

Sanpodo: a context-dependent activator and inhibitor of Notch signaling during asymmetric divisions

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Asymmetric cell divisions generate sibling cells of distinct fates ('A', 'B') and constitute a fundamental mechanism that creates cell-type diversity in multicellular organisms. Antagonistic interactions between the Notch pathway and the intrinsic cell-fate determinant Numb appear to regulate asymmetric divisions in flies and vertebrates. During these divisions, productive Notch signaling requires *sanpodo*, which encodes a novel transmembrane protein. Here, we demonstrate that *Drosophila sanpodo* plays a dual role to regulate Notch signaling during asymmetric divisions – amplifying Notch signaling in the absence of Numb in the 'A' daughter cell and inhibiting Notch signaling in the presence of Numb in the 'B' daughter cell. In so doing, *sanpodo* ensures the asymmetry in Notch signaling levels necessary for the acquisition of distinct fates by the two daughter cells. These findings answer long-standing questions about the restricted ability of Numb and Sanpodo to inhibit and to promote, respectively, Notch signaling during asymmetric divisions.

KEY WORDS: Notch, Numb, Sanpodo, Asymmetric divisions, *Drosophila*

INTRODUCTION

Asymmetric divisions – the fundamental process through which precursor cells create daughter cells of distinct fates ('A', 'B') – generate cell-type diversity throughout the animal and plant kingdoms. Antagonistic interactions between the Notch signaling pathway and the phosphotyrosine-binding (PTB)-domain-containing protein Numb regulate asymmetric divisions in higher metazoans (reviewed by Gonczy, 2008; Matsuzaki, 2000; Posakony, 1994; Schweisguth, 2004). During these divisions Numb segregates into one daughter cell, where it inhibits Notch signaling (Rhyu et al., 1994). The absence of Notch signaling in this cell permits it to adopt the default 'B' fate. In the other daughter cell, the absence of Numb allows high-level Notch signaling, which induces this cell to acquire the Notch-dependent 'A' fate.

In *Drosophila* the Notch pathway regulates many other developmental decisions, including wing blade formation, wing vein formation and lateral inhibition, during which individual precursor cells are selected from groups of equivalent cells (reviewed by Simpson, 1998). Cells undergoing these Notch-dependent events also co-express *Notch* and *numb* (Fehon et al., 1991; Matsuzaki, 2000). However, loss-of-function studies reveal that *numb* inhibits Notch signaling only during asymmetric divisions (Dye et al., 1998; Lear et al., 1999; Salzberg et al., 1994; Skeath and Doe, 1998; Uemura et al., 1989), suggesting that additional factors restrict the inhibitory action of Numb on Notch signaling to 'B' daughter cells during asymmetric divisions.

sanpodo (*spdo*) encodes a novel transmembrane protein, and like *numb*, regulates Notch signaling only during asymmetric divisions (Hutterer and Knoblich, 2005; O'Connor-Giles and Skeath, 2003).

However, in contrast to *numb*, *spdo* promotes the Notch-dependent 'A' fate, as both daughter cells adopt the default 'B' fate in the absence of *spdo* (Dye et al., 1998; Salzberg et al., 1994; Skeath and Doe, 1998). *spdo* is expressed exclusively in asymmetrically dividing cells, and physically associates with Notch and Numb (Hutterer and Knoblich, 2005; O'Connor-Giles and Skeath, 2003). Numb inhibits the ability of Spdo to localize to the cell membrane, leading to differential subcellular localization of Spdo between the daughter cells: in the 'A' cell Spdo localizes to the cell membrane, whereas in the 'B' cell Spdo localizes primarily to cytoplasmic vesicles (Hutterer and Knoblich, 2005; Langevin et al., 2005; O'Connor-Giles and Skeath, 2003). The ability of Numb to regulate Spdo localization, together with the requirement for *spdo* to promote productive Notch signaling during asymmetric divisions, led to the model that Numb acts through Spdo to inhibit Notch signaling in the 'B' daughter cell (O'Connor-Giles and Skeath, 2003; Hutterer and Knoblich, 2005).

The abilities of Numb and Spdo to regulate Notch signaling specifically during asymmetric divisions raise two important questions. Why does *numb* inhibit Notch signaling only during asymmetric divisions? And, why does productive Notch signaling require *spdo* function during asymmetric divisions, but no other Notch-dependent event? Here, through loss-of-function and gemisexpression studies on *spdo*, we demonstrate that Numb converts *spdo* from an activator to an inhibitor of Notch signaling: in cells that lack Numb protein, Spdo expression potentiates Notch signaling, whereas in cells that contain Numb, Spdo expression dampens Notch signaling. During normal development *spdo* is only expressed in asymmetrically dividing cells, and within asymmetrically dividing cells Numb segregates exclusively into the 'B' daughter cell. Thus, under wild-type conditions, Notch-dependent 'A' daughter cells are the only cells that express both Notch and Spdo, but not Numb, whereas 'B' daughter cells are the only cells that express Notch, Numb and Spdo. By exerting opposite effects on Notch signaling in a Numb-dependent manner, Spdo then simultaneously directs Notch signaling to exceed threshold levels in the 'A' daughter cell and to remain well below such levels in the 'B' daughter cell, thus ensuring the faithful execution of asymmetric divisions.

