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The *Drosophila* Numb protein inhibits signaling of the Notch receptor during cell–cell interaction in sensory organ lineage

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ABSTRACT **Specification of unequal daughter cell fates in the *Drosophila* external sense organ lineage requires asymmetric localization of the intrinsic determinant Numb as well as cell–cell interactions mediated by the Delta ligand and Notch receptor. Previous genetic studies indicated that *numb* acts upstream of *Notch*, and biochemical studies revealed that Numb can bind Notch. For a functional assay of the action of Numb on Notch signaling, we expressed these proteins in cultured *Drosophila* cells and used nuclear translocation of Suppressor of Hairless [Su(H)] as a reporter for Notch activity. We found that Numb interfered with the ability of Notch to cause nuclear translocation of Su(H); both the C-terminal half of the phosphotyrosine binding domain and the C terminus of Numb are required to inhibit Notch. Overexpression of Numb during wing development, which is sensitive to Notch dosage, revealed that Numb is also able to inhibit the Notch receptor *in vivo*. In the external sense organ lineage, the phosphotyrosine binding domain of Numb was found to be essential for the function but not for asymmetric localization of Numb. Our results suggest that Numb determines daughter cell fates in the external sense organ lineage by inhibiting Notch signaling.**

Asymmetric cell division is a fundamental developmental process for the generation of two daughter cells with distinct fates. One way to achieve this difference is to segregate an intrinsic determinant preferentially to one of the daughter cells. Alternatively, the daughter cells can become different through a cell-extrinsic mechanism via cell–cell interactions (reviewed in ref. 1). The *Drosophila* peripheral nervous system uses a combination of these two types of mechanisms.

In the *Drosophila* peripheral nervous system, the cells that comprise an external sense (es) organ arise from asymmetric cell divisions of a single sensory organ precursor (SOP) cell (2–4). This SOP cell is singled out from a cluster of neural-competent cells via lateral inhibition, a cell–cell communication process between the future SOP and the surrounding cells (reviewed in refs. 5 and 6). The SOP cell then gives rise to two different daughter cells, IIa and IIb. The IIa cell divides again asymmetrically to form the hair (tricogen) cell and socket (tormogen) cell, whereas the IIb cell generates a neuron and sheath (thecogen) cell (see Fig. 5*A* and refs. 2–4).

One of the key determinants in these asymmetric divisions is the *numb* gene (7). The product of the *numb* gene is a membrane-associated protein that is asymmetrically localized during mitosis in the sensory organ lineage, and segregates into only one of the two daughter cells (8, 9). Loss of *numb* function causes the IIb cell to be transformed into the IIa cell, whereas overexpression results in the opposite cell fate transformation (7, 8). A mammalian homolog of Numb (mouse–Numb) can functionally substitute for Numb in transgenic flies, and is also asymmetrically localized (10). A phosphotyrosine binding (PTB) domain near the N terminus of Numb is conserved between fly and mouse Numb (11, 12). In the Shc protein the PTB domain has been demonstrated to bind to the phosphorylated tyrosine of an activated receptor and to become phosphorylated in turn (13). It is not known, however, whether the PTB domain contributes to Numb function or to its asymmetric localization.

In addition to the Numb-mediated cell-intrinsic mechanism, cell–cell communication via *Notch* (*N*) and *Delta* (*Dl*) is also crucial for es (external sense) organ cell fate determination (14–16). *N* encodes a transmembrane protein with epidermal growth factor-like repeats in the extracellular domain and ankyrin repeats in the intracellular domain (17, 18). *Dl* also encodes a transmembrane protein with epidermal growth factor-like repeats (19, 20). Extensive evidence indicates that Delta binds to Notch and functions as a Notch ligand (21). Notch and Delta are known to serve a key function for cell–cell communication during neurogenesis (22), eye development (23), muscle development (24), oogenesis (25), and wing development (26, 27). Their homologs have been found in several species (see ref. 28 for a review). During neurogenesis, including the process of selecting an SOP from a cluster of neural-competent cells, Notch and Delta mediate the lateral inhibition process that allows an emerging neural precursor such as an SOP to suppress neighboring cells from becoming neural precursors (29). Consequently, loss of either *N* or *Dl* function results in supernumerary SOP cells. Moreover, studies of temperature sensitive mutants reveal that reductions of *N* or *Dl* function during sensory organ formation cause both SOP daughter cells to become IIb cells, which then divide symmetrically into four neurons (14, 15). In fact, either gain or loss of *N* function results in symmetric cell fate, as in the case of *numb* mutations, except that the *N* loss-of-function (LOF) phenotype resembles the *numb* gain-of-function (GOF) phenotype and vice versa. This indicates that, in addition to lateral inhibition during neurogenesis, *N* and *Dl* also function during asymmetric divisions. In addition, recently it was shown that *numb* acts upstream of *N* during embryonic sense organ development (16). A similar antagonism of *N* and numb was also observed in the MP2 lineage in the *Drosophila* central nervous system (30). Further, physical interactions have been reported between Notch and Numb (16) as well as between the mammalian homologs of Notch and Numb (10).

An important downstream target of the Notch receptor signaling pathway is the Suppressor of Hairless [*Su(H)*] gene (31–33). Like Notch, loss-of-*Su(H)* function during lateral inhibition results in additional SOP cells at the expense of

Abbreviations: es, external sense; PTB, phosphotyrosine binding; SOP, sensory organ precursor; N^{intra}, intracellular Notch domain; GOF, gain of function; LOF, loss of function

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epidermal cells (31). Reduction or overexpression of Su(H) during es organ development causes the IIa cell to divide symmetrically into two hair cells or two socket cells, respectively (34). Furthermore, it was shown that Notch and Su(H) interact genetically and molecularly (33): Su(H) can bind directly to the Notch protein, and a possible functional significance of this binding has been shown in cell culture. When Su(H) is transfected into *Drosophila* Schneider S2 cells, the protein localizes to the nucleus. Coexpression of Su(H) with Notch causes Su(H) to remain in the cytoplasm. When cells expressing Notch and Su(H) are aggregated with cells which express the Notch ligand Delta, Su(H) is translocated into the nucleus. Su(H) is the *Drosophila* homolog of the mammalian RBP-Jκ DNA binding protein (31, 32) which binds DNA and acts as a transcriptional activator (32, 35, 36). The role of Su(H) as a downstream target of Notch signaling is further substantiated by *in vivo* studies. Overexpression of activated Notch in transgenic flies results in transcriptional activation of genes of the *E(spl)* complex. These genes contain Su(H) binding sites in their promotor; activation of these *E(spl)* genes requires the function of Su(H) (35, 36), supporting the importance of Su(H) in Notch signaling.

