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Numb Inhibits Membrane Localization of Sanpodo, a Four-Pass Transmembrane Protein, to Promote Asymmetric Divisions in *Drosophila*

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Summary

Cellular diversity is a fundamental characteristic of complex organisms, and the *Drosophila* CNS has proved an informative paradigm for understanding the mechanisms that create cellular diversity. One such mechanism is the asymmetric localization of Numb to ensure that sibling cells respond differently to the extrinsic Notch signal and, thus, adopt distinct fates (A and B). Here we focus on the only genes known to function specifically to regulate *Notch*-dependent asymmetric divisions: *sanpodo* and *numb*. We demonstrate that *sanpodo*, which specifies the *Notch*-dependent fate (A), encodes a four-pass transmembrane protein that localizes to the cell membrane in the A cell and physically interacts with the Notch receptor. We also show that Numb, which inhibits Notch signaling to specify the default fate (B), physically associates with Sanpodo and inhibits Sanpodo membrane localization in the B cell. Our findings suggest a model in which Numb inhibits Notch signaling through the regulation of Sanpodo membrane localization.

Introduction

Cellular diversity is fundamental to the development of multicellular organisms. Conserved, general mechanisms for creating cellular diversity utilize extrinsic cues, the asymmetric segregation of intrinsic cell-fate determinants, or a combination of both mechanisms to create distinct cellular fates (reviewed in Horvitz and Herskowitz, 1992; Greenwald and Rubin, 1992; Knoblich, 2001). The developing *Drosophila* CNS employs all of these strategies to create the wide diversity of neurons and glia that comprise the mature CNS. This fact, combined with the genetic manipulability of *Drosophila*, has made the *Drosophila* embryonic CNS a valuable model system in which to study the genetic and molecular mechanisms that generate cellular diversity.

Drosophila embryonic CNS development initiates in the ventrolateral ectoderm, where *Notch*-mediated cell interactions regulate the selection of individual neural stem cells or neuroblasts (NBs) from neural equivalence groups (reviewed in Skeath and Thor, 2003). Each NB undergoes a series of intrinsically asymmetric divisions to regenerate itself and produce a smaller, secondary precursor cell known as a ganglion mother cell (GMC). Regenerated NBs continue to divide, with each round of division yielding a regenerated NB and a uniquely-specified GMC. Finally, via the synthesis of intrinsic and extrinsic cues, each GMC divides asymmetrically to produce sibling neurons that acquire distinct fates (e.g., Spana and Doe, 1996; Skeath and Doe, 1998; Buescher et al., 1998).

This work focuses on asymmetric GMC divisions to investigate the general mechanism of integrating intrinsic and extrinsic cues to create sibling cells of different fates (A and B). The ability of GMCs to divide asymmetrically depends on the presence of active Notch signaling in one sibling (A) and the absence of Notch signaling in the other (B). The differential activation of Notch signaling in sibling neurons requires the asymmetric localization of the intrinsic determinant Numb in GMCs, its subsequent segregation to only one daughter cell (B), and the ability of Numb to block the Notch signal (e.g., Spana et al., 1995; Spana and Doe, 1996; Skeath and Doe, 1998; Buescher et al., 1998; Schuldt and Brand, 1999; Lear et al., 1999).

Extensive genetic and molecular studies in the *Drosophila* PNS, CNS, and mesoderm have led to the following model of *Notch*/*numb*-mediated regulation of asymmetric precursor divisions (reviewed in Posakony, 1994; Jan and Jan, 1998). During precursor division Numb segregates exclusively into one daughter cell, the B cell. Following division, the Notch ligand Delta signals both progeny to adopt the A cell fate. In the A cell, Delta activation of Notch induces the cleavage of the Notch receptor and the subsequent translocation of the Notch intracellular domain to the nucleus, where it regulates transcription of specific target genes and executes the A fate (reviewed in Greenwald, 1998; Mumm and Kopan, 2000). In the B cell, Numb blocks reception and/or transduction of the Notch signal. The absence of active Notch signaling in this cell allows it to adopt the B fate. Recent work demonstrates that Numb—a phosphotyrosine binding domain protein—binds the endocytic protein α-Adaptin, leading to the model that Numb blocks Notch signaling in the B cell by mediating the endocytosis of Notch (Berdnik et al., 2002). However, a caveat of this model is that the levels and localization of Notch appear equivalent in the A and B cells (Berdnik et al., 2002). In fact, no members of the Notch pathway are known to be asymmetrically localized between the A and B cells in a *numb*-dependent manner.

Genetic screens identified *sanpodo* (*spdo*) as an essential regulator of asymmetric divisions (Salzberg et al., 1994; Skeath and Doe, 1998). These and subsequent studies demonstrate that *spdo* specifies the A, or *Notch*-dependent, fate in asymmetric divisions in the CNS, PNS, and mesoderm (Dye et al., 1998; Skeath and Doe, 1998; Park et al., 1998; Ward and Skeath, 2000). Thus, the asymmetry of precursor divisions depends upon integrating *spdo* and *Notch* pathway function with polarized Numb localization.

Spdo was identified as the homolog of the actin-associated protein Tropomodulin (Tmod; Dye et al., 1998), a protein that regulates actin filament length (reviewed in Fowler, 1996). The identity of the *Notch* and *spdo* phenotypes suggests that *spdo* mediates asymmetric divisions as a member of the Notch pathway. However,

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neither the genetic nor molecular placement of *spdo* function within the Notch pathway has been investigated. As observed for other Notch pathway members, *spdo* functions genetically downstream of *numb* (Dye et al., 1998; Skeath and Doe, 1998). However, the mechanism of *numb* regulation of *spdo* remains unknown.

spdo and *numb* appear to regulate Notch signaling specifically during asymmetric divisions, as neither is known to control Notch pathway activity in any other developmental context (Rhyu et al., 1994; Skeath and Doe, 1998; Lear et al., 1999). In fact, *spdo* and *numb* are the only genes known to function exclusively in the context of *Notch*-dependent asymmetric divisions. Given this, we investigated how Spdo and Numb regulate one another and the Notch pathway to promote asymmetric divisions in the *Drosophila* CNS. We find that *spdo* does not encode *tmod*, but rather a four-pass transmembrane protein that acts upstream of *Notch* and downstream of *Delta* to specify the A cell fate. Spdo colocalizes and physically associates with the Notch receptor in vivo. Spdo also exhibits differential subcellular localization between A and B cells during asymmetric divisions, localizing primarily to the cell membrane of the A cell and to the cytoplasm of the B cell. We demonstrate that Numb inhibits the cell membrane localization of Spdo in the B cell and that Numb and Spdo physically associate in vivo. These findings support a model in which Numb acts in the B cell to block Notch activity by preventing Spdo from localizing to the cell membrane, likely through its link to the endocytic machinery. In the A cell, the absence of Numb allows Spdo to localize to the cell membrane, where it promotes Notch signaling and the A cell fate, likely through a direct association with Notch.

Results

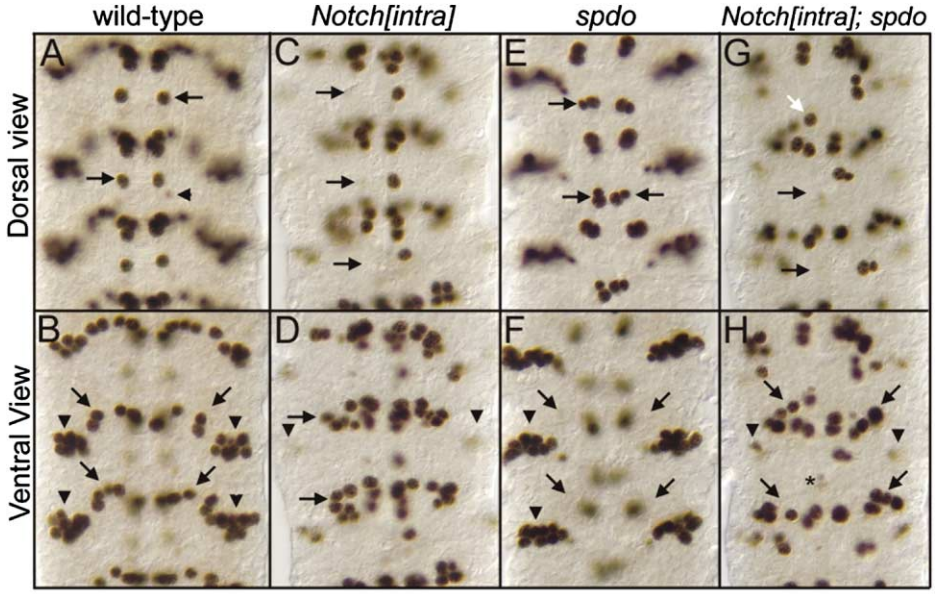
spdo Functions Upstream of *Notch* and Downstream of *Delta*

