y., Radovic, A., Woods, D. F., Hough, C. D., Parmentier, M. L., O'Kane, C. J., Bryant, P. J., and Schweisguth, F. (2001b). The Partner of Inscuteable/Discs-large

complex is required to establish planar polarity during asymmetric cell division in Drosophila. Cell *106*, 355-366.

Berdnik, D., Torok, T., Gonzalez-Gaitan, M., and Knoblich, J. A. (2002b). The endocytic protein alpha-Adaptin is required for numb-mediated asymmetric cell division in Drosophila. Dev Cell *3*, 221-231.

Berezovska, O., McLean, P., Knowles, R., Frosh, M., Lu, F. M., Lux, S. E., and Hyman, B. T. (1999). Notch1 inhibits neurite outgrowth in postmitotic primary neurons. Neuroscience *93*, 433-439.

Berezovska, O., Xia, M. Q., and Hyman, B. T. (1998). Notch is expressed in adult brain, is coexpressed with presenilin-1, and is altered in Alzheimer disease. J Neuropathol Exp Neurol *57*, 738-745.

Betschinger, J., Mechtler, K., and Knoblich, J. A. (2003). The Par complex directs asymmetric cell division by phosphorylating the cytoskeletal protein Lgl. Nature *422*, 326-330.

Bilder, D., Li, M., and Perrimon, N. (2000). Cooperative regulation of cell polarity and growth by Drosophila tumor suppressors. Science *289*, 113-116.

Bilder, D., Schober, M., and Perrimon, N. (2003). Integrated activity of PDZ protein complexes regulates epithelial polarity. Nat Cell Biol *5*, 53-58.

Blair, S. S. (2000). Notch signaling: Fringe really is a glycosyltransferase. Curr Biol *10*, R608-612.

Brand, A. H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401-415.

Bray, S., and Furriols, M. (2001). Notch pathway: making sense of suppressor of hairless. Curr Biol *11*, R217-221.

Brennecke, J., Hipfner, D. R., Stark, A., Russell, R. B., and Cohen, S. M. (2003). bantam Encodes a Developmentally Regulated microRNA that Controls Cell Proliferation and Regulates the Proapoptotic Gene hid in Drosophila. Cell *113*, 25-36.

Bruckner, K., Perez, L., Clausen, H., and Cohen, S. (2000). Glycosyltransferase activity of Fringe modulates Notch-Delta interactions. Nature *406*, 411-415.

Chambers, C. B., Peng, Y., Nguyen, H., Gaiano, N., Fishell, G., and Nye, J. S. (2001). Spatiotemporal selectivity of response to Notch1 signals in mammalian forebrain precursors. Development *128*, 689-702.

Chan, Y. M., and Jan, Y. N. (1998). Roles for proteolysis and trafficking in notch maturation and signal transduction. Cell *94*, 423-426.

Cornell, M., Evans, D. A., Mann, R., Fostier, M., Flasza, M., Monthatong, M., Artavanis-Tsakonas, S., and Baron, M. (1999). The Drosophila melanogaster Suppressor of deltex gene, a regulator of the Notch receptor signaling pathway, is an E3 class ubiquitin ligase. Genetics *152*, 567-576.

Couso, J. P., Bishop, S. A., and Martinez Arias, A. (1994). The wingless signalling pathway and the patterning of the wing margin in Drosophila. Development *120*, 621-636.

Cubas, P., de Celis, J. F., Campuzano, S., and Modolell, J. (1991). Proneural clusters of achaete-scute expression and the generation of sensory organs in the Drosophila imaginal wing disc. Genes Dev *5*, 996-1008.

Deblandre, G. A., Lai, E. C., and Kintner, C. (2001). Xenopus neuralized is a ubiquitin ligase that interacts with XDelta1 and regulates Notch signaling. Dev Cell *1*, 795-806.

Deng, W. M., Althauser, C., and Ruohola-Baker, H. (2001). Notch-Delta signaling induces a transition from mitotic cell cycle to endocycle in Drosophila follicle cells. Development *128*, 4737-4746.