Given that Notch, Delta, Su(H) and Numb are vital components for determining asymmetric cell fate during es organ differentiation and a functional link between Notch, Delta, and Su(H) has been demonstrated using cultured *Drosophila* S2 cells, we have chosen to use the same experimental system to study the function of Numb. We show that coexpression of Numb with Notch suppresses nuclear translocation of Su(H) upon binding of Delta to Notch, and report that the PTB domain and C terminus of Numb are required for this inhibitory activity. Moreover, we test the biological relevance of the findings from cell culture studies with *in vivo* experiments.

MATERIALS AND METHODS

Numb Deletion Constructs. To facilitate cloning, pSKnb was derived by cloning a *Eco*RI/*Xba*I fragment from pUAST-nb (S. Wang, personal communication) into pSK+ Bluescript. The coding sequence of Numb in pSKnb was tagged at the 3′ end with a human Myc epitope QGTEQKLISEEDLN (37) by PCR giving pSKnb-Myc.

The Δ1 to Δ4 and ΔPTB deletion mutants of Numb were constructed in a two-step PCR. First, two PCRs were performed using one primer composed of sequences at either end of the deletion and another primer at the 5′ or 3′ end of the *numb* coding sequence respectively (see Fig. 2 legend for primer locations). The resulting two PCR products were combined and another PCR was performed. The resulting PCR product was cut with restriction enzymes, and used to replace full-length *numb* in pSKnb-Myc.

Δ5 was constructed by digesting pSKnb-Myc first with *Kas*I, then followed by a partial digest with *Xho*I cutting the 3′ *Xho*I site only, blunting the overhangs, and religating.

In all cases the sequences of the deletion products were verified by sequencing of double-stranded templates using United States Biochemical Sequenase 2.0 according to instructions from the manufacturer.

Expression Constructs. The full-length Notch expression construct was pMtNMgII (21) and Su(H)-Myc was pSu(H)4 (33). The full-length *numb* coding sequence was cloned from pWHI-numb (8) into either pCaSpeR-hs (38) for the heat shock construct (hs-numb) or pRmHa-3 (39) for the *Metallothionein* construct (Mt-numb). Deletion constructs Δ1 to Δ4 and ΔPTB were cloned as *Eco*RI/*Nar*I fragments from the pSKnb-Myc deletion constructs into pSKnb and then inserted as *Eco*RI/*Xba*I fragment into pCaSpeR-hs. Δ5 was derived from the Δ5 clone in pSKnb-Myc by PCR using primers from the middle and the 3′ end of the Numb coding sequence. The PCR product was inserted into pSKnb, sequenced, and cloned into pCaSpeR-hs as described above.

The *Metallothionein* lacZ construct was made by cloning an *Eco*RI fragment containing cytoplasmic lacZ from pC4AUG (40) into pRmHa-3.

The intracellular Notch domain (N^{intra}) was constructed first by PCR of pMtNMgII with a primer with an *Eco*RI site fused to 18-bp upstream sequence and the *N* start codon and a part of the intracellular *N* sequence starting with the first A after the transmembrane segment and another primer in the intracellular Notch domain. The PCR fragment was sequenced, cut with *Eco*RI/*Sal*I, and cloned into the pRmHa-3 *Metallothionein* vector. The intracellular *Sal*I fragment and then the intracellular *Xho*I fragment of the cytoplasmic domain of Notch were then taken from pMtNMgII and inserted into the pRmHa-3 construct to generate the N^{intra} construct, which was comparable to the one used in ref. 41 for *in vivo* studies.

***Drosophila* S2 Cell Expression Experiments.** Cells were maintained at 25°C in M3 insect medium (Sigma) supplemented with 10% heat inactivated fetal calf serum (University of California, San Francisco, cell culture facility) and 100 units/ml penicillin and 100 μg/ml streptomycin (University of California, San Francisco, cell culture facility). Expression constructs were transfected into S2 cells in Falcon 6-well dishes as described (42), except that Lipofectin (GIBCO/BRL) was used as the transfection reagent and M3 medium without supplements was used as the serum-free medium. If the transfected cells were not aggregated, a sterile coverslip was placed into the dishes prior to plating the cells. After transfection cells were allowed to recover for 24 h, expression of the *Metallothionein* constructs was induced by addition of $CuSO_4$ to 0.7 mM for 12 to 16 h and then expression of the heat shock constructs was induced by heat shock treatment as described (42).

Aggregation was done by incubating cells immediately after heat shock treatment with cells stably or transiently transfected with the Delta expression construct pMtDl1 (21, 43) in 25 ml Erlenmeyer flasks on a shaker in an air incubator at 25°C for 4 h. Afterwards, cells were collected by centrifugation using an IEC clinical centrifuge and fixed in a solution of 3% formaldehyde and 0.1% glutaraldehyde in PBS for 5 min. The cells were then rinsed twice with PBS and aliquots were left overnight at 4°C on Nunc Lab-Tek 8-chamber slides coated with poly-L-lysine. Staining was done in the chambers essentially as described (21).

Cells that were not aggregated were left after heat shock for 4 h at 25°C and then fixed and stained on the coverslip as described above.

Primary antibodies were rabbit anti-Notch (E. Giniger, personal communication), guinea-pig anti-Delta (T. Parody and M. A. T. Muskavitch, personal communication), rabbit anti-Numb (8), and monoclonal mouse anti-myc 9E10 (Santa Cruz Biotechnologies). Secondary antibodies were either fluorescein-conjugated, rhodamine-conjugated, or Cy5-conjugated. Cells were mounted in glycerol (21) and examined on a Zeiss Axioplan microscope. Confocal images were obtained on a MRC-600 confocal microscope (Bio-Rad), processed with Adobe PHOTOSHOP version 3.0.5 and assembled with Adobe ILLUSTRATOR version 6.0.

Genetics and Ectopic Expression of *numb* and *Notch*. Transgenic flies with full length and deletions Δ2 and ΔPTB of myc-tagged Numb were generated by subcloning from the pSKnb-Myc constructs as *Eco*RI/*Xba*I fragments into pUAST and transforming the resulting constructs into *w* flies using standard techniques. Targeted ectopic expression of Numb and Notch were accomplished using the Gal4 system (44) where the Gal4 source was from enhancer-trap lines *patched*-Gal4 (45), $Gal4^{109-68}$ (S.Y.-S. and Y.N.J. unpublished), or *hairy*-$Gal4^{IJ3}$ (44). For wild-type Numb we used pUAST-*numb* (S. Wang, personal communication), which can functionally substitute for *numb* (10), and for wild-type Notch we used

pUAST-*N* (gift from L. Seugnet, M. Haenlin, and P. Simpson, Centre National de la Recherche Scientifique, Strasbourg, France).