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MATERIALS AND METHODS

Fly strains

Wild type is Oregon R or *w¹¹¹⁸*, *spdo^{G104}*, *spdo^{AC81}*, *spdo^{ZZ27}*, *mastermind^{NN46}* (O'Connor-Giles and Skeath, 2003; Skeath and Doe, 1998); *numb¹*, *numb²* (Uemura et al., 1989); *Dl⁷*, *Dl³* (Lindsley, 1992; Micchelli et al., 1997); *Dl^{RevF10}* *Ser^{VX82}* (Micchelli et al., 1997); *neur^{9L119}* (Jurgens, 1984); *Su(H)^{SF8}* (Schweisguth and Posakony, 1992); *E(spl)^{rv1}* (Lindsley, 1992); *gro^{E48}* (Preiss et al., 1988); *N^{81K1}* (Lindsley, 1992); *svp-lacZ* (Mlodzik et al., 1990).

Gene-misexpression studies were carried out using the GAL4/UAS system (Brand and Perrimon, 1993). GAL4 lines: *prospero-GAL4* (Shiga, 1996), *scabrous-GAL4* (Nakao and Campos-Ortega, 1996), *twist-GAL4* (Greig and Akam, 1993), *patched-GAL4* (Wilder and Perrimon, 1995), *nubbin-GAL4* (Calleja et al., 1996).

UAS transgenes: *UAS-Spdo^{G14}*, *UAS-Spdo^{G1}*, *UAS-Spdo^{G14} UAS-Spdo^{G1}* (O'Connor-Giles and Skeath, 2003); *UAS-Notch* (Giniger, 1998); *UAS-Fringe* (Okajima and Irvine, 2002); *UAS-Delta* and *UAS-GFP* (Bloomington Stock Center), *UAS-numbrNAi* (Tang et al., 2005), *UAS-neuralized* (Lai et al., 2001); *UAS-neuralized UAS-Delta* (Pavlopoulos et al., 2001); *UAS-Notch^{ΔECN}* (Struhl et al., 1993).

Genotypes generated for the study: *twist-GAL4; svp-lacZ spdo^{ZZ27}/TM3*, *twist-GAL4; spdo^{ZZ27}/TM3*, *numb² twist-GAL4/CyO*, *prospero-GAL4 spdo^{G104}/TM3*, *scabrous-GAL4; spdo^{ZZ27}/TM3*, *numb²; prospero-GAL4 spdo^{G104}/TM3*, *numb² twist-GAL4*; *spdo^{G104}/TM3*, *scabrous-GAL4*; *Dl⁷/TM6*, *twist-GAL4*; *Dl^{RevF10} Ser^{VX82}/TM6*, *UAS-spdo^{G14}*; *spdo^{G104}/TM3*, *UAS-Notch*; *spdo^{G104}/TM3*, *UAS-Notch spdo^{G104}/TM3*, *UAS-fringe*; *spdo^{G104}/TM3*, *UAS-Delta*; *spdo^{G104}/TM3*, *UAS-neuralized*; *spdo^{G104}/TM3*, *UAS-neuralized UAS-Delta*; *spdo^{G104}/TM3*, *UAS-spdo^{G14}*; *spdo^{G104}/TM3*, *numb² UAS-spdo^{G14}/CyO*; *UAS-spdo^{G1}*, *numb² UAS-Notch/CyO*; *spdo^{G104}/TM3*, *numb² UAS-fringe/CyO*; *spdo^{G104}/TM3*, *UAS-Delta*; *Dl⁷/TM6*, *UAS-fringe*; *Dl⁷/TM6*, *UAS-Notch*; *Dl⁷/TM6*, *UAS-Delta*; *Dl^{RevF10} Ser^{VX82}/TM6*, *UAS-fringe*; *Dl^{RevF10} Ser^{VX82}/TM6*, *UAS-Notch*; *Dl^{RevF10} Ser^{VX82}/TM6*, *numb² UAS-spdo^{G14}/CyO*; *Dl⁷ UAS-spdo^{G1}/TM6* and *numb² twist-GAL4/CyO*; *Dl⁷/TM6*.

Immunohistochemistry

Immunohistochemistry and immunofluorescence analysis were performed essentially as described (Skeath and Carroll, 1992) using the following antibodies: anti-Eve (Frasch et al., 1987), anti-Odd (Ward and Coulter,

2000), 22C10 (1:20; Developmental Hybridoma Studies Bank), anti-Nmr1 (1:2000) (Leal et al., 2009), anti-Spdo (1:1000) and anti-Numb (1:500) (O'Connor-Giles and Skeath, 2003), anti-β-gal (1:1000; Promega and ICN), anti-Senseless (1:800) (Nolo et al., 2000) and anti-Mef-2 (1:500) (Lilly et al., 1995).