Prior studies suggest that *spdo* acts in the Notch pathway to mediate asymmetric divisions (Dye et al., 1998; Skeath and Doe, 1998). However, as these studies did not order *spdo* function relative to members of the Notch pathway, the placement of *spdo* within the pathway remains uncertain. To order the action of *spdo* relative to the intramembranous S3 cleavage event that releases the Notch intracellular domain (NICD) from the membrane, we used two distinct constitutively active forms of *Notch, Notch*^{Intra} and *Notch*^{ECN} (Struhl et al., 1993; Schroeter et al., 1998). While both *Notch* constructs function in a ligand-independent manner, *Notch*^{ECN} contains the NICD and the Notch transmembrane domain and requires proper execution of the S3 cleavage to activate transcription of *Notch* target genes. *Notch*^{Intra}, which comprises only the NICD, functions independently of the S3 cleavage.

In these experiments we focus on the development of eight pairs of sibling neurons that arise from *spdo/Notch/numb*-dependent asymmetric divisions: RP2/RP2sib, dMP2/vMP2, aCC/pCC, and five pairs of U/Usib neurons. Molecular markers can distinguish unambiguously the fate of each of these sibling neurons from their sisters (Broadus et al., 1995; Skeath and Doe, 1998). RP2/RP2sib develop from the Even-skipped (Eve)-expressing GMC4-2a. After division RP2 retains, while RP2sib extinguishes, Eve expression. Similarly, the U/Usib neurons develop from five Eve-positive GMCs; each GMC divides to produce two initially Eve-positive neurons. The five U neurons retain Eve expression, while the five Usib neurons extinguish Eve. The dMP2/vMP2 interneurons develop from the Odd-skipped (Odd)-positive MP2 precursor. After MP2 division, dMP2 retains Odd expression and extends an axon posteriorly, while vMP2 extinguishes Odd and extends an axon anteriorly. aCC/pCC develop from the Eve-positive GMC1-1a. Both aCC and pCC retain Eve expression; however, aCC expresses 22C10 and extends a motor axon out the intersegmental nerve, while pCC is an interneuron that extends a 22C10-negative axon anteriorly. The RP2sib, pCC, vMP2, and U neurons (A fates) require *spdo/Notch* function for their specification, while their siblings (B fates) require *numb*-mediated inhibition of *spdo/Notch* activity for their development.

We expressed the two constitutively active *Notch* constructs throughout the CNS of wild-type and *spdo* mutant embryos using the Gal4/UAS system and followed the development of the RP2/RP2sib, dMP2/vMP2, and U/Usib neurons. We reasoned that, if *spdo* acts upstream of *Notch*, we should observe the *Notch* gain-of-function phenotype (A/A). Conversely, if *spdo* acts downstream of *Notch*, we should see the *spdo* phenotype (B/B). The placement of *spdo* function upstream of *Notch*^{Intra}, but downstream of *Notch*^{ECN}, would indicate a requirement for *spdo* in the S3 cleavage of the Notch receptor. In a wild-type background, we find that misexpression of either *Notch* construct is sufficient to induce cells that would normally acquire the *numb*-dependent B fate to adopt the A fate at a moderate to high frequency depending upon the sibling pair examined (Figures 1A–1D and1I). We find that misexpression of each *Notch* construct in *spdo* embryos yields identical cell fate transformations at frequencies essentially equal to those observed in wild-type embryos misexpressing each construct (Figures 1E–1H and I). These results indicate that *spdo* functions genetically upstream of the S3 cleavage of Notch during asymmetric divisions.

We next assayed the placement of *spdo* function relative to *Delta*. To do this, we misexpressed *Delta* throughout the CNS of *spdo* embryos and assayed U/Usib and RP2/RP2sib neuron development (we confirmed misexpression of Delta by anti-Delta antibody staining [data not shown]). We reasoned that, if *spdo* acts downstream of, or in parallel to, *Delta*, then misexpression of *Delta* would not rescue the *spdo* phenotype. However, if *spdo* acts upstream of *Delta*, we would observe rescue of the *spdo* CNS phenotype. We find that misexpressing *Delta* does not rescue the *spdo* phenotype (data not shown), indicating that *spdo* acts genetically downstream of, or in parallel to, *Delta* to promote asymmetric divisions. Together with our placement of *spdo* function upstream of the S3 cleavage of Notch, this result suggests that *spdo* functions at or near the membrane to promote Notch signaling during asymmetric divisions.



(I)

	Notch-intra		*Notch-ECN*	
	wild-type[a]	*spdo*	*wild-type*[a]	*spdo*
RP2/RP2Sib[b]	29.6% (318)	26.2% (744)	10.1% (316)	10.0% (936)
dMP2/vMP2[b]	99.2% (252)	99.0% (388)	93.9% (132)	93.0% (400)
U/Usib[c]	91.6 % (256)	98.5% (399)	97.2% (288)	98.1% (486)

[a] We use $spdo^{G104}$/+ as wild-type
[b] Percentages refer to percent transformation of RP2 to RP2Sib fate or dMP2 to vMP2 fate
[c] Percentages refer to percent of hemisegments exhibiting an increase in U neurons

Figure 1. *spdo* Acts Upstream of the Intramembranous Cleavage of Notch to Regulate Asymmetric Divisions

Dorsal and ventral views of wild-type (A and B) and *spdo* (E and F) nerve cords and, otherwise, wild-type (C and D) and *spdo* (G and H) nerve cords in which $Notch^{intra}$ was expressed throughout the CNS stained for Eve. Genotypes of embryos shown in (C) and (D) and in (G) and (H) are *UAS-*N^{intra} /+;*pros-Gal4/*$spdo^{G104}$ and *UAS-*N^{intra} /+; *pros-Gal4,* $spdo^{G104}$/$spdo^{G104}$, respectively.

(A and B) Each wild-type hemisegment contains one RP2 ([A], arrows), a cluster of five to six U neurons ([B], arrows), and a large cluster of Eve lateral (EL) neurons. The arrowhead in (A) points to an RP2sib that still has residual Eve expression.

(C and D) Expression of $Notch^{intra}$ throughout the CNS results in a loss of RP2 neurons ([C], arrows), an increase in U neurons ([D], arrows), and a decrease in EL neurons ([D], arrowheads).

(E and F) Each hemisegment in a *spdo* embryo contains two RP2s ([E], arrows), no U neurons ([F], arrows), and normal numbers of ELs ([F], arrowheads).