To see if Numb could inhibit Notch activity in wing margin formation, we overexpressed Numb using UAS-*numb* crossed to *patched*-Gal4. We collected eggs in vials over a period of 24 h (at 22°C), incubated the vials at 22°C until the larvae had developed to second instar, and then shifted the vials to 29°C for 24 h. To test for enhancement of the *patched*-Gal4/UAS-*numb* wing phenotype by reduction of *Notch* function, we crossed w^a N^{55e11}/yw; *patched*-Gal4 females to UAS-*numb* males. Eggs were collected at room temperature.

In Numb and Notch overexpression experiments, UAS-*numb* and UAS-*N* males were mated to $Gal4^{109-68}$ females in vials and raised at 29°C. In LOF experiments using UAS-FLP (a gift from K. Bunier and K. Golic, University of Utah), all flies were mated and maintained at 25°C as follows: for *numb*, *y w*; p[y^+] *numb*^{2} *ck* FRT^{40A} $Gal4^{109-68}$ males to *y w*; P[*mini-w*$^+$; hsπM] FRT^{40A}; UAS-FLP females; for *N*, w^a N FRT^{18A}/*FM6*; UAS-FLP/*TM3* females to *y w* FRT^{18A}; $Gal4^{109-68}$/*CyO* males.

All flies were raised on standard cornmeal-agar medium. Mutations not specifically discussed here are described in ref. 46.

RESULTS

Nuclear Translocation of Su(H) upon Notch Activation in Cell Culture Is Inhibited by Numb. By using a *Drosophila* cell culture assay system originally described by Fortini and Artavanis-Tsakonas (33), we examined potential effects of Numb on Notch function.

As reported before (33), after transfection into Schneider S2 cells, a myc epitope tagged Su(H) protein is localized predominantly in the nucleus (Fig. 1*A*). When coexpressed with full-length Notch under *Metallothionein* promotor control, Su(H) is found in the cytoplasm (Fig. 1*B* and ref. 33). However, if these cells are aggregated with Delta-expressing cells, the Su(H)-Myc protein translocates into the nucleus (Fig. 1*D* and ref. 33). Our statistical analysis of the aggregated cell clusters showed that more than 70% of the clusters of 2 to ≈10 cells had at least one cell with Su(H) localized to the nucleus (Fig. 1*F*). Thus Su(H) provides a readout for ligand dependent function of the Notch receptor in this aggregation assay.

To investigate the effects of Numb in this assay system, we included Numb expression constructs into the next set of experiments. Endogenous Numb expression in S2 cells was below the level for detection in immunohistochemical experiments (Fig. 1*A*) and barely detectable in Western analysis (data not shown). We placed full-length Numb under the control of the *hsp70* heat shock promotor (hs-numb) or *Metallothionein* promotor (Mt-numb). When either construct was transfected into S2 cells and Numb expression was induced, Numb was found associated with the membrane (Fig. 1*C*) as seen *in vivo* (8, 9), although with the Mt-numb construct Numb was frequently irregularly distributed around the entire membrane even after 12 h of induction (data not shown). To test if Numb can change either the expression or localization of the Su(H) protein, we cotransfected S2 cells with Su(H) and either hs-numb or Mt-numb. We found that Numb altered neither the nuclear localization of Su(H) in the absence of Notch (Fig. 1*C*) nor the cytoplasmic distribution of Su(H) in the presence of Notch (data not shown). These controls indicate that Numb does not have any direct effect on subcellular distribution of Su(H).

To examine the effects of Numb on the signaling of the Notch receptor upon ligand binding, we cotransfected S2 cells with Notch, Su(H), and either hs-numb or Mt-numb and found that the transfected cells aggregated with Delta-expressing cells. We still observed capping of Notch at the part of the membrane adjacent to the Delta-expressing cell (as shown in case of the N- and Su(H)-expressing cell in Fig. 1*D* and ref. 21), suggesting that the protein interaction between Notch and Delta was not suppressed by Numb. However, in the majority of aggregated cell clusters, Su(H) failed to translocate to the