RESULTS

Only during asymmetric divisions does the Notch signaling pathway require *spdo*, and respond to *numb* function. The genetic and molecular mechanisms that underlie the specificity of these interactions remain unclear. In this paper, we assess the potential of *spdo* function to influence Notch pathway activity in the presence and absence of *numb* in two ways. We used misexpression approaches to investigate *spdo* function during Notch-dependent events that do not normally require *spdo*, including lateral inhibition and wing formation. We used loss-of-function approaches, combined with overexpression of Notch pathway members, to assess *spdo* function in 'A' and 'B' daughter cells during asymmetric divisions.

Misexpression of *spdo* dampens Notch signaling activity during lateral inhibition

To determine the effect of expressing *spdo* in cells that normally express *Notch* and *numb* but not *spdo*, we focused on the process of lateral inhibition. During lateral inhibition, the Notch pathway restricts the ability of cells within equivalence groups (gray circles, Fig. 1A) from adopting the precursor fate (black circles, Fig. 1A), such that only one precursor normally arises from a given equivalence group (Fig. 1A). Reduction of Notch signaling during lateral inhibition results in the formation of additional precursors, whereas heightened Notch activity results in the failure of precursors to segregate from equivalence groups (Simpson, 1998). To test the hypothesis that Spdo enables Numb to inhibit Notch signaling, we focused on two such processes – the formation of sensory organ

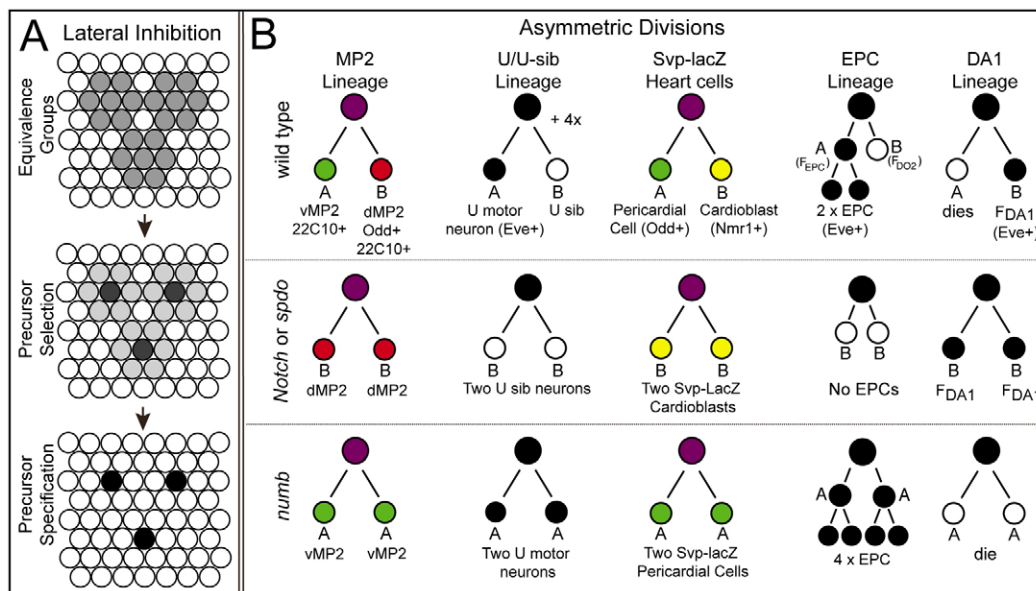


Fig. 1. Schematics of Notch-mediated lateral inhibition and the lineages of five pairs of sibling cells. (A) Initially all cells within three equivalence groups acquire the potential (gray) to adopt the precursor fate. One cell (gray) is singled out as the presumptive precursor from each cluster. This cell then acts through the Notch pathway to inhibit all other cells in the group (light gray) from adopting the precursor fate, such that individual precursor cells (black) segregate from each group (right). **(B)** Cell lineages of the five sibling pairs assayed in this paper shown for one hemisegment of wild type, *Notch/spdo* or *numb* mutant embryos. Lineages shown in color represent those in which we follow the fate of both sibling cells. Lineages shown in black/white are those in which we use eve expression (black) to follow the fate of one of the two sibling cells in each lineage.

precursors and heart precursors in the *Drosophila* peripheral nervous system and mesoderm, respectively. Specifically, we followed the formation of these precursors and the structures and cell types to which they give rise to determine the effect of *spdo* misexpression on Notch signaling levels during lateral inhibition.

Cells undergoing lateral inhibition normally express both *Notch* and *numb*, but not *spdo*. However, loss of *numb* function does not affect Notch signaling during lateral inhibition (Dye et al., 1998; Lear et al., 1999; Salzberg et al., 1994; Skeath and Doe, 1998; Uemura et al., 1989), indicating that Numb does not inhibit Notch signaling in this context. We took advantage of these circumstances to ask what happens when we express *spdo* in cells undergoing lateral inhibition. We reasoned that if *numb* acts through *spdo* to inhibit Notch pathway function, then expressing *spdo* in these cells should inhibit Notch signaling in a *numb*-dependent manner, and result in the formation of ectopic precursors and the structures/cells to which they give rise. In support of this model, ectopic *spdo* expression in the scutellar region of the wing imaginal disc led to an approximate doubling of scutellar bristles and their sensory organ precursors relative to wild type (compare Fig. 2A,C with 2F,G).