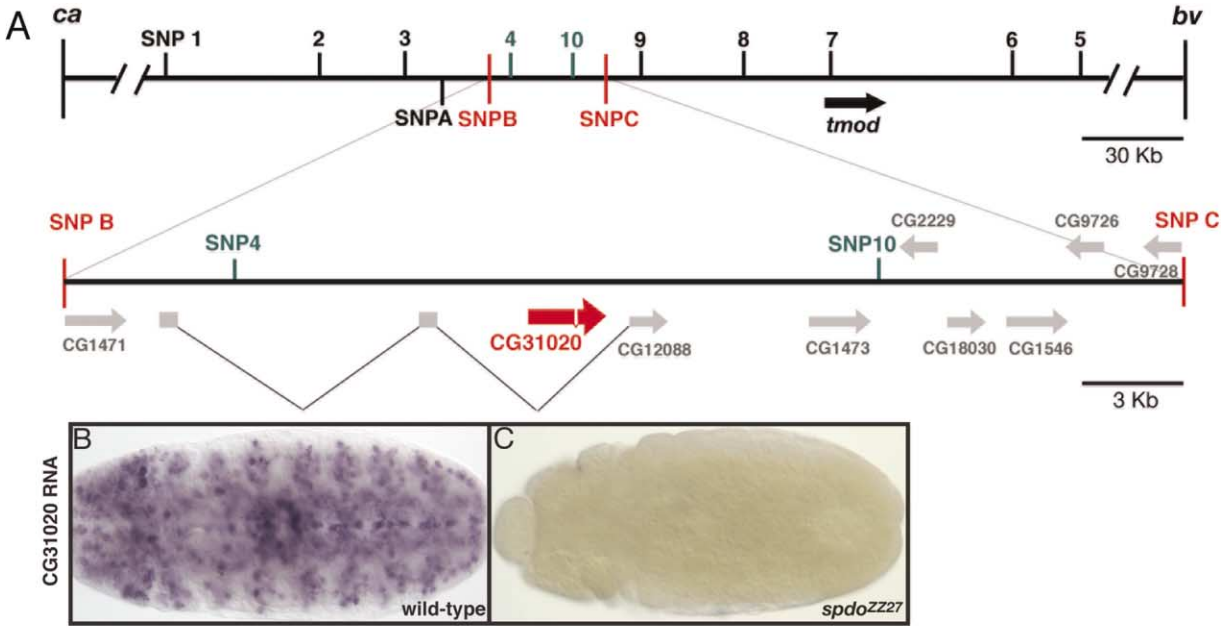
(G and H) $Notch^{intra}$ expression throughout the CNS of *spdo* embryos results in a loss of RP2 neurons, an increase in U neurons ([H], arrows), and a decrease in EL neurons ([H], arrowheads). In (G), black arrows mark hemisegments with no RP2s; white arrow marks hemisegment with one RP2. The asterisk in H marks two RP2sibs with residual Eve expression. In all panels, anterior is up.

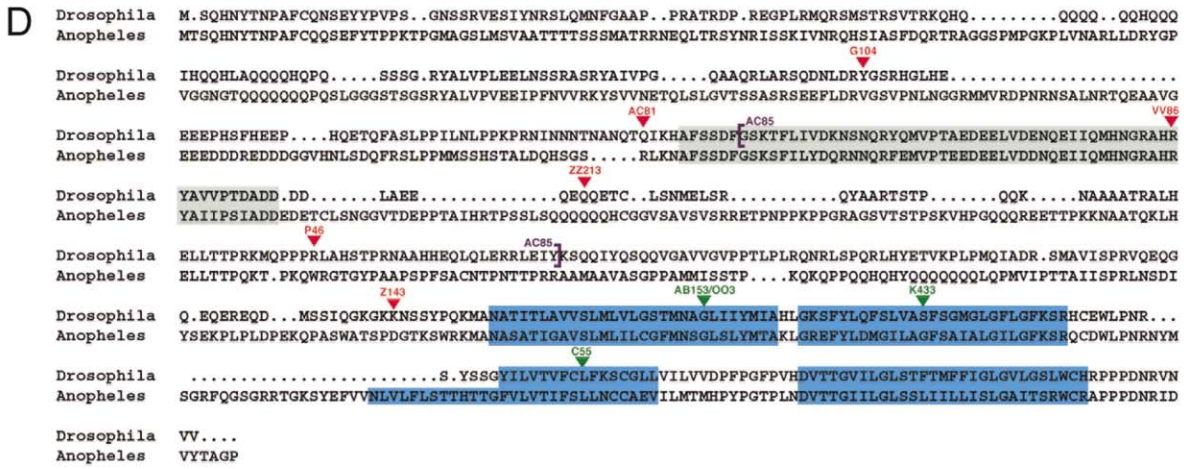
(I) Table showing the transformation percentage of B daughter cells into A daughter cells upon generalized expression of $Notch^{intra}$ or $Notch^{ECN}$ in the CNS of wild-type and *spdo* mutant embryos for the indicated sibling neurons.

Molecular Identification of *spdo*

Spdo was identified as the homolog of the actin-associated protein Tmod (Dye et al., 1998), a protein that regulates actin filament length (reviewed in Fowler, 1996). As no previous role for *tmod* in regulating cell fate had been identified, we wanted to determine whether *spdo* function during asymmetric divisions was dependent upon, or separable from, its role in actin regulation. Since chemically induced mutations often cluster in functionally critical protein domains, we sought to address this question by identifying the molecular lesions in our EMS-induced *spdo* alleles. However, despite sequencing the entire coding region, including three alternative 5′ exons, the 5′ and 3′ UTRs, and all splice sites in five alleles, as well as the majority of these sequences in four additional alleles, we failed to identify molecular lesions in *tmod*. Since the vast majority of EMS-induced mutations associated with observable phenotypes are found in the coding region of the affected gene, these data suggested that *spdo* encodes a gene other than *tmod*.

To identify *spdo*, we used genetic mapping with single nucleotide polymorphisms (SNPs) to localize the molecular lesions responsible for the *spdo* phenotype to a narrow molecular region (see Supplemental Data at http://www.developmentalcell.com/cgi/content/full/5/2/231/DC1; Jakubowski and Kornfeld, 1999). Using this approach, we localized the molecular lesion in $spdo^{AC85}$ to an 85 kb region and the lesion in $spdo^{YY233}$ to an





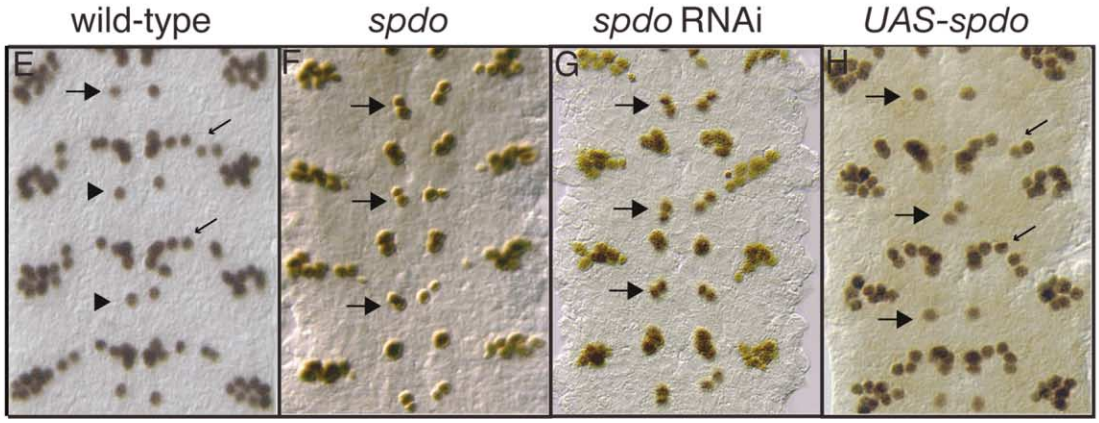


Figure 2. Molecular Identification of *spdo*

(A) Schematic of genomic region between *claret* (*ca*) and *brevis* (*bv*) with relevant SNPs indicated. SNPs B and C define the *spdo* genomic region, which contains nine genes.

(B and C) The CG31020 transcript is detected in the CNS, PNS, and mesoderm of wild-type, but not homozygous $spdo^{ZZ27}$ embryos.

(D) Amino acid alignment and predicted topology of *Drosophila* and *Anopheles* Spdo. Red triangles, nonsense mutations; green triangles, missense mutations; purple brackets, internal deletion; blue boxes, predicted transmembrane domains; gray shading, 60-amino acid conserved region.

overlapping 80 kb region. As the two alleles likely map close to one another, we focused our efforts on the 30 kb region of overlap (Figure 2A).

Sequence analysis of the *spdo* interval identified nine genes (Figure 2A; Adams et al., 2000). RNA whole-mount in situ hybridization of the nine genes revealed one gene, CG31020, specifically expressed in the CNS, PNS, and mesoderm during the stages when *spdo*-dependent cell fate decisions occur in these tissues (Figure 2B). Furthermore, $spdo^{ZZ27}$ embryos are transcript null for CG31020 (Figure 2C). To determine whether CG31020 encodes *spdo*, we sequenced its open reading frame (ORF) in our nine remaining *spdo* alleles as well as three independently generated *spdo* alleles (Salzberg et al., 1994; Hummel et al., 1999) and identified molecular lesions in all twelve alleles (Figure 2D). Ten alleles contain point mutations. The single base pair changes in six of these alleles—$spdo^{AC81}$, $spdo^{G104}$, $spdo^{VV86}$, $spdo^{Z143}$, $spdo^{ZZ213}$ and $spdo^{P46}$—result in the introduction of premature stop codons (Figure 2D, red triangles). Two alleles, $spdo^{OO3}$ and $spdo^{AB153}$, contain the identical missense mutation that converts an evolutionarily conserved glycine to an arginine, while $spdo^{C55}$ and $spdo^{K433}$ contain missense mutations that convert a conserved leucine to an arginine and a serine to a phenylalanine, respectively (Figure 2D, green triangles). Our two remaining alleles contain deletions: $spdo^{AC85}$ contains a 405 bp in-frame internal deletion (Figure 2D, purple brackets), and $spdo^{YY233}$ contains a larger deletion that extends beyond the 3′ terminus of the transcript. $spdo^{ZZ27}$ contains a large multigenic deletion that removes both CG31020 and *tmod*.

