Unequal Segregation of Neuralized Biases
Notch Activation during Asymmetric Cell Division

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Summary

In Drosophila, Notch signaling regulates binary fate decisions at each asymmetric division in sensory organ lineages. Following division of the sensory organ precursor cell (pl), Notch is activated in one daughter cell (pI) and inhibited in the other (pIIb). We report that the E3 ubiquitin ligase Neuralized localizes asymmetrically in the dividing pl cell and unequally segregates into the pIIb cell, like the Notch inhibitor Numb. Furthermore, Neuralized upregulates endocytosis of the Notch ligand Delta in the pIIb cell and acts in the pIIb cell to promote activation of Notch in the plia cell. Thus, Neuralized is a conserved regulator of Notch signaling that acts as a cell fate determinant. Polarization of the pl cell directs the unequal segregation of both Neuralized and Numb. We propose that coordinated upregulation of ligand activity by Neuralized and inhibition of receptor activity by Numb results in a robust bias in Notch signaling.

Introduction

During metazoan development, different cell fates are generated by cell-cell interactions or by the unequal segregation of cell fate determinants during asymmetric cell divisions. Both mechanisms contribute to the plia/pIIb decision during sensory organ development in Drosophila. In the pupal thorax, each sensory organ precursor cell (pl) divides along the anterior-posterior axis of the fly to generate a posterior plia cell and an anterior pIIb cell (Gho et al., 1999). The cell fate determinant Numb localizes asymmetrically at the anterior cortex of the dividing pl cell and segregates into the anterior daughter cell (Rhyu et al., 1994). Genetic analysis indicates that Numb antagonizes Notch (N) signaling and that Numb-mediated inhibition of N requires the α-adaptin (Berdnik et al., 2002; Guo et al., 1996). Numb binds both the intracellular domain of N (Guo et al., 1996) and the α-adaptin ear domain (Berdnik et al., 2002; Santolini et al., 2000). This suggests that Numb may directly regulate N endocytosis. Signaling by N receptors involves three successive cleavages (for review, see Fortini, 2001). N is first processed in the trans-Golgi network at the extracellular S1 site to produce a functional heterodimeric receptor. A second ligand-dependent cleavage of N at the extracellular S2 site generates a membrane-bound activated form which is then processed at an intramembraneous S3 site, thereby releasing the active Notch intracellular domain (NICD). Numb genetically acts upstream of S3 cleavage (Guo et al., 1996). Thus, Numb may promote internalization and degradation of N prior to S3 cleavage. Alternatively, the importance of dynamin activity for N signal transduction (Seugnet et al., 1997) raises the possibility that S3 cleavage depends on dynamin-dependent endocytosis with Numb inhibiting endocytosis of membrane-bound activated N. Regardless of the mechanism of Numb action, trafficking of N is suggested to play an important role in generating asymmetry.

Two ligands of N are known in Drosophila, Delta (Dl), and Serrate (Ser). These two ligands act redundantly to activate N during the plia/pIIb decision (Zeng et al., 1998). Recent studies have indicated that endocytosis of Dl is critical for N activation. First, dynamin-dependent endocytosis is not only required for signal transduction as mentioned above but is also required in signal-sending cells to promote N activation (Seugnet et al., 1997). Second, endocytosis-defective Dl proteins have reduced signaling capacity (Parks et al., 2000). Third, the E3-ubiquitin ligases Neuralized (Neur) in Drosophila and Mind bomb (Mib) in zebrafish promote endocytosis of Dl and appear to be required for efficient activation of N by Dl (Deblandre et al., 2001; Itoh et al., 2003; Lai et al., 2001; Pavlopoulos et al., 2001; Yeh et al., 2001). It has been proposed that Dl endocytosis facilitates the S2 cleavage of N at the surface of the signal-receiving cell (Parks et al., 2000). Here we show that Neur is unequally segregated during asymmetric division of the pl cell, upregulates endocytosis of Dl in the pIIb cell, and plays a critical role in generating cell fate diversity. We propose that Neur acts as a cell fate determinant during asymmetric cell divisions.