To determine whether this finding was specific to the nervous system or generalizable to lateral inhibition throughout development, we expressed *spdo* ubiquitously in the embryonic mesoderm and assayed cardioblast development. As observed for sensory organ precursors, the cardioblast precursors are selected from equivalence groups via Notch-mediated lateral inhibition, and reduction of Notch signaling function leads to excess cardioblast precursors and cardioblasts (Carmena et al., 1998; Hartenstein et al., 1992). In this experiment, we focused on Seven-up-lacZ⁺ (Svp-lacZ⁺) cardioblast precursors and their resultant progeny. In wild-

type embryos, two Svp-lacZ⁺ heart precursors develop per hemisegment (arrows, Fig. 3B); each precursor then divides asymmetrically to produce an Svp-lacZ⁺ cardioblast and an Svp-lacZ⁻ pericardial cell (Fig. 3A; arrows, Fig. 3B') (Ward and Skeath, 2000). The remaining four cardioblasts per hemisegment derive from two Svp-lacZ⁻ precursors; each of which produces two cardioblasts (Fig. 3A,B') (Ward and Skeath, 2000). As observed for bristles and their precursors, generalized *spdo* expression in the mesoderm led to the formation of extra Svp-lacZ⁺ precursors and their progeny (arrowheads, Fig. 3C,C'). Although we did not assay the development of Svp-lacZ⁻ cardioblast precursors, the formation of extra Svp-lacZ⁻ cardioblasts (arrow, Fig. 3C') indicated that their numbers also increased in this background. Thus, misexpression of *spdo* is also sufficient to inhibit Notch signaling during lateral inhibition in the mesoderm. Note that essentially all Svp-lacZ⁺ precursors divided asymmetrically in this experiment (99.8%, $n=829$; see Table S3 in the supplementary material). Thus, generalized *spdo* expression in the mesoderm did not alter the ability of precursors to divide asymmetrically during Notch-mediated asymmetric divisions.

To determine whether expressing *spdo* during other Notch-dependent events also compromised Notch signaling activity, we expressed *spdo* throughout the wing blade, and assayed for defects in three additional Notch-mediated events: wing blade growth, wing patterning and wing vein formation (reviewed by Blair, 2000; De Celis, 2003). *spdo* misexpression in the wing resulted in reduced wing size, notched wings and vein thickening (Fig. 2J) – phenotypes indicative of reduced Notch signaling (see Blair, 2000; De Celis, 2003). In agreement, when *Notch* function is reduced by 50% the wing and lateral inhibition phenotypes observed upon *spdo*

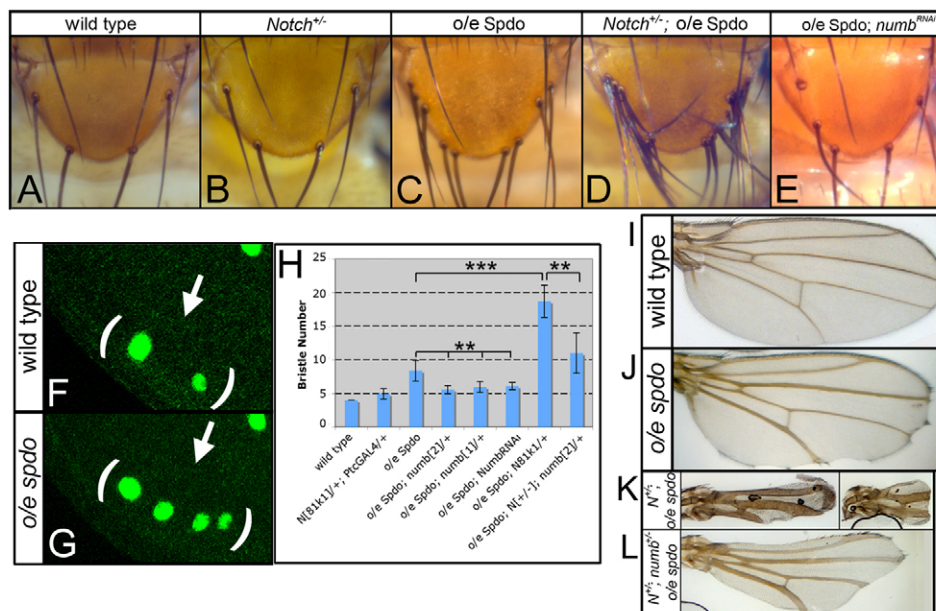


Fig. 2. *sanpodo* misexpression inhibits Notch signaling during lateral inhibition and wing development in a *numb*-dependent manner.