To confirm that CG31020 encodes *spdo*, we conducted RNA interference (RNAi) and gene rescue experiments. We find that injection of double-stranded CG31020 RNA into wild-type embryos yields a CNS phenotype essentially identical to that of *spdo* (Figures 2E–2G). In reciprocal experiments using the Gal4/UAS system to express *CG31020* throughout the CNS of otherwise *spdo* mutant embryos, we observe complete to near-complete rescue of the *spdo* CNS phenotype (Figure 2H). Our identification of molecular lesions in all *spdo* alleles analyzed together with the RNAi and gene rescue experiments demonstrates that CG31020 identifies *spdo*.

Spdo Encodes a Four-Pass Transmembrane Protein

Conceptual translation of CG31020 indicates that *spdo* encodes a 565-amino acid protein with four predicted transmembrane domains at its extreme C terminus (Figure 2D, blue). Protein topology prediction algorithms indicate that Spdo is likely a type IIIa transmembrane protein, with a 431-amino acid N-terminal cytoplasmic domain (Krogh et al., 2001). Consistent with this, we find that Spdo protein accumulates abnormally in the cytoplasm and exhibits minimal membrane targeting in embryos homozygous for *spdo* alleles containing nonsense mutations prior to the predicted transmembrane domains (Figure 3C). Except for the transmembrane domains and a glutamine-rich N-terminal domain (amino acids 71–94), Spdo contains no characterized protein motifs.

We identified Spdo orthologs in *Drosophila pseudoobscura* and *Anopheles gambiae* via comparative sequence analysis. The two *Drosophila* proteins share 80% identity, while *D. melanogaster* and *Anopheles* Spdo are 32% identical and 46% similar at the amino acid level (Figure 2D). Most of the conservation resides in the transmembrane and intervening loop domains, as well as in a 60-amino acid N-terminal region that maintains 75% identity and 93% similarity (Figure 2D, gray).

Spdo Localizes Uniformly around the Cell Membrane and to Cytoplasmic Puncta in Asymmetrically Dividing Cells

To follow the expression and subcellular localization of Spdo, we generated antibodies specific to two overlapping regions of the predicted cytoplasmic domain of Spdo (see Experimental Procedures). Using either antibody, we find that Spdo is expressed in all NBs, all GMCs, and transiently in most, if not all, neurons in the CNS (Figures 3D–3G). In the PNS, Spdo is expressed in all SOPs and their progeny (Figure 3J). In the mesoderm, Spdo is expressed in heart and somatic muscle precursors that undergo *spdo*-dependent asymmetric divisions (Figures 3H and 3I). Spdo is also expressed in the asymmetrically dividing cells of the posterior midgut (data not shown). Thus, all embryonic cells known to undergo asymmetric divisions, even those thought to divide asymmetrically in a *spdo*-independent manner, appear to express Spdo. Consistent with Spdo playing a role to regulate asymmetric NB divisions, we observe a weak, but consistent, duplication of GMC1-1a in *spdo* mutant embryos (see Supplemental Data).

We observe several notable attributes with respect to the subcellular localization of Spdo. First, Spdo localizes to the cell membrane as well as to small, intermediate, and large puncta that appear to reside interior to the cell membrane. The relative location of these puncta is consistent with their being cytoplasmic vesicles (Figures 3A, 3B, and 3D–3J). For simplicity, from here on we refer to these as cytoplasmic puncta or accumulations of Spdo. Second, cells that localize Spdo primarily to the cell membrane generally exhibit weak cytoplasmic accumulation of Spdo, while cells that localize Spdo primarily to the cytoplasm generally exhibit weak accumulation of Spdo at the membrane (see Figure 6A). Third,

(E–H) Stage 15 nerve cords of indicated genotype stained for Eve.
(E) Wild-type hemisegments contain one RP2 neuron (large arrows) and five to six U neurons (arrows).
(F) *spdo* mutant hemisegments contain two RP2s (large arrows) and no U neurons.
(G) CG31020 RNAi-treated wild-type embryo exhibits an Eve CNS phenotype identical to that of *spdo* (compare to [F]).
(H) *spdo* embryo in which CG31020 was expressed in the CNS exhibits a wild-type Eve CNS pattern (compare to [E]); large arrows mark RP2, and small arrows mark the U neurons. In (H), the genotype of the embryo is *sca-Gal4/UAS-spdo;* $spdo^{G104}$. Anterior is left in (B) and (C) and up in (E)–(H).

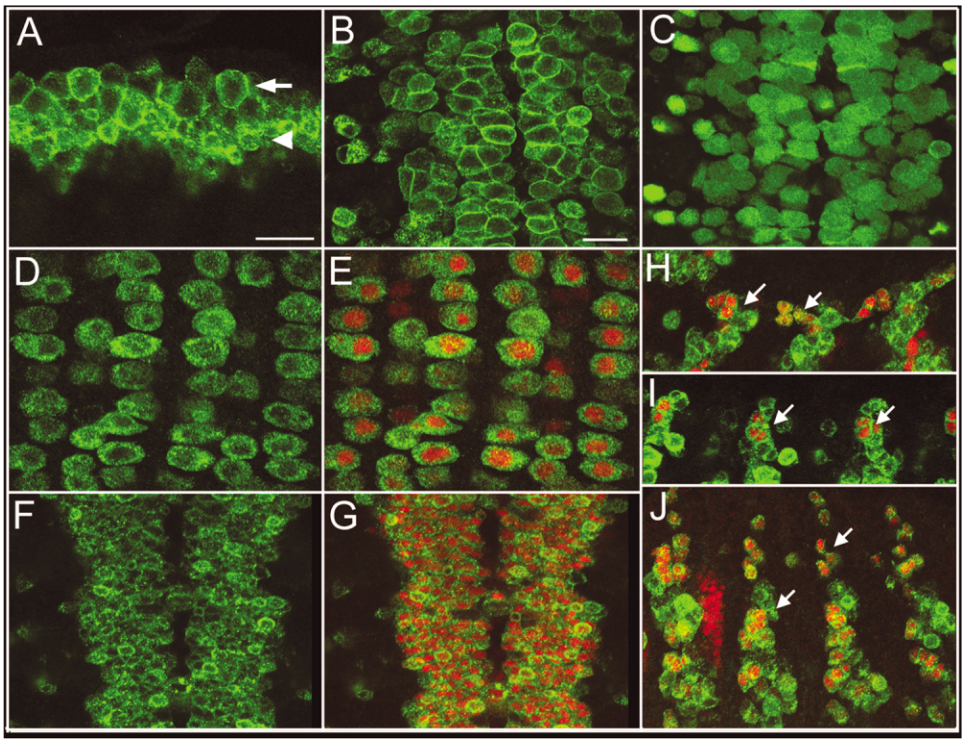


Figure 3. Spdo Appears to Be Expressed in All Embryonic Cells Known to Undergo Asymmetric Divisions

Stage 11 (A–C and F–I), stage 10 (D and E), and stage 13 (J) wild-type (A, B, and D–J) and *spdo*^{Z143} (C) embryos labeled for Spdo (green), Hunchback ([E], red), Prospero ([G], red), Svp-lacZ ([H], red), Eve ([I], red), and Cut ([J], red).

(A) Spdo protein localizes to the apical and basal side of the cell membrane of NBs and GMCs (the arrow marks the NB layer, and the arrowhead marks GMC layer).

(B) Spdo protein localizes uniformly around the medial and lateral extent of NBs and to small- and intermediate-sized cytoplasmic puncta in most expressing cells (A, B, and D–J).