Doherty, D., Feger, G., Younger-Shepherd, S., Jan, L. Y., and Jan, Y. N. (1996). Delta is a ventral to dorsal signal complementary to Serrate, another Notch ligand, in Drosophila wing formation. Genes Dev *10*, 421-434.

Eddison, M., Le Roux, I., and Lewis, J. (2000). Notch signaling in the development of the inner ear: lessons from Drosophila. Proc Natl Acad Sci U S A *97*, 11692-11699.

Etienne-Manneville, S., and Hall, A. (2001). Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKCzeta. Cell *106*, 489-498.

Etienne-Manneville, S., and Hall, A. (2003). Cdc42 regulates GSK-3beta and adenomatous polyposis coli to control cell polarity. Nature *421*, 753-756.

Fichelson, P., and Gho, M. (2003). The glial cell undergoes apoptosis in the microchaete lineage of Drosophila. Development *130*, 123-133.

Fortini, M. E. (2001). Notch and presenilin: a proteolytic mechanism emerges. Curr Opin Cell Biol *13*, 627-634.

Frise, E., Knoblich, J. A., Younger-Shepherd, S., Jan, L. Y., and Jan, Y. N. (1996). The Drosophila Numb protein inhibits signaling of the Notch receptor during cell-cell interaction in sensory organ lineage. Proc Natl Acad Sci U S A *93*, 11925-11932.

Furukawa, T., Mukherjee, S., Bao, Z. Z., Morrow, E. M., and Cepko, C. L. (2000). rax, Hes1, and notch1 promote the formation of Muller glia by postnatal retinal progenitor cells. Neuron *26*, 383-394.

Gaiano, N., Nye, J. S., and Fishell, G. (2000). Radial glial identity is promoted by Notch1 signaling in the murine forebrain. Neuron *26*, 395-404.

Gateff, E. (1978). Malignant neoplasms of genetic origin in Drosophila melanogaster. Science *200*, 1448-1459.

Gho, M., Bellaiche, Y., and Schweisguth, F. (1999). Revisiting the Drosophila microchaete lineage: a novel intrinsically asymmetric cell division generates a glial cell. Development *126*, 3573-3584.

Ghysen, A., and Dambly-Chaudiere, C. (1989). Genesis of the Drosophila peripheral nervous system. Trends Genet *5*, 251-255.

Grosshans, J., and Wieschaus, E. (2000). A genetic link between morphogenesis and cell division during formation of the ventral furrow in Drosophila. Cell *101*, 523-531.

Gundelfinger, E. D., Kessels, M. M., and Qualmann, B. (2003). Temporal and spatial coordination of exocytosis and endocytosis. Nat Rev Mol Cell Biol *4*, 127-139.

Guo, M., Jan, L. Y., and Jan, Y. N. (1996). Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. Neuron *17*, 27-41.

Gupta-Rossi, N., Le Bail, O., Gonen, H., Brou, C., Logeat, F., Six, E., Ciechanover, A., and Israel, A. (2001). Functional interaction between SEL-10, an F-box protein, and the nuclear form of activated Notch1 receptor. J Biol Chem *276*, 34371-34378.

Hartenstein, V., and Posakony, J. W. (1990). A dual function of the Notch gene in Drosophila sensillum development. Dev Biol *142*, 13-30.

Heitzler, P., Bourouis, M., Ruel, L., Carteret, C., and Simpson, P. (1996). Genes of the Enhancer of split and achaete-scute complexes are required for a regulatory loop between Notch and Delta during lateral signalling in Drosophila. Development *122*, 161-171.

Heitzler, P., and Simpson, P. (1991). The choice of cell fate in the epidermis of Drosophila. Cell *64*, 1083-1092.

Hicks, C., Johnston, S. H., diSibio, G., Collazo, A., Vogt, T. F., and Weinmaster, G. (2000). Fringe differentially modulates Jagged1 and Delta1 signalling through Notch1 and Notch2. Nat Cell Biol *2*, 515-520.

Hipfner, D. R., Weigmann, K., and Cohen, S. M. (2002). The bantam gene regulates Drosophila growth. Genetics *161*, 1527-1537.