(A) Four scutellar bristles arise in wild type. (B) Removing one copy of *Notch* has little effect on bristle number. (C–E) Expression of *spdo* by *patched-GAL4* (*o/e spdo*) in the scutellar region promotes ectopic bristle formation (C), a phenotype enhanced by reducing *Notch* function 50% (D), and suppressed by co-expression of a *numb-RNAi* transgene (E) (Tang et al., 2005). (F) In wild type two sensory organ precursors, as labeled by anti-senseless (Nolo et al., 2000), develop in the scutellar region (arrows, brackets) of the late third instar wing imaginal disc. (G) *spdo* overexpression in this region promotes the formation of ectopic sensory organ precursors (arrow). (H) Average number of scutellar bristles per indicated genotype. Error bars indicate s.d. $**P < 10^{-4}$; $***P < 10^{-10}$. (I) *nubbin-GAL4*-mediated expression of *spdo* in the wing (*o/e spdo*) leads to wing notching, vein thickening and reduced wing size. (J) Reduction of Notch function by 50% ($N^{+/-}; o/e spdo$), using the *Notch*^{81k1} allele, enhanced these phenotypes. (K,L) Simultaneously reducing *numb* function by 50% ($N^{+/-}; numb^{+/-}; o/e spdo$, L) suppressed the phenotypes observed in $N^{+/-}; o/e spdo$ flies (K). Images in I–K are shown at identical magnification.

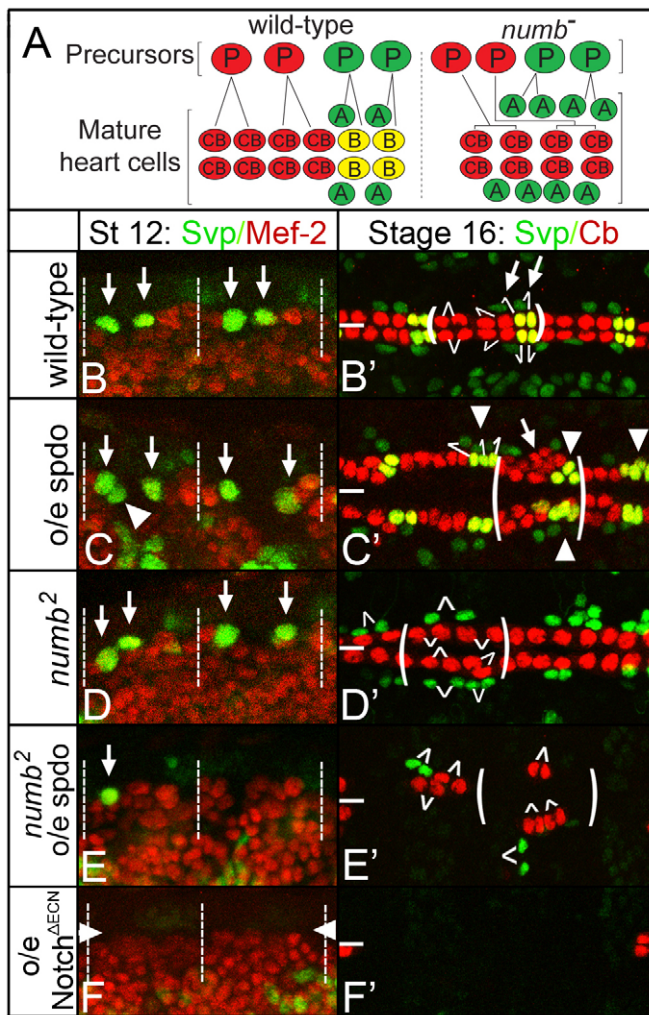


Fig. 3. *sanpodo* exerts a context-dependent effect on Notch signaling during lateral inhibition. (A) Model of heart precursors and differentiated (mature) heart cells. Left, in a wild-type hemisegment two cardioblast precursors (red cells labeled 'P') each divide to produce two cardioblasts (red cells labeled 'CB'), and two Svp-lacZ⁺ heart precursors (green cells labeled 'P') each divide to produce one Svp-lacZ⁺ pericardial cell (green, 'A' daughter cell) and one Svp-lacZ⁺ cardioblast (red, 'B' cell). Right, in *numb*^{-/-} mutant embryos heart precursors form normally but Svp-lacZ⁺ heart precursors produce only pericardial cells (green, 'A' cells). Precursors are shown for one hemisegment, whereas mature heart cells are shown for one full segment. **(B-F)** High-magnification views of two hemisegments of the dorsal mesoderm of embryos of indicated genotype labeled for Svp-lacZ⁺ (green) or Mef-2 (red), which labels mesodermal cells. **(B'-F')** High-magnification views of three segments of the heart of embryos of indicated genotype labeled for Svp-lacZ⁺ (green) and Nmr-1 (red), Nmr-1 specifically labels cardioblasts (Leal et al., 2009). (B) In wild-type stage 12 embryos, two Svp-lacZ⁺ heart precursors arise per hemisegment (arrows; dotted lines demarcate segment-sized regions in B-F). (B') By stage 16, two rows of cardioblasts (red; yellow for Svp-lacZ⁺ cardioblasts) and Svp-lacZ⁺ pericardial cells (green) align on either side of the dorsal midline (thick white line). In B'-E' one segment is bracketed and white lines indicate defined or inferred sibling relationships. (C) *twist-GAL4*-mediated expression of *spdo* in a wild-type embryo generates extra Svp-lacZ⁺ heart precursors (arrowhead) by stage 12, and extra Svp-lacZ⁺ pericardial cells and cardioblasts (arrowheads, C') as well as extra Svp-lacZ⁻ cardioblasts (arrow, C') by stage 16. (D) In *numb* mutant embryos two Svp-lacZ⁺ precursors arise normally (arrows), but each divides to produce two pericardial cells (green cells, D'), no Svp-lacZ⁺ cardioblasts develop in this background. (E) Expressing *spdo* throughout the mesoderm of a *numb* mutant embryo inhibits the formation of Svp-lacZ⁺ precursors (arrow), which leads to fewer Svp-lacZ⁺ pericardial cells (green cells, E'); note also the reduction in cardioblasts (red cells, E'). (F,F') In wild-type mesodermal expression of a constitutively active form of Notch (Notch^{Δ^{ECN}}) leads to a near complete loss of Svp-lacZ⁺ heart precursors (arrowheads, F) and heart cells (F'). Anterior, left.