Results

A numb-Independent Asymmetry in Delta Endocytosis

To examine whether asymmetry in N ligands distribution may play a role in generating cell fate diversity during asymmetric divisions, we have analyzed the subcellular distribution of Dl and Ser in the sensory organ lineage. In mitotic pl cells, Dl and Ser were uniformly distributed around the cell cortex and were equally partitioned into both daughter cells (data not shown). In both pl daughter cells, Dl and Ser accumulated at the apical cell cortex as well as in intracellular dots of 0.5 ± 0.2 μm in diameter (Figures 1A–1A* and E–E*; see Supplementary Figure S1 at http://www.developmentalcell.com/cgi/content/full/5/1/139/DC1). These dots were coated by Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) (Figures 1C–1D*). Hrs binds ubiquitinated proteins via its ubiquitin-interacting motif and sorts endocytic cargos into the lumen of multivesicular bodies (MVBs) (Lloyd et al., 2002; Raiborg et al., 2002; Shih et al., 2002). Therefore, these Dl-positive vesicles appeared to
be large endocytic vesicles that probably correspond to MVBs. These DI-positive vesicles also contained Notch extracellular domain (NECD) and NICD epitopes (Figures 1A–1A′ and data not shown). Strikingly, a higher number of large DI-positive vesicles was seen in the anterior signal-receiving pIIb cell (5.0 ± 2.2, n = 130) than in the posterior signal-receiving pIIa cell (2.0 ± 1.5, n = 130; Figure 3C). This difference was significant (p < 0.001; Student’s t test). This asymmetry in DI endocytosis is established independently of the unequal partitioning of Numb. Indeed, anterior pI daughter cells are shown to accumulate a higher number of DI-positive vesicles than posterior pI daughter cells in numb mutants and numb mutant clones (Figures 1B–1B′; numb: 5.1 ± 1.8 versus 2.8 ± 1.6, n = 24, p < 0.001; numb: 5.7 ± 2.5 versus 3.1 ± 1.9, n = 16, p < 0.001). Thus, asymmetry in DI endocytosis did not depend on Numb.

Neuralized Upregulates the Endocytosis of Delta in the pIIb Cell

Recent studies have suggested that endocytosis of DI is promoted by the ubiquitination of DI by Neur, a RING finger-type E3-ubiquitin ligase required for N signaling (Deblandre et al., 2001; Lai et al., 2001; Pavlopoulos et al., 2001; Yeh et al., 2001). Neur is found in a complex with DI and is required for DI ubiquitination. Finally, Neur stimulates the accumulation of DI into intracellular vesicles in imaginal disc cells. The latter conclusion was, however, based on the analysis of steady-state levels of DI, making it difficult to unambiguously conclude whether Neur promotes DI endocytosis or favors direct sorting from the Golgi to intracellular vesicles. To discriminate between these two possibilities and to test whether Neur regulates DI trafficking in sensory cells, we developed an ex vivo assay for endocytosis. Internalization of DI was followed in living epithelial cells using antibodies recognizing the extracellular part of DI. Briefly, the single-layered epithelium corresponding to the pupal notum was dissected and cultured in presence of anti-DI antibodies (Figure 2A). Following medium changes and fixation, the uptake of anti-DI antibodies was revealed using secondary antibodies. Anti-DI antibodies were specifically internalized in the pIIa and pIIb cells (Figures 2B and B′). Internalized anti-DI antibodies co-localized with DI into large DI-positive vesicles (Figures 2B–2B′). Internalization of anti-DI required dynamin activity (Figure 2C) and was not observed at 4°C (data not shown). Together, these results indicate that anti-DI interacts with DI at the cell surface and that DI-anti-DI complexes are endocytosed in sensory cells.

We then used this assay to examine the function of neur. Clones of neur mutant cells have been shown to exhibit a neurogenic phenotype with too many pI cells being specified. The progeny of these mutant pI cells produced no external sensory structures indicative that pIIa cells have been transformed into pIIb-like cells (Lai and Rubin, 2001a; Yeh et al., 2000). These cell fate transformations are associated with defects in DI trafficking. High levels of anti-DI remain at the surface of neur mutant clones and internalization of anti-DI was drastically reduced (Figures 2D and 2D′). We conclude that neur is required for the endocytosis of DI in sensory cells.