(C) Spdo exhibits largely cytoplasmic localization in *spdo*^{Z143} embryos.

(D–G) All NBs ([E], red) and all GMCs ([G], red) express Spdo.

(H–J) Spdo is also expressed in Svp-lacZ ([H], red) and Eve ([I], red)-positive mesodermal cells and in all Cut-positive PNS cells ([J], red). Anterior is up in (B)–(G) and left in (A) and (H)–(J); apical is up in (A); scale bar, 20 μm.

Spdo localizes uniformly around the cell membrane of cells that localize Spdo predominantly to the cell membrane (Figures 3A and 3B). The apparent dynamic subcellular localization of Spdo raises the possibility that modulation of Spdo localization may regulate the ability of Spdo to promote Notch signaling during asymmetric divisions.

Spdo Colocalizes with Notch and Delta

To examine the potential relevance of the subcellular localization of Spdo, we performed colocalization studies between Spdo and Notch, Delta, and Numb. We find that Spdo exhibits extensive colocalization with Notch at the cell membrane and in small and large puncta throughout the cytoplasm (Figures 4A–4F). We detect strong Spdo and Notch colocalization in large cytoplasmic puncta in NBs (Figures 4A–4C) as well as in smaller puncta near and at the cell membrane of GMCs (Figures 4D–4F). Although we observe that a significant majority of Notch-expressing puncta in the CNS colocalize with Spdo, this is not an obligate relationship, as some Notch puncta do not colocalize with Spdo, and many Spdo puncta do not colocalize with Notch. However, the significant overlap between Spdo and Notch suggests that Spdo promotes Notch signaling during asymmetric divisions through a close association with Notch.

The relative localization of Spdo and Delta is more complex than that observed for Spdo and Notch. In general, we observe that Spdo and Delta are expressed in largely complementary patterns in and around the CNS (data not shown). This is in agreement with a prior report demonstrating that Delta is expressed at high levels in the mesoderm and at lower levels in NBs and the neurectoderm, but not in GMCs or neurons (Spana and Doe, 1996). However, in regions of close contact between GMCs, neurons, and neighboring Delta-expressing cells, we observe tight juxtaposition of Spdo-expressing and Delta-expressing puncta at or near cell membranes (Figures 4G–4I). In most instances, Spdo- and Delta-expressing puncta reside immediately adjacent to one another and exhibit partial overlap (Figures 4G–4I). As with Notch, the apposition of Spdo and Delta is not obligate. Most Delta-expressing puncta in these regions are associated with Spdo expression; however, many

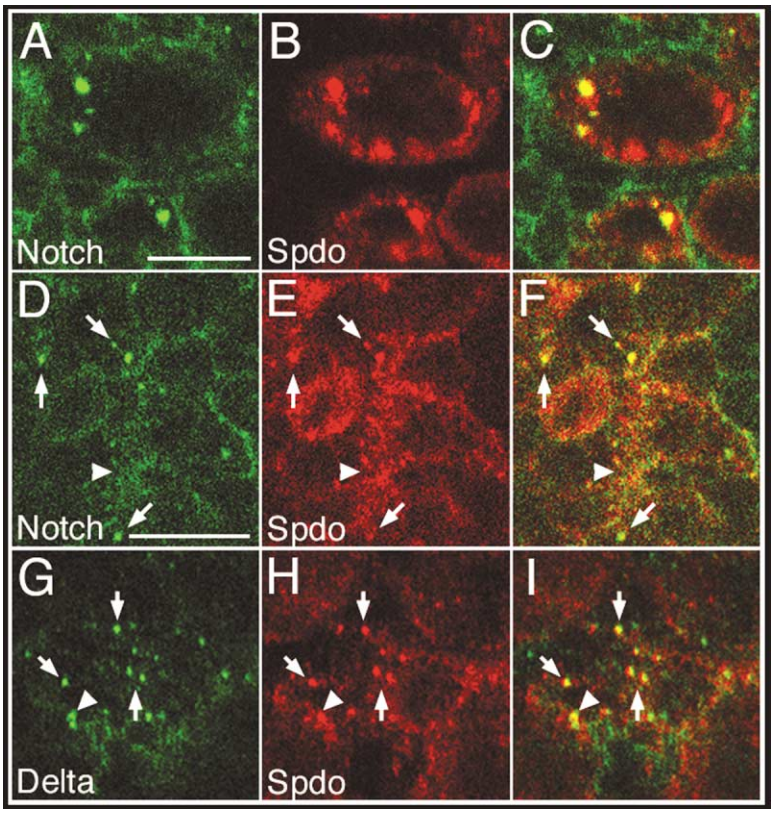


Figure 4. Spdo Colocalizes with Notch and Delta

High-magnification views of stage 9 (A–C) and stage 11 (D–I) embryos labeled for Spdo (red) and Notch ([A–F], green) or Delta ([G–I], green).

(A–C) In early NBs Notch (A and C) and Spdo (B and C) colocalize in large puncta near the cell membrane.

(D–F) In GMCs, Notch (D and F) and Spdo (E and F) colocalize in smaller puncta near the cell membrane (arrows) and more diffusely throughout the cytoplasm (arrowheads).

(G–I) In the GMC layer Delta-positive puncta (G and I) at the membrane reside in tight apposition to (arrows), or colocalize with (arrowhead), Spdo. Scale bars, 10 um; scale bar in (D) applies for (D)–(I). Anterior is up in all panels.

are not, and most Spdo-positive puncta are not associated with Delta expression. However, the significant colocalization of Spdo with Notch and the frequent juxtaposition of Spdo- and Delta-expressing puncta at or near the cell membrane suggest that Spdo functions in close association with Notch and its ligand Delta to promote productive signaling during asymmetric divisions. Interestingly, we do not observe any gross changes in the expression or localization of Notch or Delta in *spdo* mutant embryos (data not shown).

Spdo Physically Associates with Notch In Vivo

Our genetic, molecular, and expression data suggest that Spdo promotes productive Notch signaling through a close association with Notch. To determine whether Spdo physically associates with the Notch receptor, we immunoprecipitated Notch and assayed for the coprecipitation of Spdo. We find that Spdo coprecipitates at roughly equivalent efficiencies with antibodies specific to either the intracellular or extracellular domain of Notch (Figure 5A), suggesting that Spdo associates with the full-length Notch receptor. As a control, we find that the EGF receptor (EGFR) does not coprecipitate with Notch (Figure 5A), even though Notch and EGFR are coexpressed at the membrane of the same cells at a significantly greater frequency than Notch and Spdo (data not shown). In addition, we find that Spdo does not coprecipitate with EGFR (Figure 5C) which coexpresses with Spdo in a pattern similar to Notch, though to a somewhat lesser degree (data not shown). These data indicate that Spdo associates with the Notch receptor in vivo and suggest that Spdo promotes Notch signaling during asymmetric divisions through a physical association with the Notch receptor.

Numb Inhibits Spdo Membrane Localization

We also observe significant colocalization between Spdo and Numb at the cell membrane and in the cytoplasm. However, these studies also reveal a general inverse correlation between the presence of Numb and the membrane localization of Spdo. For example, CNS, PNS, and mesodermal cells that express low levels of Numb generally localize Spdo largely to the cell membrane, whereas cells that express high levels of Numb generally localize Spdo largely to the cytoplasm (Figures 6A–6C). The correlation is not absolute; however, together with the genetic placement of *numb* as an upstream negative regulator of *spdo,* it raises the possibility that *numb* inhibits Notch signaling during asymmetric divisions by regulating the subcellular localization of Spdo.