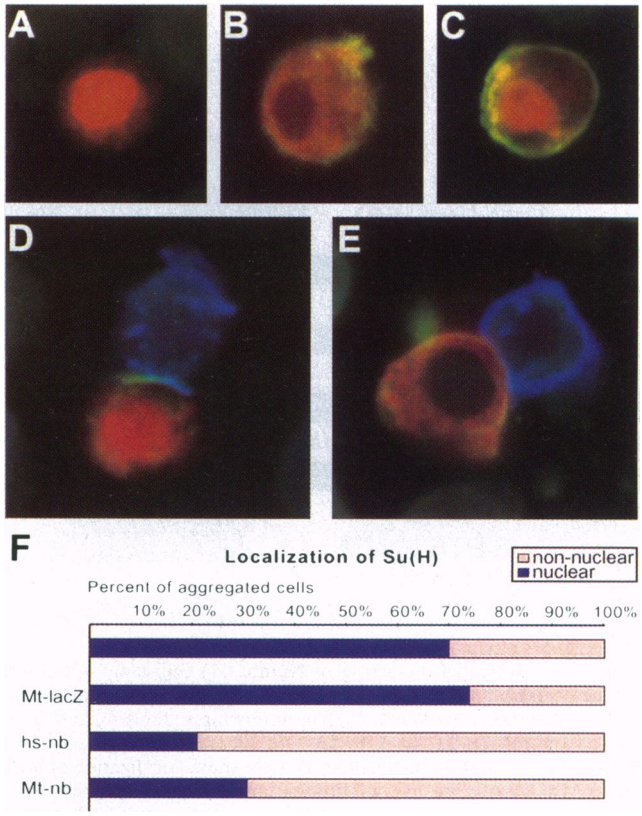

FIG. 1. Numb inhibits the translocation of Su(H) into the nucleus in *Drosophila* S2 cells. Numb and Notch are depicted in green, Su(H) in red, and Delta in blue. In *D* and *E*, the transfected cells were aggregated with transiently transformed Delta-expressing cells (42) (*A*) Cell transfected with Su(H)-Myc and stained with anti-Numb (green) and anti-myc (red) shows nuclear localization of Su(H) and the absence of endogenous Numb. (*B*) Cell transfected with Su(H), Notch (green), and *lacZ* (not shown) shows cytoplasmic distribution of the Su(H) protein (red). (*C*) Cell transfected with Su(H) and Numb shows nuclear localization of Su(H) (red) and membrane association of Numb (green). (*D*) Cell transfected with Su(H) (red), Notch (green), and *Metallothionein lacZ* (not shown) is aggregated with a Delta (blue)-expressing cell. Su(H) is translocated to the nucleus. Expression of *lacZ* was confirmed in cells from the same assay using anti-β galactosidase instead of anti-Notch antibodies. (*E*) Cell transfected with Su(H) (red), Notch (not shown), and Numb (green) under *Metallothionein* promotor control aggregated with a Delta (blue)-expressing cell. Su(H) is no longer translocated to the nucleus but stays in the cytoplasm. Notch expression was verified in cells from the same assay using anti-Notch instead of anti-Numb antibodies. (*F*) Numerical analysis of the localization of Su(H) in Notch-expressing cells aggregated with Delta-expressing cells. Included in this analysis are only aggregates in which Delta-expressing cells clearly contacted the Notch-expressing cells. Each aggregate was counted only once, regardless of the number of Notch-expressing cells in the cluster. If one or more cells in a cluster had nuclear Su(H) the cluster was counted as "nuclear." In a number of aggregates Su(H) was distributed roughly equally between cytoplasm and nucleus. Since Su(H) normally does not localize into the nucleus in a Notch-expressing cell (see *B* and ref. 33), we scored such cases as "nuclear." Each experiment was done twice independently and 50–100 aggregates were scored each time. The percentage of aggregated cells with nuclear localization is indicated in dark blue and the rest with cytoplasmic localization is indicated in red. Data are shown for cells transfected with N/Su(H)-, N/Su(H)/Mt-lacZ-, N/Su(H)/hs-numb (hs-nb)-, and N/Su(H)/Mt-numb (Mt-nb)-expressing cells.

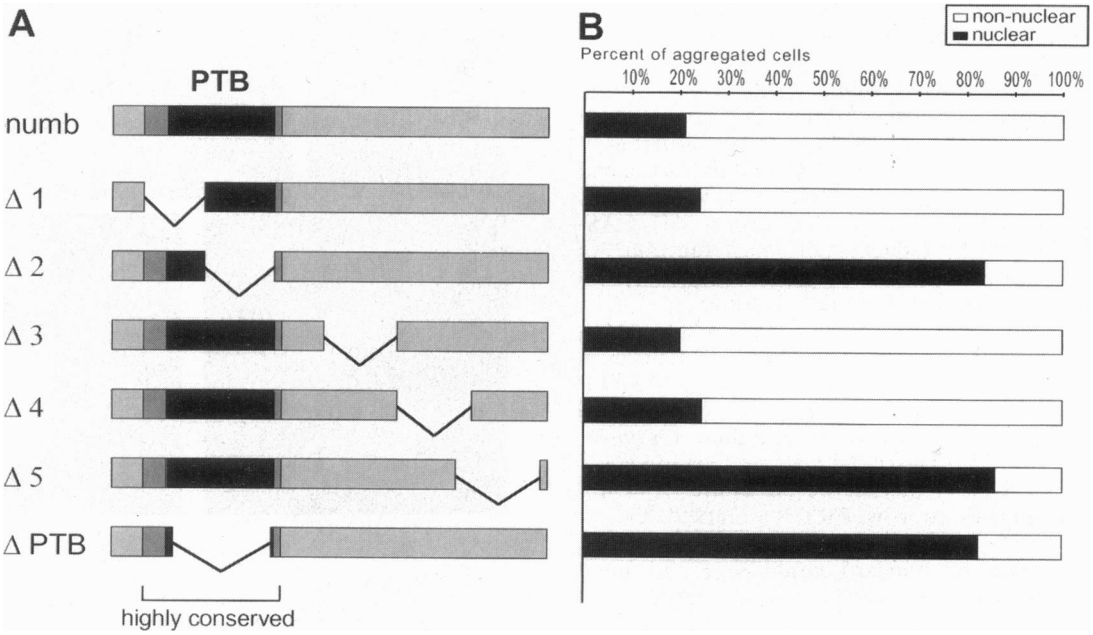

FIG. 2. Functional dissection of Numb. (*A*) Full-length Numb and six deletion constructs are shown schematically. The domain conserved between the fly and mammalian Numb is shown in dark gray, and the PTB domain within the conserved domain in black. Borders of the PTB domain were taken from ref. 12. The following regions were deleted in the constructs (numbering follows the deduced amino acid sequence of Numb from ref. 7): Δ1, 40–118; Δ2, 119–206; Δ3, 270–363; Δ4, 364–458; Δ5, 426–546; and ΔPTB, 78–204. (*B*) Numerical analysis of the effects of the deletions in *A* on the nuclear/cytoplasmatic localization of Su(H) in the cell culture assay. Cells were transfected transiently with pMtNMgII, Su(H)-Myc, and the Numb deletion mutants under heat shock control, aggregated with stably transfected Delta-expressing cells (42) and scored as described in Fig. 1*F*. Parallel to each aggregation with cells expressing the deletion mutants, aggregation with cells transfected with full-length Numb or *lacZ* was performed as control. Because stably transfected Delta-expressing cells were used instead of the transient transfection as in Fig. 1*F*, the number of cells with nuclear Su(H) is slightly higher. Expression of the Numb deletion mutants was verified either by immunohistochemical staining with rabbit anti-Numb (8) or by Western blot analysis with rat anti-Numb (7) for the Δ5 mutant.

nucleus (Fig. 1 *E* and *F*). Thus Numb inhibited the Delta-dependent function of Notch. The heat shock Numb construct was more effective than the *Metallothionein* Numb construct in inhibiting Su(H) nuclear translocation (Fig. 1*F*). This may be due to the initially higher levels of expression of Numb driven by the heat shock promotor (data not shown) or to the uneven distribution of the Numb protein when expressed from the *Metallothionein* promotor.

To investigate whether the inhibition of Notch is specific to Numb, we cotransfected S2 cells with Notch, Su(H), and full-length cytoplasmic *lacZ* under *Metallothionein* promotor control and performed the aggregation assay. No alteration of the nuclear translocation efficiency of Su(H) was observed (Fig. 1*F*). The inhibitory effect of Numb is therefore unlikely to be due to nonspecific effects of protein overexpression but, rather, arises from specific interactions between Numb and Notch.