This defect in DI endocytosis was quantified on fixed tissues. neur mutant pI cells and pIIb-like progeny cells were found to accumulate high levels of DI at the cell surface (Figures 3A–3B′). Accumulation of DI at the cell surface is consistent with the proposed function of Neur in the internalization and degradation of DI (Deblandre et al., 2001; Lai et al., 2001; Pavlopoulos et al., 2001). Quantification of DI-positive vesicles in neur mutant clones revealed that mutant pIIb-like cells contained much fewer DI-positive vesicles (1.5 ± 0.9, n = 135; Figure 3C) than wild-type pIIb cells (5.0 ± 2.2, n = 130). Thus, in the absence of neur function, both pI daughter cells have the same reduced number of DI-positive vesicles. Furthermore, a similar distribution of DI-containing
Unequal Segregation of Neuralized into the pIIb Cell

Upregulation of DI endocytosis in the pIIb cell may result from higher levels of Neur in this cell. To test this hypothesis, we examined the localization of Neur. The Neur protein was detectable in the pI cell and in its progeny cells, but not in epidermal cells. Neur was perinuclear in prophase and localized asymmetrically at the anterior cortex during prometaphase (Figures 4A–4B’). At telophase, Neur specifically segregated into the anterior daughter cell (Figures 4C–4C’). At cytokinesis, Neur uniformly redistributed at the cortex and in the cytoplasm in the pIIb cell (Figures 4D–4D’). Localization of Neur at mitosis is identical to the one described for Partner of Numb (Pon) (Bellaïche et al., 2001a; Lu et al., 1998). Consistently, Neur colocalized with Pon-GFP throughout mitosis (Figures 4A–4D’). Asymmetric localization of Neur was also seen in the pIIb and pIIa dividing cells (see Supplementary Figure S2 at http://www.developmentalcell.com/cgi/content/full/5/1/139/DC1).

Specificity of anti-Neur antibodies was demonstrated by absence of staining in neur mutant plI cells (Figures 4E–4F’). Unequal segregation of Neur did not depend on numb activity (Figures 4G–4I). Conversely, unequal segregation of Numb did not depend on neur activity (Figures 4J–4K’). Thus, the numb-independent unequal segregation of Neur into the pIIb cell provides a simple explanation for the upregulation of DI endocytosis in the pIIb cell.

To test the functional significance of Neur unequal segregation, Neur was overexpressed in plI cells. Overexpression of Neur using neur17GAL4 (Bellaïche et al., 2001a) failed to affect the unequal partitioning of Neur at plI mitosis (see Supplementary Figure S3A–S3C at http://www.developmentalcell.com/cgi/content/full/5/1/139/DC1) and the pIIa/pIIb decision (data not shown) but resulted in a weak double-socket phenotype associated with a shaft-to-socket transformation (Supplementary Figures S3H–S3K at http://www.developmentalcell.com/cgi/content/full/5/1/139/DC1). This fate transformation is known to result from high levels of Delta-Notch.
Figure 3. neur Upregulates DI Endocytosis in the pIIb Cell

(A–B″) Localization of DI (red) and NECD (blue vesicular staining) was analyzed in neur^{F65} mutant cells (marked by loss of nlsGFP in green) at a stage when pairs of pIIa/pIIb cells (nuclear Cut in blue) are seen in surrounding wild-type tissue. (B–B″) are enlargements of the region boxed in (A′). Loss of neur activity resulted in a dramatic increase in DI accumulation at the cell surface (compare the wild-type and neur mutant sensory cells indicated by arrowheads in [A″]) and in a reduction in the number of DI-positive (arrowheads in [B and B″]) and NECD-positive (B″) vesicles. In both wild-type and neur mutant cells, most DI-positive vesicles were coated by Hrs (data not shown). A low level of neur-independent trafficking of DI toward Hrs-positive vesicles was expected since epidermal cells, that did not express neur, also contained a few Hrs-positive DI-containing vesicles (see Figure 1 and data not shown). Loss of neur function also led to the specification of a large excess of pI cells. Bar is 5 μm in (B–B″).

(C) Plot showing the distribution of the number of DI-positive vesicles in wild-type pIIa (blue bars) and pIIb (red bars) cells as well as in neur^{F65} mutant pIIb-like (green bars) cells. Counting of DI-positive vesicles in neur pIIb-like (green bars) cells. Counting of DI-positive vesicles in neur pIIb-like (green bars) cells was performed on confocal z-sections only within rows containing both dividing pl cells and pIIa/pIIb pairs outside the clone. The mutant pIIb-like cells were distinguished from pl cells based on their small nuclei.

signaling (Bang and Posakony, 1992; Schweisguth and Posakony, 1994) and is opposite to the socket-to-shaft transformation seen in neur mutant clones (see below). Moreover, this shaft-to-socket transformation may result from the equal partitioning of Neur (but not Numb) in the two pIIa daughter cells which can also be observed at low frequency (Supplementary Figures S3G–S3G″ at http://www.developmentalcell.com/cgi/content/full/5/1/139/DC1). Thus, these observations support the notion that unequal segregation of Neur is functionally important.