Hoppe, T., Matuschewski, K., Rape, M., Schlenker, S., Ulrich, H. D., and Jentsch, S. (2000). Activation of a membrane-bound transcription factor by regulated ubiquitin/proteasome-dependent processing. Cell *102*, 577-586.

Hoppe, T., Rape, M., and Jentsch, S. (2001). Membrane-bound transcription factors: regulated release by RIP or RUP. Curr Opin Cell Biol *13*, 344-348.

Hubbard, E. J., Wu, G., Kitajewski, J., and Greenwald, I. (1997). sel-10, a negative regulator of lin-12 activity in Caenorhabditis elegans, encodes a member of the CDC4 family of proteins. Genes Dev *11*, 3182-3193.

Hurd, T. W., Gao, L., Roh, M. H., Macara, I. G., and Margolis, B. (2003). Direct interaction of two polarity complexes implicated in epithelial tight junction assembly. Nat Cell Biol *5*, 137-142.

Irvine, K. D. (1999). Fringe, Notch, and making developmental boundaries. Curr Opin Genet Dev *9*, 434-441.

Jackson, P. K., Eldridge, A. G., Freed, E., Furstenthal, L., Hsu, J. Y., Kaiser, B. K., and Reimann, J. D. (2000). The lore of the RINGs: substrate recognition and catalysis by ubiquitin ligases. Trends Cell Biol *10*, 429-439.

Joberty, G., Petersen, C., Gao, L., and Macara, I. G. (2000). The cell-polarity protein Par6 links Par3 and atypical protein kinase C to Cdc42. Nat Cell Biol *2*, 531-539.

Johnson, K., and Wodarz, A. (2003). A genetic hierarchy controlling cell polarity. Nat Cell Biol *5*, 12-14.

Justice, N., Roegiers, F., Jan, L. Y., and Jan, Y. N. (2003). Lethal giant larvae acts together with numb in notch inhibition and cell fate specification in the Drosophila adult sensory organ precursor lineage. Curr Biol *13*, 778-783.

Justice, N. J., and Jan, Y. N. (2002). Variations on the Notch pathway in neural development. Curr Opin Neurobiol *12*, 64-70.

Kagami, M., Toh-e, A., and Matsui, Y. (1998). Sro7p, a Saccharomyces cerevisiae counterpart of the tumor suppressor l(2)gl protein, is related to myosins in function. Genetics *149*, 1717-1727.

Kalmes, A., Merdes, G., Neumann, B., Strand, D., and Mechler, B. M. (1996). A serine-kinase associated with the p127-l(2)gl tumour suppressor of Drosophila may regulate the binding of p127 to nonmuscle myosin II heavy chain and the attachment of p127 to the plasma membrane. J Cell Sci *109 (Pt 6)*, 1359-1368.

Kiernan, A. E., Ahituv, N., Fuchs, H., Balling, R., Avraham, K. B., Steel, K. P., and Hrabe de Angelis, M. (2001). The Notch ligand Jagged1 is required for inner ear sensory development. Proc Natl Acad Sci U S A *98*, 3873-3878.

Klueg, K. M., and Muskavitch, M. A. (1999). Ligand-receptor interactions and trans-endocytosis of Delta, Serrate and Notch: members of the Notch signalling pathway in Drosophila. J Cell Sci *112 (Pt 19)*, 3289-3297.

Koyama, K., Fukushima, Y., Inazawa, J., Tomotsune, D., Takahashi, N., and Nakamura, Y. (1996). The human homologue of the murine Llglh gene (LLGL) maps within the Smith-Magenis syndrome region in 17p11.2. Cytogenet Cell Genet *72*, 78-82.

Lai, E. C. (2002). Protein degradation: four E3s for the notch pathway. Curr Biol *12*, R74-78.

Lai, E. C., Deblandre, G. A., Kintner, C., and Rubin, G. M. (2001). Drosophila neuralized is a ubiquitin ligase that promotes the internalization and degradation of delta. Dev Cell *1*, 783-794.