Domains of the Numb Protein Necessary for Notch Inhibition. To identify the domains of Numb which are important for inhibiting Notch signaling, we deleted different regions of Numb and tested the deletion derivatives of Numb in the aggregation assay described above. The N-terminal part of Numb is highly conserved between *Drosophila* and mouse (10) and has been shown to contain a PTB domain (11, 12). We constructed two partial deletions of the PTB domain, three deletions in other parts of Numb, and a deletion of the entire PTB domain (Fig. 2), and placed these deletion mutants under *hsp70* promotor control. Deletion of either the entire PTB domain or just the C-terminal portion completely abolished Numb function (Fig. 2, constructs ΔPTB and Δ2). By contrast, deletion of the N-terminal portion of the PTB domain had no significant effect on Numb inhibition of Notch (Fig. 2, construct Δ1). In addition, deletion of the most C terminus of Numb eliminated the ability of the mutant Numb to prevent translocation of Su(H)-Myc to the nucleus upon Notch activation (Fig. 2, construct Δ5). Other deletions of Numb had no effect on Numb function in this assay.

Numb Changes the Localization of the Intracellular Notch Domain. Deletion of the Notch extracellular domain results in a constitutively active, GOF mutation (41, 43). To investigate the effects of Numb on constitutively active Notch in cell culture, we made a Notch construct consisting of the intracellular domain only (referred to as N^{intra}). As found *in vivo* (41, 47), N^{intra} colocalizes with Su(H) in the nuclei of S2 cells (Fig. 3*A*). However when hs-numb was coexpressed with N^{intra} and Su(H)-Myc in S2 cells, the N^{intra} and Su(H) proteins were colocalized at the membrane and in the cytoplasm in more than 76% of the cells (Fig. 3 *C* and *D*). In 17% of the cells no nuclear N^{intra} and Su(H) was observed but in the majority of cases, some of the Su(H) and N^{intra} proteins remained in the nucleus (Fig. 3 *A–C*). These observations raise the possibility that N^{intra} and Su(H) are localized to the membrane and cytoplasm as a result of a direct interaction between Notch and Numb.

Numb Acts To Inhibit Notch *in Vivo*. Having observed that Numb inhibits the Notch receptor signaling in S2 cells, we wondered if Numb can also inhibit Notch activity *in vivo*, and used the UAS Gal4 system to misexpress Numb during wing development. Normally, wing margin development requires *N* but not *numb* function. Further, wing margin formation is sensitive to *N* gene dosage; a reduction of *N* dosage by half leads to a "Notched wing" phenotype (Fig. 4*B* and ref. 46). Using *patched*-Gal4 (45), we ectopically expressed UAS-*numb* (S. Wang, personal communication) in a stripe of cells crossing the wing margin at the anterior/posterior boundary (indicated by an arrow in the wild-type wing in Fig. 4*A*). When Gal4 expression levels were raised by a temperature shift, these flies exhibited loss of distal wing margin at the anterior/posterior boundary (Fig. 4*C*), which mimics that of a reduction of *N* function (Fig. 4*B*). An even stronger phenotype was observed

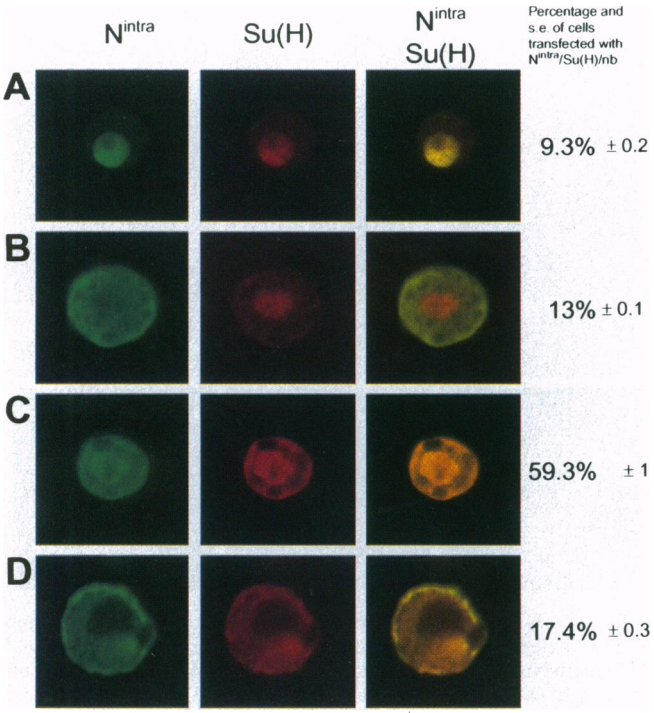

FIG. 3. Localization of the intracellular Notch domain (N^{intra}) is altered by Numb. N^{intra} under *Metallothionein* promotor control and *Metallothionein* lacZ or hs-numb were transiently transfected and evaluated in two independent experiments. N^{intra} is depicted in green (*Left*), Su(H) in red (*Middle*), and the superimposition of these two panels is shown at the *Right*. (*A*) In cells expressing N^{intra}, Su(H), and Mt-lacZ both N^{intra} and Su(H) are almost exclusively localized to the nucleus. When cells are transfected with N^{intra}, Su(H), and Numb, the intracellullar localization of N and Su(H) is seldomly nuclear, as in *A*, but more often partially membrane associated, as in *B* and *C* or entirely cytopasmic or membrane associated as in *D*. The percentage and standard errors (SE) of contransfected cells with each of the four types of protein distributions are shown to the right of the panels in *A–D*. (*B*) N^{intra} is predominantly at the membrane and Su(H) in the nucleus. (*C*) Both N^{intra} and Su(H) are found both at the membrane and in the nucleus. In this case the intensities of nuclear staining of N^{intra} and Su(H) are not closely related. (*D*) N^{intra} and Su(H) are colocalized at the membrane and the cytoplasm but excluded from the nucleus.

when we misexpressed Numb in flies with only one copy of *N* without temperature shift (Fig. 4*D*, compare with a sibling from the same cross with two copies of *N* in Fig. 4*E*), as expected from the inhibitory effect of Numb on Notch activity. This also indicates that the relative abundance of Notch with respect to Numb is important.