A Similar Mode of Asymmetric Distribution for Neuralized and Numb

We next investigated the mechanisms by which Neur localized at the anterior cortex of the dividing pl cell. The role of the cytoskeleton was studied by applying drugs to cultured nota. Colcemid, a microtubule-depolymerizing agent, was found to have no significant effect (Figures 5A and 5D). In contrast, both Latrunculin A, an agent that depolymerizes actin microfilaments, and the myosin motor inhibitor butanedione-2-monoxime (BDM) strongly impaired (Figures 5B and 5C) or completely
Unequal Segregation of Neuralized

Figure 4. Unequal Segregation of Neur

(A–D‘) Localization of Neur (red) and Pon-GFP (green) was studied in mitotic pl cells. Sensory cells were detected using Pon-GFP expressed under the control of neurP72GAL4. At prophase (A–A‘), Neur colocalized with Pon-GFP in a perinuclear compartment and in a weak cortical crescent at the anterior pole. At prometaphase, Neur colocalized with Pon-GFP at the anterior cortex (B–B‘) and unequally segregated into the anterior pllb daughter cell (C–C‘). At cytokinesis, Neur uniformly redistributed at the cortex and in the cytoplasm in the pllb cell (D–D‘).

(E–F‘) No anti-Neur immunoreactivity (red) was detected in mitotic neur1F65 mutant pl cells (Cut in blue; nlsGFP in green served as a clone marker). An enlarged view of the inset in (E) is shown in (F–F‘).

(G–I) Neur (red) localized asymmetrically in mitotic numb2 mutant pl cells (Cut in blue; nlsGFP in green). The localization of Neur in wild-type cells (I) was compared to that in numb mutant cells (H). Anterior is up in (G–I).

(J–L‘) Numb (blue) localized asymmetrically in mitotic neur1F65 mutant pl cells (nlsGFP in green). Note the strong and uniform cortical accumulation of Di (red) in dividing neur1F65 mutant pl cells (K); inset in (J) corresponds to (K). A wild-type control is shown in (L–L‘).

Bar is 25 μm in (E) and (J), 10 μm in (F), and 5 μm in all other panels.

inhibited (data not shown) the asymmetric localization of Neur. Thus, both myosin motor activity and an intact actin cytoskeleton are required for the formation and/or maintenance of the Neur crescent at the anterior cortex of the dividing pl cell. These requirements for Neur localization are similar to the ones seen earlier for Numb (Berdnik and Knoblich, 2002; Knoblich et al., 1997) and Pon (Lu et al., 1999). Neur also behaves similarly to Numb and Pon in that localization of Neur at the anterior cortex of the pl cell depends on planar polarity genes (data not shown) and on the polarity genes discs-large and pins (Figures 5E–5G) (Bellaiche et al., 2001b). Moreover, mispartitioning of Neur in dig and pins mutant cells correlated with a loss in asymmetric internalization of Di (Figures 5H–5I). These data indicate that Neur and Numb share part of the same molecular machinery to localize asymmetrically in the pl cell.

neuralized Activity Is Required in Signal-Sending Cells

Unequal segregation of Neur in the anterior pllb cell suggests that Neur acts in this cell to promote adoption of the plla fate by the posterior cell. To test whether neur activity is indeed required in the pllb cell, we generated clones within the sensory organ lineage. Mitotic recombination in the pl cell produces one neur mutant cell and one wild-type cell (Figure 6A). Importantly, the anterior daughter cell will inherit Neur, regardless of its genotype. Thus, when the anterior cell is neur mutant, the posterior cell is predicted to adopt a plla-like fate (Figure 6A). This should result in a bristle loss phenotype.
In contrast, if \textit{neur} acts in the signal-sending cell, the mutant posterior cell is predicted to become a \textit{plla} cell (Figure 6A). This mutant \textit{plla} cell should then produce two mutant cells unable to signal, hence leading to bristle duplication. Mitotic recombination induced at 0–6 hr before puparium formation (PF), when most microchaete \textit{pl} cells are specified but have not yet divided (Huang et al., 1991), produced flies with double-shaft bristles on the head, thorax and at the wing margin (Figures 6B and 6C). No macrochaete loss was detectable. This double-shaft phenotype appears to result from wild-type \textit{pllb}/mutant \textit{plla} pairs because sensory organs composed of two mutant shaft cells and wild-type \textit{pllb} progeny cells were detected at 20 hr after PF (Figures 6D–6E; \(n = 9\)). Reciprocally, a sheath-to-neuron transformation was observed in mutant \textit{pllb}/wild-type \textit{plla} pairs (data not shown; \(n = 6\)). These data show that \textit{neur} is required for the socket/shaft and neuron/sheath fate decisions and further indicate that \textit{neur} acts in the \textit{pllb} cell to specify the \textit{plla} cell.