Lai, E. C., and Rubin, G. M. (2001). neuralized functions cell-autonomously to regulate a subset of notch-dependent processes during adult Drosophila development. Dev Biol *231*, 217-233.

Le Borgne, R., Bellaiche, Y., and Schweisguth, F. (2002). Drosophila E-cadherin regulates the orientation of asymmetric cell division in the sensory organ lineage. Curr Biol *12*, 95-104.

Lee, E. C., Yu, S. Y., and Baker, N. E. (2000). The scabrous protein can act as an extracellular antagonist of notch signaling in the Drosophila wing. Curr Biol *10*, 931-934.

Lee, E. C., Yu, S. Y., Hu, X., Mlodzik, M., and Baker, N. E. (1998). Functional analysis of the fibrinogen-related scabrous gene from Drosophila melanogaster identifies potential effector and stimulatory protein domains. Genetics *150*, 663-673.

Lee, R. C., and Ambros, V. (2001). An extensive class of small RNAs in Caenorhabditis elegans. Science *294*, 862-864.

Lee, T., and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron *22*, 451-461.

Lehman, K., Rossi, G., Adamo, J. E., and Brennwald, P. (1999). Yeast homologues of tomosyn and lethal giant larvae function in exocytosis and are associated with the plasma membrane SNARE, Sec9. J Cell Biol *146*, 125-140.

Levison, S. W., and Goldman, J. E. (1993). Both oligodendrocytes and astrocytes develop from progenitors in the subventricular zone of postnatal rat forebrain. Neuron *10*, 201-212.

Lin, D., Edwards, A. S., Fawcett, J. P., Mbamalu, G., Scott, J. D., and Pawson, T. (2000). A mammalian PAR-3-PAR-6 complex implicated in Cdc42/Rac1 and aPKC signalling and cell polarity. Nat Cell Biol *2*, 540-547.

Lu, B., Jan, L., and Jan, Y. N. (2000). Control of cell divisions in the nervous system: symmetry and asymmetry. Annu Rev Neurosci *23*, 531-556.

Lu, B., Rothenberg, M., Jan, L. Y., and Jan, Y. N. (1998). Partner of Numb colocalizes with Numb during mitosis and directs Numb asymmetric localization in Drosophila neural and muscle progenitors. Cell *95*, 225-235.

Mata, J., Curado, S., Ephrussi, A., and Rorth, P. (2000). Tribbles coordinates mitosis and morphogenesis in Drosophila by regulating string/CDC25 proteolysis. Cell *101*, 511-522.

Mechler, B. M., McGinnis, W., and Gehring, W. J. (1985). Molecular cloning of lethal(2)giant larvae, a recessive oncogene of Drosophila melanogaster. Embo J *4*, 1551-1557.

Mechler, B. M., Torok, I., Schmidt, M., Opper, M., Kuhn, A., Merz, R., and Protin, U. (1989). Molecular basis for the regulation of cell fate by the lethal (2) giant larvae tumour suppressor gene of Drosophila melanogaster. Ciba Found Symp *142*, 166-178; discussion 178-180.

Miklos, G. L., and Rubin, G. M. (1996). The role of the genome project in determining gene function: insights from model organisms. Cell *86*, 521-529.

Milan, M., Diaz-Benjumea, F. J., and Cohen, S. M. (1998). Beadex encodes an LMO protein that regulates Apterous LIM-homeodomain activity in Drosophila wing development: a model for LMO oncogene function. Genes Dev *12*, 2912-2920.

Moloney, D. J., Panin, V. M., Johnston, S. H., Chen, J., Shao, L., Wilson, R., Wang, Y., Stanley, P., Irvine, K. D., Haltiwanger, R. S., and Vogt, T. F. (2000). Fringe is a glycosyltransferase that modifies Notch. Nature *406*, 369-375.

Moore, A. W., Jan, L. Y., and Jan, Y. N. (2002). hamlet, a binary genetic switch between single- and multiple- dendrite neuron morphology. Science *297*, 1355-1358.