Next we investigated the role of Numb and Notch in the adult es organ, where both proteins are necessary for asymmetric cell fate specification. To allow for a direct comparison between Numb and Notch, we utilized the UAS-Gal4 system to target both LOF and GOF of both genes to the SOP lineage (Fig. 5*A*), and used the Gal4 enhancer-trap line $Gal4^{109-68}$, which expresses Gal4 specifically in the SOP and its daughter cells. LOF mutations were generated by mitotic recombination using UAS-FLP (K. Bunier and K. Golic, personal communication) and *numb FRT* or *N FRT*, which yields one daughter cell with two copies of the null mutation and the other daughter cell with two copies of the wild-type gene. GOF phenotypes were induced by $Gal4^{109-68}$ driving either UAS-*numb* or UAS wild-type *N*. When either LOF or GOF mutations were directed to the IIa daughter cell of the SOPs of adult external sense organ, the daughters no longer had different fates. N^{GOF} (Fig. 5*D*) and $numb^{LOF}$ (Fig. 5*C*) yielded the double socket phenotype (two sockets, no hair) whereas N^{LOF} (Fig. 5*B*) and $numb^{LOF}$ (Fig. 5*E*) gave rise to the the twinned

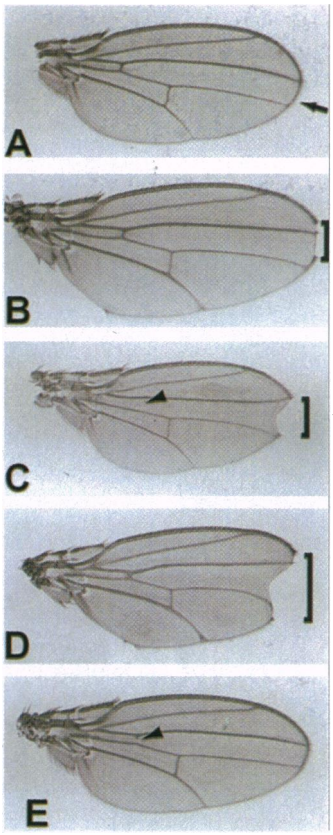

FIG. 4. Numb inhibits Notch function in the formation of the wing margin. (*A*) Wild-type wing (arrow marks the anterior/posterior boundary). (*B*) Wing from a w^a N^{55e11}/*y w*; *patched*-Gal4/+ female displaying the typical *Notch* haplo-insufficient "Notched wing" phenotype (bracket). (*C*) Wing from *y w*; *patched*-Gal4/+; UAS-*numb*/+ female. To increase Gal4 expression levels the temperature was shifted to 29°C during second instar larval stage (early in wing development). Note the"Notched wing" (bracket) and missing cross vein (arrowhead). (*D*) Wing from a w^a N^{55e11}/*y w*; *patched*-Gal4/+; UAS-*numb*/+ female grown at room temperature. Note the increased wing notching (bracket) in contrast to the less severe wing notching phenotype in the sibling shown in *E*. (*E*) Wing from a *y w*; *patched*-Gal4/+; UAS-*numb*/+ male sibling of the female used in *D*. Because the temperature was not shifted to 29°C as in *C*, the UAS-*numb* phenotype is limited to the missing cross vein (arrowhead).

hair phenotype (two hairs, no sockets); both phenotypes arise from cell fate transformation so that the two daughter cells of the IIa cell adopt symmetric cell fates. This result is consistent with the previous finding of *numb* and *N* function (8, 14) and further substantiates the antagonistic relationship between these two genes.

The PTB domain of Numb Is Necessary for *numb* Function *in Vivo*. To further test the significance of the effect of Numb on Notch as revealed in our cell culture experiments, we used the Gal4-UAS system to express two inactive Numb deletions in the SOP lineage. Deletion Δ2 and ΔPTB (Fig. 2) and wild-type Numb were each tagged with a human c-Myc epitope (37), placed under UAS Gal4 promotor control, and transformed into flies. Introduction of the $Gal4^{109-68}$ into these transgenic flies caused the Myc-tagged Numb proteins to be expressed in SOP cells. At 25°C, expression of wild-type Numb caused both the absence of micro- and macrochaete in the notum and scutellum and socket to hair transformations, as described earlier in this work (Fig. 6*A*; compare with Fig. 5*E*). The wing margins also showed a similar bristle phenotype (Fig. 6*B*). At 30°C, expression of wild-type Numb resulted in lethality, presumably due to increased Gal4 expression resulting in higher UAS-*numb* expression levels. By contrast, neither

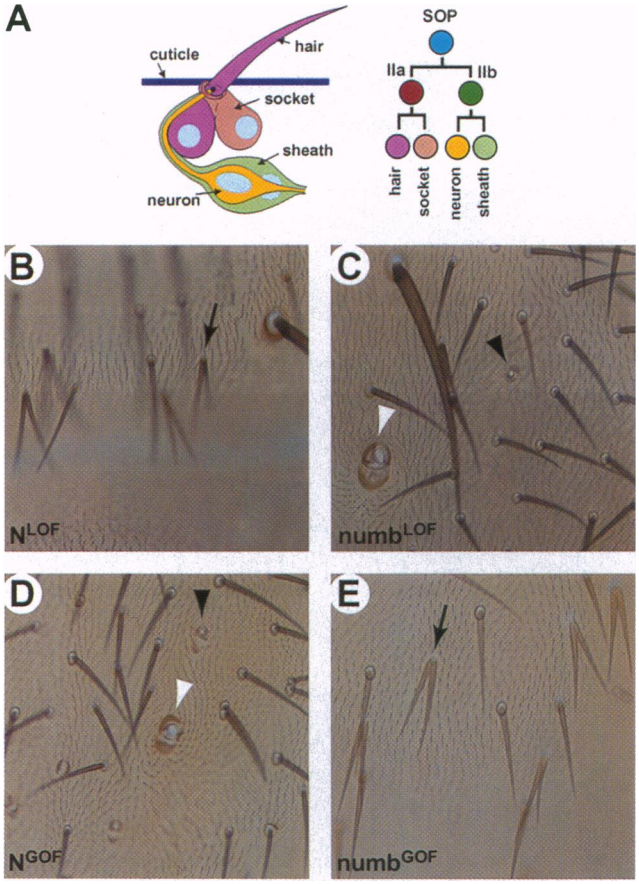

FIG. 5. Involvement of Numb and Notch in the SOP lineage. (*A*) Schematic view of a bristle (*Left*) and its development from a single SOP cell (*Right*). (*B*) Micro- and macrochaete from notum of a *y w* FRT/w^a N^{55e11} FRT; Gal4$^{109-68}$/+; UAS-FLP/+ female. Note twinned microchaete (arrow) due to N^{LOF} in the IIa cell, resulting in the socket to hair transformation. (*C*) Micro- and macrochaete from notum of y^+ $numb^2$ *ck* FRT Gal4$^{109-68}$/FRT; UAS-FLP/+ female. Note double socket phenotype (indicated by a white arrowhead for a macrochaete and a black arrowhead for a microchaete) due to $numb^{LOF}$ in the IIa cell resulting in the hair to socket transformation. (*D*) Micro- and macrochaete from notum of a *y w*; UAS-N^{+22}/ Gal4$^{109-68}$ fly. Double socket phentype due to N^{GOF} (white and black arrowheads as in *C*). (*E*) Micro- and macrochaete from notum of a *y w*; Gal4$^{109-68}$/+; UAS-$numb^{+34a}$/+ fly. Twinning (arrow) is due to $numb^{GOF}$.