Previous studies on the cell autonomy of \textit{neur} have led to contradictory results (Lai and Rubin, 2001a; Pavlopoulos et al., 2001; Yeh et al., 2000) (see Discussion). Thus, we further tested the autonomy of \textit{neur} activity during the \textit{pl}/epidermal fate decision in the developing notum. Analysis of cell fate decisions at the clone border has previously been shown to be extremely powerful to distinguish activities required for signal reception, like \textit{N}, from activities required for signal production, like \textit{DI} (Heitzler and Simpson, 1991). Mutant cells unable to receive inhibitory signaling autonomously adopt the \textit{pl} fate and inhibit their wild-type neighbors to become \textit{pl} cells. Thus, most of the \textit{pl} cells at the clone border are mutant. Conversely, mutant cells unable to produce inhibitory signals fail to inhibit their wild-type neighbors but still receive inhibitory signaling produced by neighboring wild-type cells. Thus, most of the \textit{pl} cells at the clone border are wild-type. We have used this assay to study the autonomy of \textit{neur}. The genotype of the \textit{pl} cells that contact the border of \textit{neur}/mutant clones running across microchaete rows 1–5 was examined (Figures 6A and A'). One position per microchaete row was scored. Wild-type \textit{pl} cells were found in 79% of these positions (blue arrows, Figure 3A', \(n = 73\); 34 clones). Thus, \textit{neur} mutant cells are not defective in receiving the inhibitory signal sent by wild-type cells but fail to efficiently inhibit wild-type cells from becoming \textit{pl} cells. We conclude that \textit{neur} activity is primarily required in signal-sending cells.

\section*{Discussion}

Notch signaling regulates binary cell fate decisions in a wide variety of developmental contexts. Following asymmetric division, up- or downregulation of N signaling in one daughter cell may generate an asymmetry in fate. In this study, we identify \textit{Neur} as an important factor that establishes an asymmetry in N signaling in the sensory bristle lineage. First, loss of \textit{neur} activity in dividing \textit{pl} and \textit{plla} precursor cells results in \textit{plla}-to-\textit{pllb} and socket-to-shaft transformations, respectively. Conversely, overexpression of \textit{Neur} in the bristle lineage may result in the opposite socket-to-shaft transformation. Thus, \textit{Neur} regulates the binary \textit{plla}/\textit{pllb} and

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure5.png}
\caption{Requirements for \textit{Neur} Asymmetric Localization. (A–D) Asymmetric localization of \textit{Neur} (red) was examined in dividing \textit{pl} cells (Cut in blue in the small insets) from dissected nota cultured in control medium (A) or in the presence of 2 \(\mu\)M Latrunculin A (B), 5 mM BDM (C), or 1 \(\mu\)M colcemid (D). Depolymerization of microtubules did not affect Neur localization (\(n = 39\)). In contrast, depolymerization of microfilaments by Latrunculin A and inhibition of myosin motor activity by BDM strongly impaired (B), \(n = 4/17\); (C), \(n = 5/19\) or completely abolished \textit{Neur} crescent formation (data not shown; Latrunculin A, \(n = 13/17\) and BDM, \(n = 14/19\)). Pron-GFP behaved similarly to Neur in these assays (data not shown). (E–G) \textit{Neur} (red) was mostly cytoplasmic in \textit{dlg}mutant (EF, \(n = 18\)) and \textit{pins} mutant \textit{pl} cells (Cut in blue). A wild-type control is shown in (E). (H–I) Similar numbers of \textit{Dl} (red) and NECD-containing (green; arrowheads in [H' and I']) vesicles were seen in both \textit{pl} daughter cells of \textit{dlg}mutant ([H and H']; 2.4 \(\pm\) 1.7 and 2.7 \(\pm\) 1.6 vesicles in the anterior and posterior \textit{pl} daughter cells, respectively; \(n = 19\)) and \textit{pins} mutant ([I and I']; 3.2 \(\pm\) 1.3 and 3.0 \(\pm\) 1.5 vesicles in the anterior and posterior \textit{pl} daughter cells, respectively; \(n = 32\)). Sensory cells were detected using Cut (green nuclear staining). TRITC-phalloidin staining (shown in blue) was used to outline the cells. Bar is 5 \(\mu\)m.}
\end{figure}
socket/shaft decisions. Second, Neur localizes asymmetrically in the dividing pl and pila cells and unequally segregates into the pllb and shaft cells, respectively. Third, endocytosis of Dl is upregulated in the pllb cell, and this upregulation depends on neur activity. Fourth, clonal analysis indicates that Neur acts nonautonomously in the pllb cell to promote activation of N in the pila cell. The mechanisms by which endocytosis of Dl in the pllb cell may be linked to the activation of N in the pila cell are discussed below. Together, these results indicate that Neur acts as a cell fate determinant in the sensory bristle lineage. Thus, two distinct pathways establish an asymmetry in N signaling in this lineage. A first pathway involves inhibition of N by Numb (Berdnik et al., 2002; Guo et al., 1996; Rhyu et al., 1994), while a second pathway involves the Neur-dependent upregulation of Dl signaling. These two parallel pathways may have evolved to reinforce fidelity and robustness in generating fate asymmetry.