Morrison, S. J., Perez, S. E., Qiao, Z., Verdi, J. M., Hicks, C., Weinmaster, G., and Anderson, D. J. (2000). Transient Notch activation initiates an irreversible switch from neurogenesis to gliogenesis by neural crest stem cells. Cell *101*, 499-510.

Nakao, K., and Campos-Ortega, J. A. (1996). Persistent expression of genes of the enhancer of split complex suppresses neural development in Drosophila. Neuron *16*, 275-286.

Negre, N., Ghysen, A., and Martinez, A. M. (2003). Mitotic G2-arrest is required for neural cell fate determination in Drosophila. Mech Dev *120*, 253-265.

Oberg, C., Li, J., Pauley, A., Wolf, E., Gurney, M., and Lendahl, U. (2001). The Notch intracellular domain is ubiquitinated and negatively regulated by the mammalian Sel-10 homolog. J Biol Chem *276*, 35847-35853.

Ohno, S. (2001). Intercellular junctions and cellular polarity: the PAR-aPKC complex, a conserved core cassette playing fundamental roles in cell polarity. Curr Opin Cell Biol *13*, 641-648.

Ohshiro, T., Yagami, T., Zhang, C., and Matsuzaki, F. (2000). Role of cortical tumour-suppressor proteins in asymmetric division of Drosophila neuroblast. Nature *408*, 593-596.

Parnavelas, J. G., and Nadarajah, B. (2001). Radial glial cells. are they really glia? Neuron *31*, 881-884.

Pavlopoulos, E., Pitsouli, C., Klueg, K. M., Muskavitch, M. A., Moschonas, N. K., and Delidakis, C. (2001). neuralized Encodes a peripheral membrane protein involved in delta signaling and endocytosis. Dev Cell *1*, 807-816.

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

Petritsch, C., Tavosanis, G., Turck, C. W., Jan, L. Y., and Jan, Y. N. (2003). The Drosophila myosin VI Jaguar is required for basal protein targeting and correct spindle orientation in mitotic neuroblasts. Dev Cell *4*, 273-281.

Plant, P. J., Fawcett, J. P., Lin, D. C., Holdorf, A. D., Binns, K., Kulkarni, S., and Pawson, T. (2003). A polarity complex of mPar-6 and atypical PKC binds, phosphorylates and regulates mammalian Lgl. Nat Cell Biol *5*, 301-308.

Powell, P. A., Wesley, C., Spencer, S., and Cagan, R. L. (2001). Scabrous complexes with Notch to mediate boundary formation. Nature *409*, 626-630.

Price, D. M., Jin, Z., Rabinovitch, S., and Campbell, S. D. (2002). Ectopic expression of the Drosophila Cdk1 inhibitory kinases, Wee1 and Myt1, interferes with the second mitotic wave and disrupts pattern formation during eye development. Genetics *161*, 721-731.

Qiu, L., Joazeiro, C., Fang, N., Wang, H. Y., Elly, C., Altman, Y., Fang, D., Hunter, T., and Liu, Y. C. (2000). Recognition and ubiquitination of Notch by Itch, a hect-type E3 ubiquitin ligase. J Biol Chem *275*, 35734-35737.

Rao, Y., Bodmer, R., Jan, L. Y., and Jan, Y. N. (1992). The big brain gene of Drosophila functions to control the number of neuronal precursors in the peripheral nervous system. Development *116*, 31-40.

Rauskolb, C., Correia, T., and Irvine, K. D. (1999). Fringe-dependent separation of dorsal and ventral cells in the Drosophila wing. Nature *401*, 476-480.

Rebay, I., Fehon, R. G., and Artavanis-Tsakonas, S. (1993). Specific truncations of Drosophila Notch define dominant activated and dominant negative forms of the receptor. Cell *74*, 319-329.

Rebay, I., Fleming, R. J., Fehon, R. G., Cherbas, L., Cherbas, P., and Artavanis-Tsakonas, S. (1991). Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. Cell *67*, 687-699.

Redmond, L., and Ghosh, A. (2001). The role of Notch and Rho GTPase signaling in the control of dendritic development. Curr Opin Neurobiol *11*, 111-117.