Δ2 nor ΔPTB showed any bristle defects in scutellum, notum, or wing margin (Fig. 6 *D*, *E*, *G*, and *H*) at either 25 or 30°C. In control experiments to verify the expression of the Numb constructs, *hairy*-Gal4 was introduced into flies. Both wild-type Numb and the deletion mutants were found to be expressed and asymmetrically localized during neuroblast divison in the embryo (Fig. 6 *C*, *F*, and *I*). Thus the inactivity of the deletion mutants was not likely to be due to complete disruption of protein structure or failure of asymmetric localization during asymmetric divisions. Instead, the loss of biological function of these Numb mutants correlated with their inability to inhibit Notch signaling.

DISCUSSION

Correct cell fate specification in the sensory organ lineage requires both the cell intrinsic Numb protein, which is asymmetrically localized prior to division and then segregated into one of the daughter cells (8), and cell extrinsic signaling by the Delta–Notch pathway (14, 15). To analyze how an intrinsic factor and extrinsic signals can work together to specify the cell

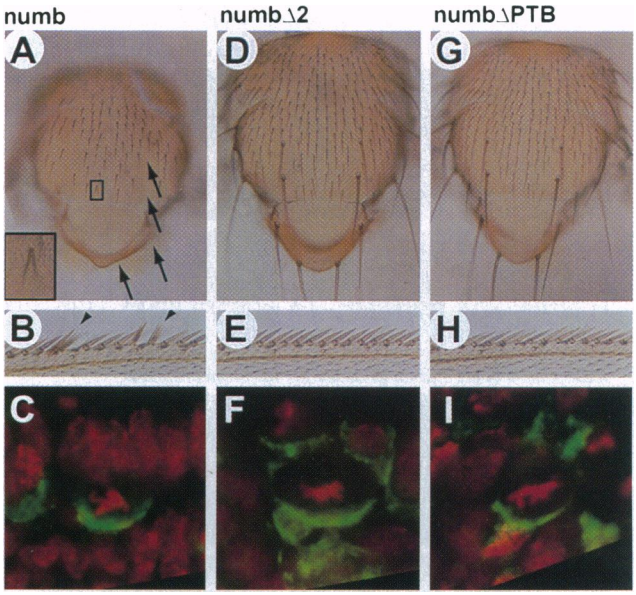

FIG. 6. *In vivo* effects of ectopic expression of full-length Numb and two deletion mutants of Numb. Depicted are myc epitope tagged full-length Numb (*A–C*), deletions Δ2 (*D–F*), and ΔPTB (*G–I*). Adult notae (*A*, *D*, and *G*) and wing margins (*B*, *E*, and *H*) are from transgenic flies with UAS-*numb*, UAS-*numb*Δ2, or UAS-*numb*ΔPTB with the Gal4$^{109-68}$ at 25°C. The notae in *A*, *D*, and *G* are composites of several focal planes processed with Adobe PHOTOSHOP. Overexpression of Numb in the notum (*A*) as in the $numb^{GOF}$ phenotype in Fig. 5*E*, results in the missing macrochaete (arrows) and double bristle without socket phenotype (boxed and shown in higher magnification) and the comparable bristle phenotype (arrowheads) at the wing margin (*B*). The macrochaete in the scutellum were missing with 100% penetrance. The deletions do not have any effects in the notum (*D* and *G*) or the wing margin (*E* and *H*). Expression of the Numb constructs was verified in neuroblasts after crossing the transgenic lines with *hairy*-Gal4 as shown in *C*, *F*, and *I*. Embryos were stained with anti-Numb using standard methods (53) and with Propidiumiodide to visualize DNA. Ectopic Numb was distinguished from the endogenous one by the significantly higher expression levels and by staining with anti-myc (data not shown). In all three cases the wild-type or mutant Numb is expressed and asymmetrically localized.

fate of the daughter cells, we analyzed the effect of Numb on Notch signaling in a cell culture system. This system uses Su(H) nuclear translocation as a reporter for the activation of Notch by Delta in *Drosophila* Schneider S2 cells (33). When S2 cells are transfected with the transcription factor Su(H), the protein localizes to the nucleus. By contrast, in S2 cells cotransfected with Notch and Su(H), Su(H) is present in the cytoplasm. Upon binding of the ligand Delta to the Notch-expressing cells, Su(H) translocates to the nucleus. We studied the consequences of the presence of Numb in the Notch-expressing cell and determined that Numb prevents the translocation of Su(H) into the nucleus upon binding of Delta to Notch. Our results suggest that Numb interferes with signaling of the ligand-bound Notch receptor but does not have any direct effect on Su(H) localization. Overexpression of Numb during wing margin and SOP development caused inhibition of Notch activity *in vivo*. We further performed a deletion analysis of Numb and found that both the PTB domain and the C terminus of Numb are required for its ability to inhibit Notch signaling.

The Interplay Between Numb and Notch. *A priori*, Numb could interfere with the Notch signaling pathway by affecting the activation of the receptor, by inhibiting the receptor directly or by inhibiting a downstream target of Notch signaling. Several observations indicate that Numb inhibits Notch function by direct protein–protein interactions. First, we showed that localization of an immediate downstream target,

Su(H), is not influenced by Numb in the absence of Notch, suggesting that the inhibition of the Notch signaling pathway is accomplished via the Notch receptor. Second, if Numb acted by preventing the activation of Notch, it should have no effect on a constitutively active receptor. The intracellular domain of Notch (N^{intra}) is constitutively active (41) and has two nuclear localization signals which may cause N^{intra} to localize into the nucleus both *in vivo* (41, 47) and in cell culture (ref. 48 and Fig. 3*A*). We found that Numb can still counteract the effect of N^{intra} and prevent Su(H) from localizing to the nucleus. Third, a direct inhibition of Notch by Numb is strongly supported by the fact that Numb can physically interact with Notch (10, 16) and by our observation that N^{intra} is no longer localized to the nucleus in Numb-expressing S2 cells.

Su(H) can bind to a region of the intracellular domain of Notch close to the transmembrane domain (33, 49), and indeed both N^{intra} and Su(H) colocalize to the nucleus (ref. 47 and Fig. 3*A*). Numb also physically interacts with this region of Notch (16). However, the interaction between Su(H) and Notch does not seem to be affected by Numb, since Su(H) colocalizes with N^{intra} at the cell membrane of Numb-expressing cells (Fig. 3 *B–D*). Thus Numb does not simply work by displacing Su(H) from Notch.