Previous studies have shown that Neur is found in a complex with Dl and is required for Dl monoubiquitination and internalization, suggesting that Dl may be a substrate for Neur (Deblandre et al., 2001; Lai et al., 2001; Pavlopoulos et al., 2001; Yeh et al., 2001). In yeast, monoubiquitination of surface membrane protein has been shown to serve as a signal for endocytosis (Galan et al., 1996; Polo et al., 2002). By analogy, Neur may ubiquitinate an endocytic motif of Dl, thereby regulating Dl endocytosis. We have used here an endocytosis assay to follow the internalization of Dl in living epithelial cells and have shown that neur is required for the endocytosis of Dl in sensory cells.

The mechanism by which Neur regulates N signaling has remained controversial. One model proposes that Neur acts in signal-receiving cells to promote signal reception. In this model, Neur antagonizes the inhibition mediated by Dl in cis on N. In this model, Neur sorts Dl away from N (Deblandre et al., 2001; Lai et al., 2001). A second model proposes that Neur acts in signal-producing cells to upregulate Dl signaling (Pavlopoulos et al., 2001). Our analysis clearly indicates that Neur is required in signal-sending cells and, therefore, supports the latter model. First, clonal analysis in the bristle lineage has revealed that neur mutant plia cells produce shaft cells,
indicating that they have been correctly specified. Similarly, following mitotic recombination in the pl cell, double-shaft bristles produced by pairs of neur mutant cells have been independently observed by Lai and Rubin (2001a). This indicates that neur is not autonomously required for the specification of the pil cell. Second, our clonal analysis indicates that neur is required in the signal-sending pl cell during lateral inhibition and therefore confirms the results of Pavlopoulos et al. (2001). Our conclusion that Neur acts in signal-sending cells to upregulate Dl signaling is further strengthened by the observation that forced expression of Neur and Dl, but not Dl alone, in clones induce activation of N target genes in cells surrounding the clones (Pavlopoulos et al., 2001). Finally, this conclusion is consistent with the observed accumulation of Neur in the signal-sending pl, pIIb, pIIb, and shaft cells (Lai and Rubin, 2001b; Yeh et al., 2000) (this study).

However, this conclusion contradicts two earlier studies (Lai and Rubin, 2001a; Yeh et al., 2000). In a first study, neur mutant bristles were found to preferentially develop at the clone border in adult flies. This led the authors to propose that neur acts cell autonomously (Yeh et al., 2000). While the approach is similar to the one used here, two methodological differences likely account for the different results. First, in order to detect mutant bristles in adult flies, a weak allele of neur was used. We therefore suggest that these mutant cells can still signal and that they overall produce a stronger inhibitory signal than the wild-type cells due to their higher density. Second, the ratio of wild-type versus mutant bristles was not corrected for the higher density of mutant bristles. By contrast, our analysis had no such bias since only one position per microchaete row was taken into account. In a second study (Lai and Rubin, 2001a), neur was proposed to act cell autonomously because mutant cells at the clone border did not differentiate bristles. This interpretation is based on the hypothesis that pil cells receive inhibitory signals from epidermal cells. There is no data to support this hypothesis (see Zeng et al., 1998). We suggest instead that neur mutant pIIb cells fail to signal to their sister cells that therefore adopt a pIIb-like fate. Finally, Lai and Rubin (2001a) also observed at the wing margin that mutant pl cells develop at the clone border, but this phenotype was not quantified. This is in contrast with the detailed study of Pavlopoulos et al. (2001) who have analyzed the same decision and have concluded that neur is primarily required in signal-sending cells. Although we do not exclude the possibility that Neur has additional cell-autonomous functions in signal-receiving cells, our analysis as well as previous published data point to the conclusion that neur is required in signal-sending cells to upregulate Dl signaling.