Rhyu, M. S., Jan, L. Y., and Jan, Y. N. (1994). Asymmetric distribution of numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. Cell *76*, 477-491.

Richmond, J. E., and Broadie, K. S. (2002). The synaptic vesicle cycle: exocytosis and endocytosis in Drosophila and C. elegans. Curr Opin Neurobiol *12*, 499-507.

Roegiers, F., Younger-Shepherd, S., Jan, L. Y., and Jan, Y. N. (2001a). Bazooka is required for localization of determinants and controlling proliferation in the sensory organ precursor cell lineage in Drosophila. Proc Natl Acad Sci U S A *98*, 14469-14474.

Roegiers, F., Younger-Shepherd, S., Jan, L. Y., and Jan, Y. N. (2001b). Two types of asymmetric divisions in the Drosophila sensory organ precursor cell lineage. Nat Cell Biol *3*, 58-67.

Rorth, P. (1996). A modular misexpression screen in Drosophila detecting tissue-specific phenotypes. Proc Natl Acad Sci U S A *93*, 12418-12422.

Rorth, P., Szabo, K., Bailey, A., Laverty, T., Rehm, J., Rubin, G. M., Weigmann, K., Milan, M., Benes, V., Ansorge, W., and Cohen, S. M. (1998). Systematic gain-of-function genetics in Drosophila. Development *125*, 1049-1057.

Ryan, T. A., Reuter, H., and Smith, S. J. (1997). Optical detection of a quantal presynaptic membrane turnover. Nature *388*, 478-482.

Sakamoto, K., Ohara, O., Takagi, M., Takeda, S., and Katsube, K. (2002). Intracellular cell-autonomous association of Notch and its ligands: a novel mechanism of Notch signal modification. Dev Biol *241*, 313-326.

Seher, T. C., and Leptin, M. (2000). Tribbles, a cell-cycle brake that coordinates proliferation and morphogenesis during Drosophila gastrulation. Curr Biol *10*, 623-629.

Sestan, N., Artavanis-Tsakonas, S., and Rakic, P. (1999). Contact-dependent inhibition of cortical neurite growth mediated by notch signaling. Science *286*, 741-746.

Seto, E. S., Bellen, H. J., and Lloyd, T. E. (2002). When cell biology meets development: endocytic regulation of signaling pathways. Genes Dev *16*, 1314-1336.

Shaye, D. D., and Greenwald, I. (2002). Endocytosis-mediated downregulation of LIN-12/Notch upon Ras activation in Caenorhabditis elegans. Nature *420*, 686-690.

Shimizu, K., Chiba, S., Saito, T., Kumano, K., Takahashi, T., and Hirai, H. (2001). Manic fringe and lunatic fringe modify different sites of the Notch2 extracellular region, resulting in different signaling modulation. J Biol Chem *276*, 25753-25758.

Skeath, J. B., and Carroll, S. B. (1991). Regulation of achaete-scute gene expression and sensory organ pattern formation in the Drosophila wing. Genes Dev *5*, 984-995.

Smith, C. M., and Luskin, M. B. (1998). Cell cycle length of olfactory bulb neuronal progenitors in the rostral migratory stream. Dev Dyn *213*, 220-227.

Spradling, A. C., and Rubin, G. M. (1982). Transposition of cloned P elements into Drosophila germ line chromosomes. Science *218*, 341-347.

Strand, D., Jakobs, R., Merdes, G., Neumann, B., Kalmes, A., Heid, H. W., Husmann, I., and Mechler, B. M. (1994a). The Drosophila lethal(2)giant larvae tumor suppressor protein forms homo-oligomers and is associated with nonmuscle myosin II heavy chain. J Cell Biol *127*, 1361-1373.

Strand, D., Raska, I., and Mechler, B. M. (1994b). The Drosophila lethal(2)giant larvae tumor suppressor protein is a component of the cytoskeleton. J Cell Biol *127*, 1345-1360.