To investigate whether *numb* regulates the subcellular distribution of Spdo, we followed Spdo localization in embryos homozygous mutant for *numb*. Because of maternal *numb* product, we focused on late stage 11 and older embryos, when we detect minimal levels of maternal Numb protein. Relative to wild-type, in *numb* embryos, we observe a significant increase in Spdo localization to the cell membrane and a corresponding decrease in Spdo-expressing cytoplasmic puncta in NBs, GMCs, neurons, and mesodermal and PNS precursors (Figures 6D and 6E; data not shown for mesoderm

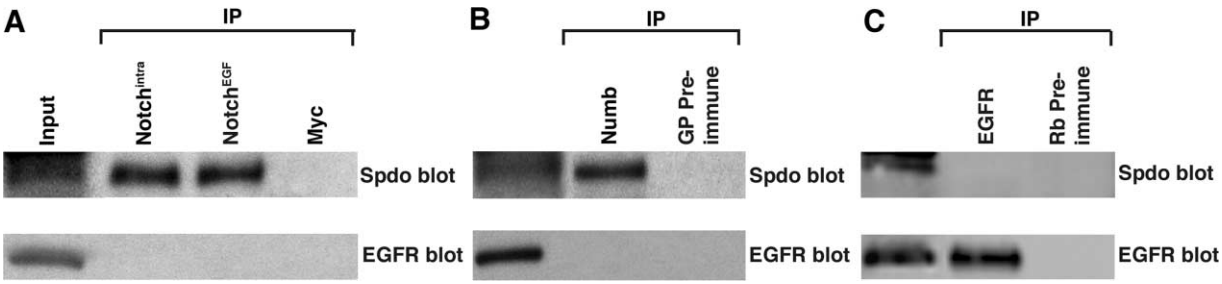


Figure 5. Spdo Physically Associates with Notch and Numb In Vivo

(A) Antibodies specific to the intracellular and extracellular domain of Notch immunoprecipitate Spdo. In control experiments mouse anti-Myc antibodies do not coprecipitate Spdo and neither Notch antibody coprecipitates EGFR.

(B) Numb-specific antisera, but not preimmune sera, coimmunoprecipitates Spdo, but not EGFR.

(C) Antibodies specific for EGFR do not immunoprecipitate Spdo. In each panel, lane 1 contains precleared embryonic lysate equal to one-tenth of the input for the immunoprecipitation assays.

and PNS). We also observe persistent expression of Spdo in *numb* embryos, as most CNS neurons in stage 13 *numb* embryos express Spdo at high levels, whereas, in stage 13 wild-type embryos, most CNS neurons express Spdo at low levels (data not shown). Thus, *numb* appears to regulate the cell membrane localization and levels of Spdo in asymmetrically dividing cells.

numb Regulates the Differential Localization of Spdo between vMP2 and dMP2

Our data together with the exclusive segregation of Numb to the B cell suggest a model in which Numb blocks Notch signaling by inhibiting the cell membrane localization of Spdo in the B cell. To test this model, we followed Spdo localization in the progeny of the CNS precursor MP2, which divides asymmetrically under the control of *spdo* and *numb*. In wild-type, MP2 produces two siblings: a larger dorsal cell, dMP2, and a smaller ventral cell, vMP2 (see Figure 6F). During this division, Numb segregates exclusively into dMP2 (the B cell), where it blocks Notch signaling and promotes the dMP2 fate. Notch signaling is active in vMP2 (the A cell) and specifies the vMP2 fate (Spana and Doe, 1996). If Numb inhibits the cell membrane localization of Spdo in the B cell, we would expect to observe strong Spdo membrane localization in vMP2 and weak membrane localization in dMP2. Using Odd-skipped expression to identify newly born d/vMP2 siblings in wild-type embryos (Spana and Doe, 1996), we find that Spdo localizes to the cell membrane of vMP2, but not dMP2 (Figures 6G and 6H). Specifically, we observe that, in 81.1% of d/vMP2 sibling pairs (n = 58), Spdo localizes predominantly to the membrane and exhibits minimal cytoplasmic accumulation in vMP2 (Figure 6G), while, in dMP2, Spdo exhibits minimal or no membrane localization and significant cytoplasmic accumulation (Figure 6H). We never detect increased Spdo membrane localization in dMP2 relative to vMP2 or increased cytoplasmic accumulation in vMP2 relative to dMP2 (n = 58). These results indicate that Spdo exhibits differential subcellular localization between sibling vMP2 (A) and dMP2 (B) cells and suggest that Numb promotes this difference by preventing Spdo from localizing to the cell membrane of dMP2.

To determine whether the differential localization of Spdo between vMP2 and dMP2 depends on *numb*, we followed Spdo localization during MP2 divisions in *numb* mutant embryos (Figures 6I and 6J). In *numb* embryos, MP2 still produces a smaller ventral cell and a larger dorsal cell; however, both cells acquire the vMP2, or A cell, fate (Spana and Doe, 1996). As in wild-type, the ventral cell always exhibits significant localization of Spdo to the cell membrane and no/minimal cytoplasmic accumulation of Spdo (Figure 6I). However, in *numb* embryos we find that, 93% of the time (n = 31), the larger dorsal cell exhibits no/minimal cytoplasmic accumulation of Spdo; this cell also exhibits increased localization of Spdo to the cell membrane (Figure 6J). Thus, the differential subcellular localization of Spdo between vMP2 and dMP2 observed in wild-type embryos appears to depend on the ability of Numb to restrict Spdo from the cell membrane in the B cell. This *numb*-dependent asymmetry in the subcellular localization of Spdo, a positive mediator of Notch signaling, suggests that Numb blocks Notch signaling in the B cell through its ability to inhibit the localization of Spdo to the cell membrane.

Numb and Spdo Physically Associate In Vivo

The ability of Numb to regulate the subcellular localization of Spdo together with the known dosage-sensitive interactions between these genes (Skeath and Doe, 1998) suggests that Numb may physically associate with Spdo to regulate its subcellular localization. To address this possibility, we assayed whether Numb and Spdo associate in vivo via coimmunoprecipitation assays. We observe that antibodies directed against Numb coprecipitate Spdo, but not EGFR, from wild-type embryonic cell lysates (Figure 5B). Thus, Spdo and Numb appear to physically associate in vivo, consistent with the idea that Numb inhibits the localization of Spdo to the cell membrane and, thus, active Notch signaling in the B cell through this association.

Discussion

Asymmetric divisions are a fundamental mechanism that creates cell diversity during development. Seminal work in *Drosophila* and more recent work in mammals reveal that antagonistic interactions between *numb* and

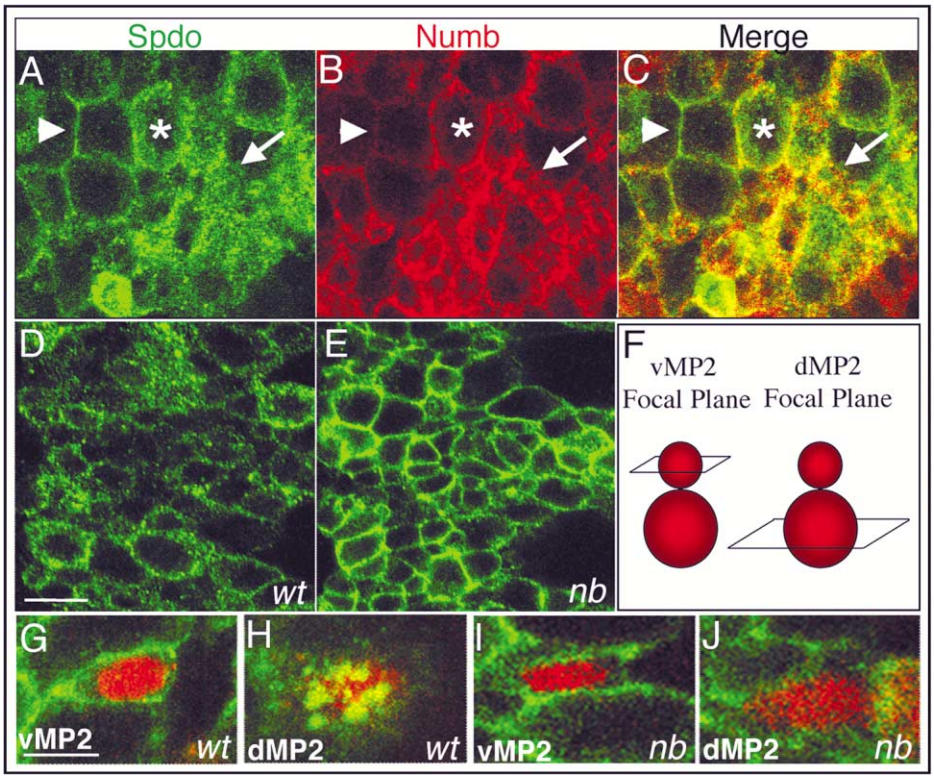


Figure 6. Numb Inhibits the Membrane Localization of Spdo

(A–C) Lateral section of the CNS of a stage 11 wild-type embryo. Cells with low levels of Numb (red) generally localize Spdo (green) to the cell membrane (arrowhead). Cells with high levels of Numb generally localize Spdo to the cytoplasm (arrow). The asterisk marks an NB with high-level Numb and largely cytoplasmic Spdo.