Our proposal that neur-mediated Dl endocytosis in the signal-sending pIIb cell promotes N receptor activation in the signal-receiving pil cell is counterintuitive. However, several observations suggest the existence of an unknown mechanism required for S2 cleavage that is associated with ligand endocytosis. First, dynamin-dependent endocytosis, which is required for both N signaling and Dl endocytosis (Parks et al., 2000; Seugnet et al., 1997), does not appear to be required for the presenilin-dependent transmembrane S3 cleavage of N in Drosophila (Struhl and Adachi, 2000). Indeed, requirement for dynamin activity can be bypassed by the deletion of the extracellular part of the receptor, which mimicks the extracellular S2 cleavage of N (Struhl and Adachi, 2000). This therefore suggests that endocytosis is required upstream of the S3 cleavage. Second, an unknown activity residing within the intracellular domain of mouse Delta1 is required for or upstream of the S3 cleavage (Shimizu et al., 2002). This observation has been interpreted to suggest that multimerization and/or endocytosis mediated by signals in the intracellular domain of Delta1 promote N activation. Third, a recent functional analysis of the mind bomb (mib) gene indicates that endocytosis of Dl is required for nonautonomous activation of N in the zebrafish neural tube (Itoh et al., 2003). Like Neur, the E3 ubiquitin ligase Mib co-immunoprecipitates with Dl, promotes Dl ubiquitination and upregulates Dl endocytosis. Interestingly, Mib is structurally distinct from Neur and both Mib and Neur have been conserved during evolution. The function of Drosophila mib is not yet known. Results form elegant cell transplantation studies indicate that mib mutant cells are not defective in receiving inhibitory signals but are less effective at producing inhibitory signals in the neural tube (Itoh et al., 2003). Thus, both Neur- and Mib-mediated internalization of Dl appear to nonautonomously activate N. Finally, endocytosis-defective Dl proteins remain accumulated at the cell surface and have reduced signaling capacity (Parks et al., 2000). Together, these results have suggested that Dl endocytosis might either expose N on the receiving cell to extracellular S2 cleavage or dissociate the cleaved ectodomain from the rest of the receptor (Parks et al., 2000; Pavlopoulos et al., 2001; Struhl and Adachi, 2000). One prediction from this model is that NEDC, but not NICD, should colocalize with Dl into endocytic vesicles. This prediction was not verified in the pIIb cell since both NEDC and NICD were found to colocalize with Dl into large endocytic vesicles. It is possible, however, that NEDC and NICD are targeted by independent mechanisms toward Hrs-positive vesicles. Whether Neur upregulates N activation by clustering N receptors in signal-receiving cells, by trans-endocytosis of Dl-NEDC complexes in signal-producing cells or by yet another mechanism, such as the targeting of Dl into signaling vesicles budding in the lumen of MVBs (exosomes), remains to be studied (Le Borgne and Schweisguth, 2003).

Homologs of Drosophila neur have been identified in frogs and mice. The regulatory function of Xneur in Dl endocytosis has been conserved in Xenopus (Deblandre et al., 2001). Xneur RNA is detectable in the developing nervous system and skin at neural plate stages (Deblandre et al., 2001). At this stage, the Xneur-expressing cells may correspond to primary neurons within the neural plate and to specialized ciliated cells in the skin. Expression of Xneur is blocked by constitutive activation of N and is upregulated by the inhibition of N (Deblandre et al., 2001). Thus, the neur gene is expressed in signal-sending cells that are selected by lateral inhibition in both Xenopus and Drosophila. However, elucidation of the exact function of Xneur in N signaling awaits Xneur inactivation. Knock out of m-neu1, one of the two murine homologs of Drosophila neur, reveals that m-neu1 is not an essential gene. m-neu1 mutant mice are viable
and show no obvious developmental defects (Ruan et al., 2001; Vollrath et al., 2001). It is possible that m-neu2 compensates for the loss of m-neu1 function. Considering the conservation of Neur function from flies to frogs, it will be of interest to examine the distribution of Neur during asymmetric cell divisions in vertebrate species. In summary, Neur and Numb are unequally segregated into the same daughter cell, independently of each other, during the asymmetric division of the pI cell. Neur promotes ligand endocytosis in the anterior cell, thereby activating N in its posterior sister. Concomitantly, Numb inhibits signal transduction in the anterior cell. We propose that Neur and Numb act in parallel to bias N activation.