The assay for Notch activity in the S2 cells is nuclear translocation of Su(H), a phenomenon evident in cultured cells but not *in vivo* (47). This raises the possibility that quantitative or qualitative differences exist between Notch signaling in cultured Schneider cells and *in vivo*. To test whether Numb also inhibits Notch activity *in vivo*, we investigated the action of Numb in wing development, where Notch, but not Numb, is normally required, and in the SOP lineage where both proteins are necessary. Overexpression of Numb causes loss of wing margin tissue very similar to that caused by reduction of Notch activity, albeit limited to the region where Numb is misexpressed. This effect is highly sensitive to *N* dosage, as expected from a direct action of Numb on Notch. Similarly, if determination of es organ cell fates depends on Notch inhibition by Numb, overexpression of Notch in the SOP lineage should nullify the effects of Numb, whereas overexpression of Numb should inhibit all available Notch, thus leading to a *N* LOF phenotype. We found the expected phenotype in both cases, suggesting that Numb functions during SOP development by inhibiting Notch activity.

It should be noted that during embryogenesis Numb expression is not restricted to those cells that require Numb for cell fate specification, and yet there does not appear to be strong effects of this Numb expression on Notch signaling (7, 8). The most likely explanation is that the levels of Numb expression are not high enough to block Notch function. Consistent with this possibility, the effects of ectopically expressed Numb on Notch function during wing margin formation depend on the relative levels of Numb and Notch expression. In the developing wing with normal levels of Notch, conditions that lead to severe overexpression of Numb are required to cause Notch inhibition, whereas a reduction of *N* dosage results in enhanced sensitivity to Numb (Fig. 4*D*).

Numb Function Involves the PTB Domain and C Terminus. The only distinct motif in Numb identified thus far is a PTB domain (11, 12). This domain is important for the interaction of the Shc protein with an activated receptor and the consequent phosphorylation of Shc (13). We have shown that the PTB domain of Numb is vital for its function both in the cell culture system and *in vivo*. Deletions of the entire PTB domain or of the C-terminal portion rendered the mutant Numb incapable of inhibiting Notch signaling in cell culture. Overexpression of these mutant Numb proteins with deletions in the PTB domain was also ineffective in producing bristle phenotypes even though the mutant proteins are still localized asymmetrically *in vivo* (Fig. 6*F* and *I*). These observations indicate that the PTB domain is not required for asymmetric localization, but is essential for Numb function. The region of Numb that contains the PTB domain is highly conserved between *Drosophila* and mammalian Numb, which when transformed into mutant flies can functionally substitute for Numb in specifying cell fates of es organ lineage (10). Furthermore, it was shown that the PTB domain of fly or mouse Numb physically interacts with the Notch receptor (10, 16). Our results indicate that the PTB domain is important for Numb function. Taken together, these findings raise the possibility that Numb functions by interacting directly with Notch via the PTB domain. In addition to the PTB domain, the C terminus of Numb is also required for inhibition of Notch signaling (see Fig. 2, deletion Δ5). This could arise from a facilitory effect of the C terminus on binding of Numb to Notch. Alternatively, it is conceivable that upon binding of Notch to Numb via the PTB domain, the C terminus of Numb may function by interfering with Notch signaling processes.

A Model for SOP Differentiation. Our results suggest that Numb can act by inhibiting the Notch signaling pathway. Based on this observation and the phenotype of *numb* and *N* during SOP differentiation, we propose a model for cell fate specification in the SOP lineage (Fig. 7). After cell division, the intrisic factor Numb segregates into one of the two daughter cells. In the daughter cell which does not inherit Numb (left cell in Fig. 7), Delta to Notch signaling takes place normally, and signal transduction causes this cell to acquire the IIa cell fate. The other cell (right cell in Fig. 7), contains Numb which inhibits Notch and prevents signal transduction. Without Notch signaling, this cell eventually assumes the IIb cell fate.

That the IIa cell fate depends on cell extrinsic Delta–Notch signaling is supported by the LOF phenotypes of *N* and *Dl*. Thus overexpression of constitutively activated Notch causes both cells to acquire the IIa cell fate. By contrast, loss of *N* function results in two IIb cells which eventually form four neurons (14), suggesting that in the absence of Notch signaling the IIb cell fate is adopted. However, the IIb cell fate requires the intrisic factor Numb, since loss of *numb* function results in two IIa cells and Numb overexpression causes both cells to acquire the IIb cell fate (8).

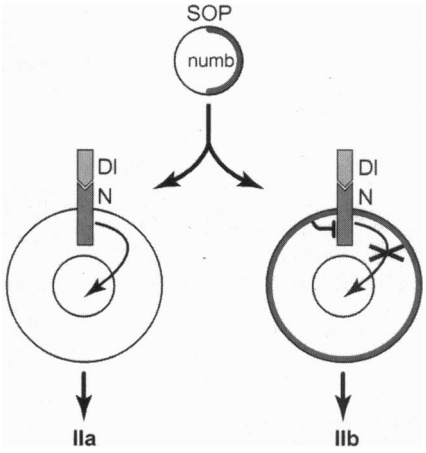

FIG. 7. Model for es organ differentiation. Each cell is symbolized as a circle and the nucleus as a smaller circle inside. The membrane-associated Numb (numb) protein shown in dark gray is asymmetrically localized to one pole of the SOP cell and segregated into the right daughter cell. Notch (N) and Delta (Dl) proteins are depicted as bars, and the signal transduction of Notch into the nucleus is depicted as an arrow pointing to the nucleus. Delta could be displayed either by the surrounding cells or the other daughter cell. In the left cell without Numb, Notch signal transduction occurs normally, and this cell therefore assumes the IIa cell fate. The Numb protein in the right cell inhibits Notch, thus prevents Notch signaling, and the cell becomes the IIb cell.

The interaction between Notch, Delta, Numb, and Su(H) constitutes part of the pathway for cell fate specification during es organ formation. In fact, a number of other genes have been already implicated for specifying the SOP cell lineage. *Hairless* acts antagonistically to *Su(H)* (34, 50), and thus could provide additional downregulation of Su(H). The *E(spl)* complex which includes several basic helix-loop-helix transcriptional regulators is necessary for epidermal cell fate (51, 52) and is directly regulated by Su(H) in response to activated Notch (35, 36). Overexpression of the *E(spl)* genes m5 and m8 leads to an es organ lineage phenotype of duplicated bristles, double sockets and other forms of aberrant outer support cells. Another gene, *tramtrack*, has a LOF phenotype opposite to that of *numb* (53) and is downstream of Delta–Notch signaling (16). Our studies indicate that Numb functions by inhibiting Notch in this pathway and thus determines the sequence of events that establish asymmetric divisions. With this knowledge it will be interesting to further examine the interaction between genes involved in the SOP lineage.

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