misexpression were dramatically enhanced (Fig. 2D,H,K). We conclude that superimposing *spdo* expression on cells undergoing these four Notch-dependent events dampens Notch signaling levels, consistent with the model that Numb normally fails to inhibit Notch signaling during these events owing to the absence of Spdo.

Misexpression of *spdo* amplifies Notch signaling in the absence of Numb

To test directly whether *spdo*-mediated inhibition of Notch signaling depends on *numb*, we misexpressed *spdo* in backgrounds with reduced *numb* function. We found that reducing *numb* function by

50% genetically (Fig. 2H, Table 1), or by co-expression of a *numb*-RNAi transgene (Fig. 2E,H), suppressed the ability of *spdo* to inhibit Notch signaling during lateral inhibition. For example, expression of *spdo* alone increased the number of scutellar bristles from four in wild type to just over eight (Fig. 2H), whereas expressing *spdo* in backgrounds compromised for *numb* function yielded an average of six scutellar bristles per fly (Fig. 2E,H). Similarly, reducing *numb* function by 50% in embryos that express *spdo* generally throughout the mesoderm restored heart cell numbers to near wild-type levels (Table 1). Thus, the ability of *spdo* to inhibit Notch signaling is *numb* dependent.

Table 1. *spdo* regulates Notch pathway activity in a *numb*-dependent manner

Genotype	Number of Svp-lacZ ⁺ heart cells	P-value	Number of Svp-lacZ ⁻ cardioblasts	P-value	Number of Eve ⁺ pericardial cells	P-value
Wild type	28.1±0.5 (n=15)		37.9±0.9 (n=15)		—	—
<i>o/e spdo</i>	31.2±2.2 (n=15)	<0.0001*	47.9±4.9 (n=15)	<0.0001*	—	—
<i>o/e spdo; numb^{2/+}</i>	27.6±2.2 (n=15)	<0.001 [†]	39.2±1.8 (n=15)	<0.0001 [†]	—	—
<i>numb²</i>	22.9±3.5 (n=20)		33.9±1.7 (n=20)		44.2±2.4 (n=20)	
<i>o/e spdo; numb²</i>	8.6±3.1 (n=20)	<1×10 ^{-10†}	27.4±3.8 (n=40)	<1×10 ^{-5‡}	18.7±9.0 (n=20)	<1×10 ^{-10‡}

Numbers are given per bilateral side of an embryo; *n* refers to the number of embryos scored. The *P*-value is derived by *t*-test. Values are given ± s.d. *o/e spdo* refers to *twist-GAL4*-mediated expression of UAS-Spdo^{G14}; UAS-Spdo^{G1} transgenes.

*Relative to wild-type embryos.

[†]Relative to *o/e Spdo* embryos.

[‡]Relative to *numb* mutant embryos.