Strand, D., Unger, S., Corvi, R., Hartenstein, K., Schenkel, H., Kalmes, A., Merdes, G., Neumann, B., Krieg-Schneider, F., Coy, J. F., and et al. (1995). A human homologue of the Drosophila tumour suppressor gene l(2)gl maps to 17p11.2-12 and codes for a

cytoskeletal protein that associates with nonmuscle myosin II heavy chain. Oncogene *11*, 291-301.

Struhl, G., and Adachi, A. (1998). Nuclear access and action of notch in vivo. Cell *93*, 649-660.

Tanentzapf, G., Smith, C., McGlade, J., and Tepass, U. (2000). Apical, lateral, and basal polarization cues contribute to the development of the follicular epithelium during Drosophila oogenesis. J Cell Biol *151*, 891-904.

Tanentzapf, G., and Tepass, U. (2003). Interactions between the crumbs, lethal giant larvae and bazooka pathways in epithelial polarization. Nat Cell Biol *5*, 46-52.

Tanigaki, K., Nogaki, F., Takahashi, J., Tashiro, K., Kurooka, H., and Honjo, T. (2001). Notch1 and Notch3 instructively restrict bFGF-responsive multipotent neural progenitor cells to an astroglial fate. Neuron *29*, 45-55.

Tsai, H., Hardisty, R. E., Rhodes, C., Kiernan, A. E., Roby, P., Tymowska-Lalanne, Z., Mburu, P., Rastan, S., Hunter, A. J., Brown, S. D., and Steel, K. P. (2001). The mouse slalom mutant demonstrates a role for Jagged1 in neuroepithelial patterning in the organ of Corti. Hum Mol Genet *10*, 507-512.

Uemura, T., Shepherd, S., Ackerman, L., Jan, L. Y., and Jan, Y. N. (1989). numb, a gene required in determination of cell fate during sensory organ formation in Drosophila embryos. Cell *58*, 349-360.

Van De Bor, V., and Giangrande, A. (2001). Notch signaling represses the glial fate in fly PNS. Development *128*, 1381-1390.

Vassin, H., Vielmetter, J., and Campos-Ortega, J. A. (1985). Genetic interactions in early neurogenesis of Drosophila melanogaster. J Neurogenet *2*, 291-308.

Wang, S., and Barres, B. A. (2000). Up a notch: instructing gliogenesis. Neuron *27*, 197-200.

Wu, G., Lyapina, S., Das, I., Li, J., Gurney, M., Pauley, A., Chui, I., Deshaies, R. J., and Kitajewski, J. (2001). SEL-10 is an inhibitor of notch signaling that targets notch for ubiquitin-mediated protein degradation. Mol Cell Biol *21*, 7403-7415.

Yamanaka, T., Horikoshi, Y., Sugiyama, Y., Ishiyama, C., Suzuki, A., Hirose, T., Iwamatsu, A., Shinohara, A., and Ohno, S. (2003). Mammalian Lgl Forms a Protein Complex with PAR-6 and aPKC Independently of PAR-3 to Regulate Epithelial Cell Polarity. Curr Biol *13*, 734-743.