(D and E) Ventral sections of the CNS in late stage 12 wild-type and *numb*^{2} embryos. In wild-type, Spdo accumulates in puncta near the cell membrane and in the cytoplasm (D). In *numb*^{2} embryos, Spdo accumulates at high levels around the entire cell membrane of most cells and exhibits reduced cytoplasmic accumulation (E). (D) and (E) were obtained from identically staged wild-type and *numb* embryos from the same staining reaction with identical parameters.

(F) Schematic of d/vMP2 sibling neurons and the focal planes of the images shown in (G)–(J).

(G–J) Sections showing newly born dMP2 or vMP2 neurons in stage 11 wild-type or *numb*^{2} embryos. In wild-type, Spdo (green) localizes to the cell membrane of vMP2 ([G], red) and to cytoplasmic puncta in dMP2 ([H], red).

(I and J) In *numb* embryos, Spdo (green) localizes to the membrane of vMP2 ([I], red) and dMP2 ([J], red) and exhibits minimal cytoplasmic accumulation in either cell. The scale bars are 10 μm in (A)–(E) and 5 μm in (G) and (H). Anterior is left and apical in (A)–(C) and up in (D)–(J).

the Notch pathway play a conserved role in promoting asymmetric divisions in metazoans (reviewed in Cayouette and Raff, 2002). In *Drosophila*, *numb* and *spdo* have been found to function specifically in the regulation of *Notch*-mediated asymmetric divisions. Below we discuss how our findings on Spdo and Numb support a revised model of *Notch*-dependent asymmetric divisions and explain the restricted ability of Numb to inhibit Notch pathway activity.

A Model of Numb/Spdo Regulation of Asymmetric Precursor Divisions

A recent model for Numb-dependent inhibition of Notch activity during asymmetric divisions suggests that Numb blocks Notch signaling by targeting Notch for endocytosis in the B cell (Berdnik et al., 2002). In support of this model, Numb can physically interact with Notch and α-Adaptin, a component of the endocytic machinery, and hypomorphic mutations in α-*adaptin* yield a *numb*-like phenotype in the PNS (Guo et al., 1996; Berdnik et al., 2002). Yet caveats to the model exist. First, if Numb targets Notch for endocytosis, one would expect to observe lower levels or differential localization of Notch in the B cell relative to the A cell. However, the levels and distribution of Notch appear equivalent between these cells during asymmetric divisions (Berdnik et al., 2002). Second, as discussed below, the presence of Numb and α-Adaptin are not sufficient to inhibit Notch pathway activity in other developmental contexts.

Our results support a revised model in which Numb interferes with Spdo function to inhibit Notch activity during asymmetric divisions (Figure 7). In this model, Numb inhibits Notch activity in the B cell by blocking the ability of Spdo to localize to the cell membrane. In the A cell the absence of Numb permits Spdo to localize to the cell membrane, where it promotes Notch signaling and the A cell fate, likely through a physical association with Notch. The ability of Numb to associate with Spdo and α-Adaptin suggests that Numb removes Spdo from the cell membrane via the endocytic machinery. As active Notch signaling appears to require Spdo at the cell membrane, the internalization of Spdo in the B cell is

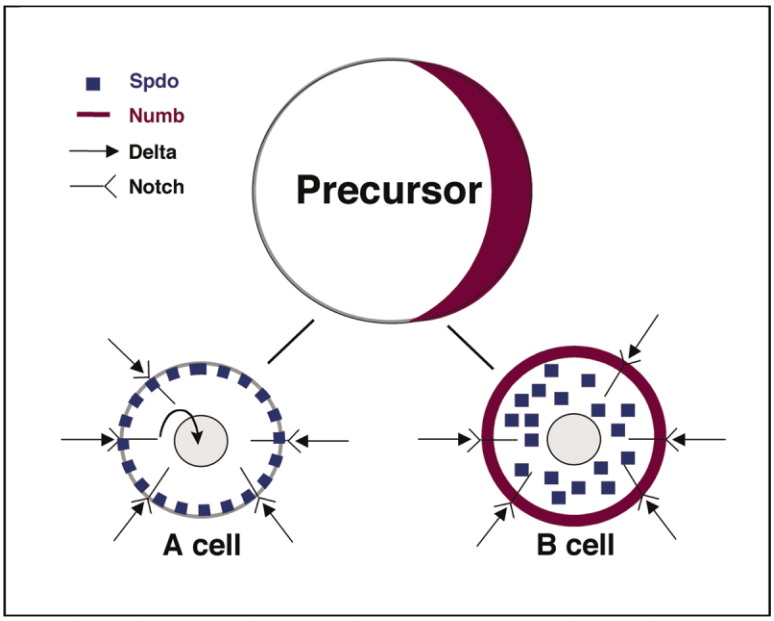


Figure 7. Model of *spdo*/*Notch*/*numb*-Dependent Asymmetric Divisions

In the A cell Spdo localizes to the cell membrane, where it promotes active Notch signaling. In the B cell, the presence of Numb inhibits the cell membrane localization of Spdo. In the absence of Spdo protein at the cell membrane, productive Notch signaling does not occur in the B cell (see text for details).

incompatible with productive Notch signaling. While our model does not preclude Notch internalization along with Spdo in the B cell, it does not rely upon differential internalization of Notch between the A and B cells—a phenomenon we have not yet seen in the embryonic CNS (unpublished data).

Spdo and Numb: Context-Specific Regulators of Notch Signaling

Our work and that of others indicate that *spdo* is generally required to promote *Notch*/*numb*-dependent asymmetric divisions. For example, *spdo* promotes the *Notch*-dependent fate in all *Notch*/*numb*-dependent CNS, heart, and mesoderm precursor divisions assayed to date (Skeath and Doe, 1998; Park et al., 1998; Ward and Skeath, 2000). *spdo* also appears to play a role in all *Notch*/*numb*-dependent asymmetric divisions in the PNS. In the canonical external sensory organ lineage, a single precursor (SOPI) and its progeny (SOPIIa, SOPIIb, and SOPIIIb) divide asymmetrically under *Notch*/*numb* control to produce the distinct cell types that make up the sensory organ (reviewed in Jan and Jan, 1994; Posakony, 1994). *spdo* has been shown to regulate the asymmetric divisions of SOPIIa and SOPIIIb (Salzberg et al., 1994; Dye et al. 1998). In addition, mitotic *spdo* clones in the eye proper and notum lack bristles (unpublished data), a phenotype indicative of *spdo* promoting the asymmetric division of SOPI. These studies indicate that *spdo* likely plays an important role in mediating all *Notch*/*numb*-dependent asymmetric divisions in *Drosophila*.

Although *spdo* and *numb* appear to regulate all *Notch*-dependent asymmetric divisions in *Drosophila*, neither has been shown to regulate Notch pathway activity in any other developmental context. The limited effect of Numb on Notch signaling cannot be explained by a restricted expression pattern, as Numb (and α-Adaptin) exhibits a relatively general expression pattern (Rhyu et al., 1994; Dornan et al., 1997; unpublished data). The apparent inability of Numb to inhibit Notch signaling in developmental contexts other than asymmetric divisions suggests that it may function through a protein or proteins specifically required for *Notch*-dependent asymmetric divisions. Critically, Notch signaling requires Spdo only during asymmetric divisions, and, in this context, Spdo appears to act at the cell membrane to promote Notch signaling. The ability of Numb to inhibit the cell membrane localization of Spdo suggests that Spdo may be the key factor that links Numb to the regulation of Notch pathway activity. If this model is correct, then Numb will only be able to inhibit Notch signaling in those developmental contexts in which Notch activity requires Spdo function—asymmetric divisions. This model then provides a rational explanation for why Numb appears to inhibit Notch signaling only during asymmetric divisions.