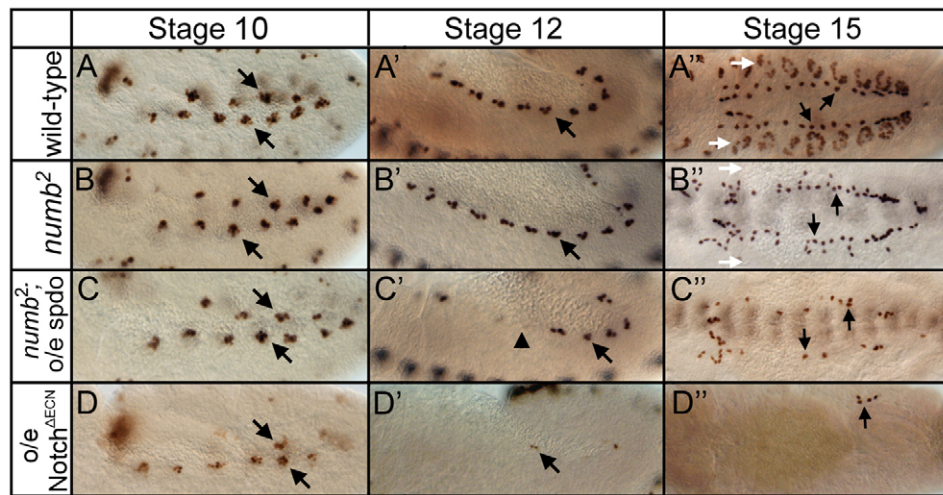


Fig. 4. *spdo* potentiates Notch signaling in the absence of *numb*. Left and middle panels show lateral view; right panel shows dorsal view of eve expression in the mesoderm. (**A-A''**) In wild type, mesodermal Eve⁺ equivalence groups (arrows, A) generate Eve⁺ precursor cells (arrows, A') that produce the EPCs (arrows, A''), and the Eve⁺ DA1 muscles (white arrows, A''). (**B-B''**) In *numb* mutant embryos, equivalence group formation and precursor selection occurs normally (arrows, B, B'); however, defects in asymmetric divisions lead to a doubling of EPC numbers (arrows, B'') and a loss of DA1 muscles (white arrows, B''). (**C-C''**) Eve⁺ equivalence groups form normally in *numb* mutant embryos that misexpress *spdo* in the mesoderm under the control of *twist-GAL4* (arrows); however, Eve⁺ precursors fail to segregate from half of these groups (arrowhead, C'), resulting in fewer EPCs (arrows, C''). (**D-D''**) Eve⁺ equivalence groups form, albeit with reduced eve expression, upon mesodermal expression of a constitutively active form of Notch (*Notch*^{ΔEΔN}) in wild-type embryos; however, few precursors and EPCs arise in this background (arrows, D', D''). Anterior, left.

If *spdo* acts through *numb* to inhibit Notch signaling, then misexpression of *spdo* in the absence of *numb* during lateral inhibition might have little effect on Notch signaling activity. To test this prediction, we expressed *spdo* throughout the mesoderm of embryos homozygous mutant for *numb*, and assayed the resulting effect on the development of differentiated heart cells and their precursors. Surprisingly, we observed a clear decrease in the formation of heart precursors and their progeny (Fig. 3D, Fig. 4C) – a phenotype indicative of increased Notch signaling. For example, in wild-type or *numb* mutant embryos, two Svp-lacZ⁺ heart precursors arise per hemisegment (Fig. 3B,D). In contrast, when *spdo* was overexpressed throughout the mesoderm of otherwise *numb* mutant embryos less than half the normal number of Svp-lacZ⁺ precursors and their progeny arose (Fig. 3E,E'; Table 1). Fewer Svp-lacZ⁻ cardioblasts also developed in this background (Fig. 3E'; Table 1), suggesting that a fraction of their precursors also failed to develop. These phenotypes are similar to but less severe than observed upon expression of a constitutively active form of Notch throughout the mesoderm of wild-type embryos, which blocks the formation of essentially all cardioblasts and their precursors (Fig. 3F,F'). We conclude that expressing *spdo* in the absence of *numb* during lateral inhibition increased Notch signaling levels, leading to the segregation of fewer precursors than normal from equivalence groups.

To test directly whether the decrease in heart cells arose owing to a failure of precursors to segregate reliably from equivalence groups, rather than from a defect in establishing equivalence groups, we followed the dynamics of *even skipped* (*eve*) expression in the mesoderm (Fig. 4). In wild type, the Eve⁺ precursors of the Eve pericardial cells (EPCs) and the Eve⁺ DA1 muscles (arrow, Fig. 4A') are selected via lateral inhibition from adjacent *eve*-expressing equivalence groups (arrows, Fig. 4A) (Carmena et al., 1998). In their respective lineages, EPCs represent 'A' daughter cells (arrows, Fig. 4A''), and Eve⁺ DA1 muscles represent 'B' daughter cells (white

arrows, Fig. 4A''); see also Fig. 1B). In *numb* mutant embryos, the formation of *eve*-expressing equivalence groups, and the segregation of Eve⁺ precursors from them both occurred normally (arrows, Fig. 4B,B'). However, defects in asymmetric divisions led to a doubling of EPCs ('A' cells) and a loss of DA1 muscles ('B' cells) (Fig. 4B; see also Fig. 1B). Eve⁺ equivalence groups also formed normally in *numb* mutant embryos that express *spdo* throughout the mesoderm (arrows, Fig. 4C). However, in this background Eve⁺ precursors failed to segregate from roughly half of the equivalence groups (arrowhead, Fig. 4C'), resulting in the formation of far fewer EPCs than in *numb* mutant embryos (compare Fig. 4B'' with 4C''). This phenotype is similar to but less severe than observed upon generalized mesodermal expression of a constitutively active form of Notch, where essentially no precursors segregate from Eve⁺ equivalence groups (Fig. 4D-D'). We conclude that expressing *spdo* in the absence of *numb* amplifies Notch signaling levels during lateral inhibition, resulting in the subsequent failure of precursors to segregate from equivalence groups. Conversely, expressing *spdo* in the presence of Numb decreases Notch signaling levels during lateral inhibition, resulting in the segregation of extra precursors.