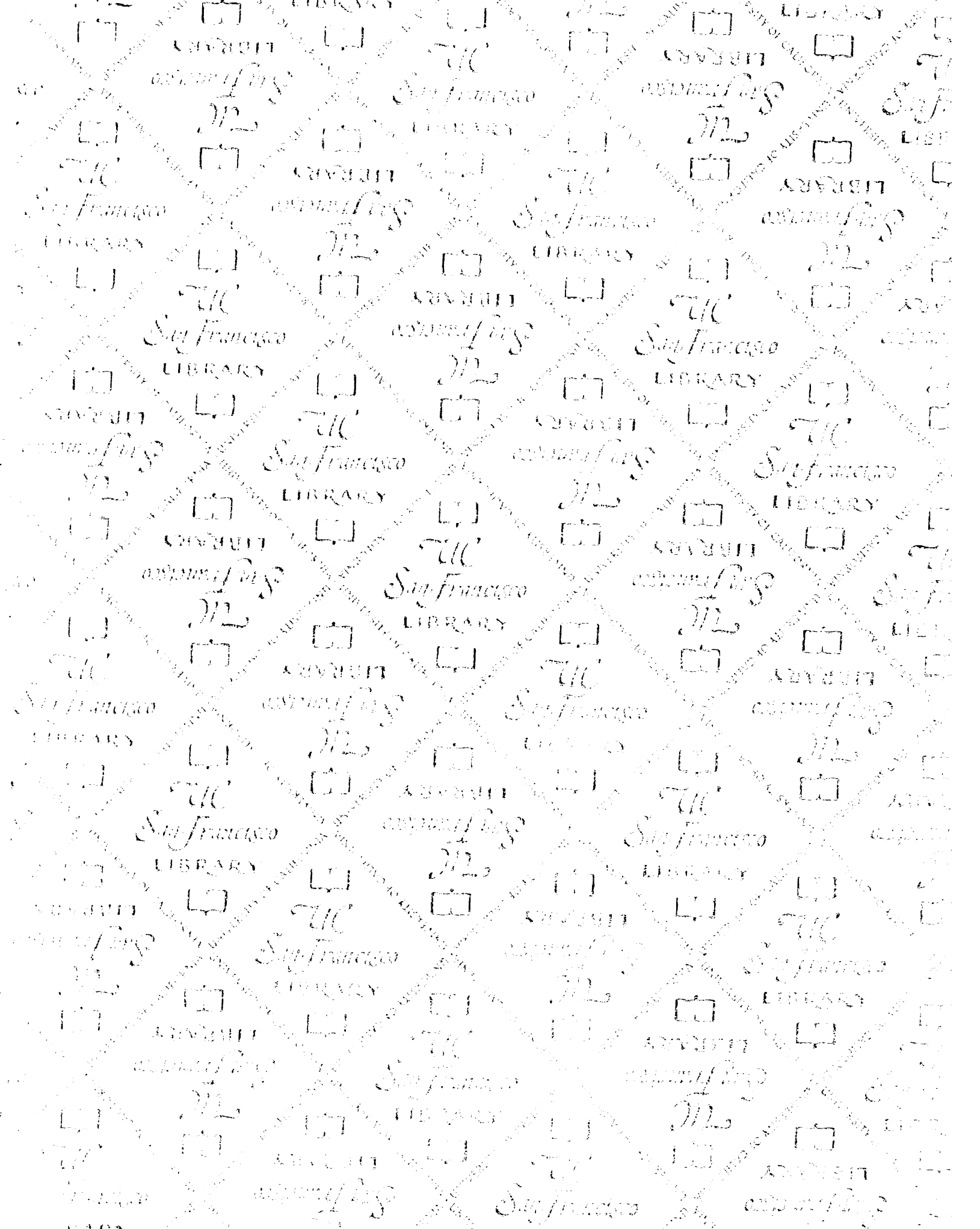
Yeh, E., Dermer, M., Commisso, C., Zhou, L., McGlade, C. J., and Boulianne, G. L. (2001). Neuralized functions as an E3 ubiquitin ligase during Drosophila development. Curr Biol *11*, 1675-1679.

Yeh, E., Zhou, L., Rudzik, N., and Boulianne, G. L. (2000). Neuralized functions cell autonomously to regulate Drosophila sense organ development. Embo J *19*, 4827-4837.

Zeng, C., Justice, N. J., Abdelilah, S., Chan, Y. M., Jan, L. Y., and Jan, Y. N. (1998a). The Drosophila LIM-only gene, dLMO, is mutated in Beadex alleles and might represent an evolutionarily conserved function in appendage development. Proc Natl Acad Sci U S A *95*, 10637-10642.

Zeng, C., Younger-Shepherd, S., Jan, L. Y., and Jan, Y. N. (1998b). Delta and Serrate are redundant Notch ligands required for asymmetric cell divisions within the Drosophila sensory organ lineage. Genes Dev *12*, 1086-1091.

Zhang, N., Martin, G. V., Kelley, M. W., and Gridley, T. (2000). A mutation in the Lunatic fringe gene suppresses the effects of a Jagged2 mutation on inner hair cell development in the cochlea. Curr Biol *10*, 659-662.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