Experimental Procedures

Drosophila Stocks

The neu[17]GAL4 (Bellaiche et al., 2001a) driver was used to express Pon-GFP (Lu et al., 1999) and UAS-Neur (Lai and Rubin, 2001a). Mitotic clones for neu[17]GAL4, numb[7], and numb[10] were induced using the FLP-FRT technique by heat shocking first instar larvae (30 min at 37°C). The following genotypes were used: (1) y w hsFLP/w; FRT82B neu[17]GAL4/FRT82B nlsGFP; (2) y w hsFLP/w; FRT40A numb[7] (or numb[10])/FRT40A nlsGFP. neu[17]GAL4, numb[7], and numb[10] (Berdnik et al., 2002) are amorphic alleles, while dig[17]GAL4 is a hypomorph allele. The amorphic pins[10] allele (Yu et al., 2000) is associated with a deletion that extends from 898 to 1210 (-1 is at the ATG) (Y. Bellaiche and F.S., unpublished data).

Immunofluorescence

Noto were dissected and processed as previously described (Bellaiche et al., 2001a). Primary antibodies used were rabbit anti-Neur antibody (a gift from E. Lai; 1:500), guinea pig anti-Dl (a gift from M. Muskavitch; 1:3000), mouse anti-Di (C594-9B, Developmental Studies Hybridoma Bank [DSHB]), rabbit anti-Ser antibody (a gift from E. Knust; 1:200), mouse anti-NECD (C458-2H, DSHB; 1:3000), mouse anti-Cut (2B10, DSHB; 1:1000), rabbit anti-Asense (a gift from Y.-N. Jan; 1:2000), guinea pig anti-Hrs (a gift from H. Bellen; 1:600), rabbit anti-Numb (a gift from Y.-N. Jan; 1:2000), rat anti-Elav (7E8, DSHB), rabbit anti-GFP (Molecular Probes; 1:1000) and rat anti-Su(H) (1:1000). All Alexa-coupled and Cy3/5-coupled antibodies (1:1000) were from Molecular Probes and Jackson Laboratories. Images were acquired on a Leica SP2 microscope and assembled using Adobe Photoshop.

Endocytosis Assay

Pupal nota were dissected in Schneider’s Drosophila medium (GIBCO-BRL, Life Technology, Carlsbad, CA) containing 1% fetal calf serum (GIBCO-BRL). After dissection, medium was replaced and supplemented with 1 μg/ml 20-OH ec dysone (Sigma, Saint Louis, MO). Pupal nota were cultured for 15 min in presence of the mouse monoclonal anti-Di antibody C594-9B that recognizes the extracellular portion of Di. Following medium changes, epithelial cells were fixed. Localization of anti-Di antibodies was revealed using secondary antibodies. Di was detected using guinea pig anti-Di.

Drug Treatment of Noto

For drug treatment, pupae were dissected as described above. Dissected nota were incubated for 1 hr at 25°C with 1 μg/ml 20-OH ec dysone (Sigma) alone or with 2 μM Latrunculin A (Sigma), 5 mM of BDM (Sigma), or 1 μM colcemid (Sigma). Pupal nota were then fixed and processed for immunofluorescence as described above.

Acknowledgments

We thank H. Bellen, W. Chia, Y.N. Jan, J. Knoblich, E. Knust, E. Lai, M. Muskavitch, and the DSHB (University of Iowa) for flies and antibodies. We thank A. Bardin, Y. Bellaiche, A. Brand, C. Goridis, D. Henrique, S. Lee, V. Orgogozo, A. Prochiantz, and I. Stüttgen for critical reading. This work was supported in part by a grant from the ARC (#4512). Received: January 21, 2003 Revised: May 2, 2003 Accepted: May 2, 2003 Published: July 7, 2003

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