It remains unclear why Spdo is required for Notch signaling only during asymmetric divisions. The context-specific requirement of *spdo* suggests that *spdo* does not promote an event generally required for Notch activity—such as Notch presentation at the membrane or Notch proteolysis—but rather an event specifically required for Notch activity during asymmetric divisions. Insight into this question may come from the observation that most *spdo*-independent *Notch*-mediated decisions occur in an epithelium, while *spdo*/*Notch*-dependent asymmetric divisions occur in nonepithelial cells. Thus, it is possible that, during asymmetric divisions, Notch signaling requires accessory proteins not needed in epithelial cells to stabilize or otherwise to promote Notch-Delta interaction and/or signaling—proteins such as Spdo. The relative expression patterns of Spdo, Notch, and Delta are consistent with this, as is the observation that asymmetric divisions that produce siblings that retain a close association with the epithelium (e.g., the SOPIIa division that produces the socket and bristle) exhibit a weaker requirement for Spdo than those that produce siblings that do not retain close contact with

the epithelium (e.g., GMCs, heart precursors, and SOPI-IIb; Dye et al., 1998; Skeath and Doe, 1998; Ward and Skeath, 2000).

Evolutionary Conservation of *spdo* Function

Notch and Numb localize asymmetrically within CNS precursors in the mammalian brain, and molecular and genetic studies indicate that Notch and Numb regulate the asymmetric division of these precursors (Chenn and McConnell, 1995; Zhong et al., 2000; Shen et al., 2002). These observations together with the apparent link Spdo provides between Numb and the Notch pathway in *Drosophila* led us to speculate that mammalian orthologs of Spdo mediate Notch/Numb-dependent asymmetric divisions in mammals.

Standard computational approaches, however, fail to identify mammalian Spdo orthologs. The *Anopheles* Spdo ortholog shares 32% amino acid identity with *Drosophila* Spdo (Figure 2D). This degree of identity is significantly lower than the average identity of 56% observed between orthologous pairs of *Drosophila* and *Anopheles* proteins (Zdobnov et al., 2002), identifying *spdo* as a fast-evolving gene with limited constraints on amino acid substitutions. Thus, it will likely be difficult to identify Spdo orthologs in distantly related species through standard computational approaches. However, additional research on the Notch pathway as well as work on vertebrate and invertebrate odorant receptors suggests that alternate strategies may identify mammalian Spdo orthologs.

LAG-3, a *C. elegans* glutamine/proline-rich protein, forms a ternary complex with the Notch pathway transcription factor LAG-1 [CSL/Su(H)] and the Notch intracellular domain to activate transcription of *Notch* target genes (Petcherski and Kimble, 2000a). Database searches do not identify LAG-3 orthologs in other species. Despite this, Petcherski and Kimble (2000b) used a modified yeast two-hybrid system to search for functional LAG-3 homologs. This work identified Mastermind, a glutamine/proline-rich protein and canonical member of the Notch signaling pathway in *Drosophila*, and a murine homolog, mMam1, as functional LAG-3 homologs (Petcherski and Kimble 2000b). The identical roles LAG-3 and Mastermind play in Notch signaling together with their similar structural composition lead to the model that LAG-3 and Mastermind share a common ancestor but that this relationship is occluded by a high rate of amino acid substitution in these proteins (Petcherski and Kimble 2000b). As with LAG-3, the identification of Spdo-interacting proteins may provide a tool for identifying functional Spdo homologs, while also elucidating the molecular basis by which Spdo regulates Notch signaling.

In vertebrates, *C. elegans* and *Drosophila* odorant receptors comprise large families of seven-pass G protein-coupled receptors. However, the vertebrate, *C. elegans*, and *Drosophila* odorant receptor families are essentially unrelated to each other at the primary sequence level. Nonetheless, the initial identification of *Drosophila* odorant receptors succeeded through the use of a multivariable computer algorithm trained to identify *Drosophila* ORFs with physicochemical properties similar to G protein-coupled receptors (Clyne et al., 1999). A similar approach that focuses on structural properties likely conserved in Spdo orthologs may help identify mammalian Spdo orthologs. As Notch/Numb-mediated asymmetric divisions likely facilitate the generation of cellular diversity in organisms ranging from flies to humans, it will be important to determine whether Spdo is an obligate member of this regulatory cassette.

Experimental Procedures

Fly Stocks and Genetics

The following fly stocks were used: $spdo^{G104}$, $spdo^{Z143}$, $spdo^{AB153}$, $spdo^{AC81}$, $spdo^{AC85}$, $spdo^{OO3}$, $spdo^{VV86}$, $spdo^{YY233}$, $spdo^{ZZ27}$, $spdo^{ZZ213}$, $spdo^{C55}$ (H. Bellen), $spdo^{K433}$ (H. Bellen), $spdo^{P46}$ (C. Klambt), $numb^{2}$ (Uemura et al., 1989), and $numb^{4}$ [l(2)06740; Berkeley *Drosophila* Genome Project]. We obtained all other stocks from the Bloomington Stock Center.

We performed *spdo* rescue experiments by crossing *sca*-Gal4; $spdo^{G104}$/TM3 *ftz-lacZ* flies to UAS-*spdo*; $spdo^{G104}$/TM3 *ftz-lacZ* flies. We used the following lines to conduct the *Notch* and *Delta* epistasis experiments: *pros*-Gal4 $spdo^{AC85}$/TM3 *ftz-lacZ*, UAS-$Notch^{Intra}$; $spdo^{G104}$/TM3 *ftz-lacZ*, UAS-$Notch^{ECN}$; $spdo^{G104}$/TM3 *ftz-lacZ*, and UAS-$Delta^{H}$; $spdo^{G104}$/TM3 *ftz-lacZ*.

Molecular Cloning of *spdo*

We used meiotic and deficiency mapping to localize *spdo* between *claret* (*ca*) and *brevis* (*bv*) in the distal tip of 3R. We then employed SNPs to map *spdo* to a 30 kb interval in this region following a modified version of the method of Jakubowski and Kornfeld (1999). We used standard PCR-based sequencing methods to identify molecular lesions in CG31020 in *spdo* alleles. For more details see Supplemental Data.

RNA Interference (RNAi)

RNAi experiments were conducted essentially as described in Misquitta and Paterson (1999).

Generation of UAS-*spdo* Lines

We amplified the *spdo* ORF from CG31020 ESTs RE23355 and RE04681 (Rubin et al., 2000). We used SOE (Horton et al., 1989) to amplify and connect the N-terminal region from RE04681 with the C-terminal region from RE23355 because RE23355 contains an apparent missense mutation at amino acid 289, and RE04681 is incompletely spliced. We then cloned the full-length ORF directionally into pUAST (Brand and Perrimon, 1993) and created germline transformants by standard protocols.

Antibody Generation and Expression Analyses

Antigen production and purification, as well as antibody generation, were carried out as described in Williams et al. (1993). Spdo antibodies were raised against regions corresponding to either amino acids 11–232 or 198–431. Numb antibodies were raised against a region corresponding to amino acids 6–537. We used the following antibodies: rabbit anti-Spdo (1:1000), rat anti-Spdo (1:100), guinea pig anti-Numb (1:500), mouse anti-22C10 (1:20; Developmental Hybridoma Studies Bank [DSHB]), mouse anti-β-gal (1:1000; Promega), rabbit anti-β-gal (1:1000; ICN), mouse anti-Cut (1:100; DSHB), mouse anti-Delta (1:20; DSHB), rabbit anti-Eve (1:1500; M. Frasch), guinea pig anti-Hunchback (1:400; D. Kosman), mouse anti-Notch C458.2H (1:10; DSHB), Rabbit anti-Odd (1:500), and mouse anti-Pros (1:4; C. Doe). We used Alexa 488 and 633 and Cy3 with appropriate species specificity for immunofluorescence (Molecular Probes and Jackson ImmunoResearch).

RNA in situ hybridization was performed as described in Lehmann and Tautz (1994).

Coimmunoprecipitations and Western Analysis

Cell extracts were prepared from 0–20 hr embryos. Immunoprecipitations were conducted with guinea pig anti-Numb (see above), mouse anti-Notch C17.9C6 (specific for the intracellular domain; DSHB), mouse anti-Notch C458.2H (specific for EGF repeats 12–20 of the extracellular domain; DSHB), and rabbit anti-EGFR (N. Baker).

For Western analysis we used rabbit anti-EGFR at 1:10,000 and rabbit anti-Spdo at 1:1,000. As predicted, Spdo runs as a band of ~64 kDa; this band corresponds to Spdo, as it is absent in Western blot analysis of lysate prepared from homozygous *spdo*^{ZZ27} embryos.

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