During asymmetric divisions Numb inhibits Spdo from localizing to the cell membrane of the 'B' daughter cell; in the 'A' daughter cell, which lacks Numb, Spdo localizes to the cell membrane (O'Connor-Giles and Skeath 2003; Hutterer and Knoblich 2005; Langevin et al., 2005). To see if *numb* maintains its ability to regulate the localization of Spdo in cells that do not normally express *spdo*, we misexpressed *spdo* throughout the ectoderm, a tissue in which *spdo* is never expressed, and then visualized Spdo localization in wild-type and *numb* mutant embryos. Spdo localized primarily to the cytoplasm in a diffuse and punctate pattern in ectodermal cells in otherwise wild-type embryos (Fig. 5A). By contrast, in *numb* mutant embryos Spdo exhibited increased localization to the cell membrane (Fig. 5B). Thus, *numb* maintained its competence to regulate the subcellular localization of Spdo in ectodermal cells, even though *spdo* is not

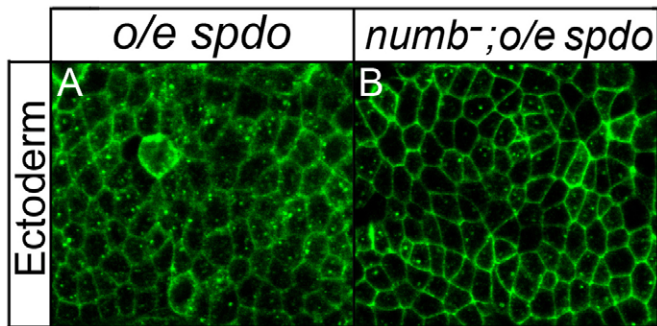


Fig. 5. *numb* regulates the subcellular localization of *Spdo*. *Spdo* localization (green) in ectodermal cells of otherwise wild-type (A) or *numb* mutant (B) embryos. (A) *Spdo* exhibits predominately diffuse and punctate localization in ectodermal cells of wild-type embryos. (B) *Spdo* exhibits increased localization to the cell membrane of ectodermal cells in *numb* mutant embryos. Each panel shows a single z-section of a stage 12 embryo taken at similar apical basal positions; both embryos were processed in parallel and imaged on a Leica SP1 confocal microscope using identical parameters. *Spdo* misexpression mediated by *scabrous-GAL4*. Anterior, left.

normally expressed in these cells. Taken together, we find that in the presence of Numb *Spdo* localizes predominately to the cytoplasm and decreases Notch signaling activity, whereas in the absence of Numb, *Spdo* exhibits increased localization to the cell membrane and increases Notch signaling.

***spdo* potentiates Notch signaling in the 'A' daughter cell**

During wild-type development, *spdo* expression is restricted to asymmetrically dividing cells. Thus, the only cells that normally express Notch and *Spdo* but not Numb are the Notch-dependent 'A' daughter cells, and the only cells that express Notch, *Spdo* and Numb are 'B' daughter cells (Fehon et al., 1991; O'Connor-Giles and Skeath, 2003; Rhyu et al., 1994). Given the above results, we set out to test the hypotheses that: (1) *Spdo* functions in 'A' cells to amplify Notch signaling during asymmetric divisions; whereas (2) in 'B' cells, *Spdo* enables Numb to inhibit Notch signaling.

We reasoned that if *Spdo* amplifies Notch signaling levels in 'A' daughter cells, then increasing Notch signaling levels through other means might bypass the requirement for *spdo* in these cells. Thus, we overexpressed wild-type forms of Notch pathway members in embryos homozygous mutant for *spdo* and assayed asymmetric divisions in four distinct cell lineages in the embryonic central nervous system (CNS) and mesoderm (see Fig. 1B). In all cases, we found that overexpression of the full-length forms of either the Notch receptor or Fringe [which has been shown to modify Notch to enhance Notch-Delta signaling during wing development (Irvine, 1999; Moloney et al., 2000)] largely or completely restored 'A' cell development in *spdo* mutant embryos. For example, in each hemisegment of the CNS, the MP2 precursor divides asymmetrically to yield vMP2 (arrows, Fig. 6A), which projects an axon anteriorly, and dMP2 (arrowheads, Fig. 6B), which expresses Odd skipped and extends an axon posteriorly (see also Fig. 1B). Similarly, each of the first five ganglion mother cells generated by neuroblast 7-1 divides asymmetrically to produce an *Eve*⁺ U motoneuron and an *Eve*⁺ U sibling neuron (Fig. 1B; arrowheads in Fig. S1A in the supplementary material) (Pearson and Doe, 2003). vMP2 and the *Eve*⁺ U neurons are 'A' daughter cells, and adopt the fate of their respective siblings at nearly 100% frequencies in the

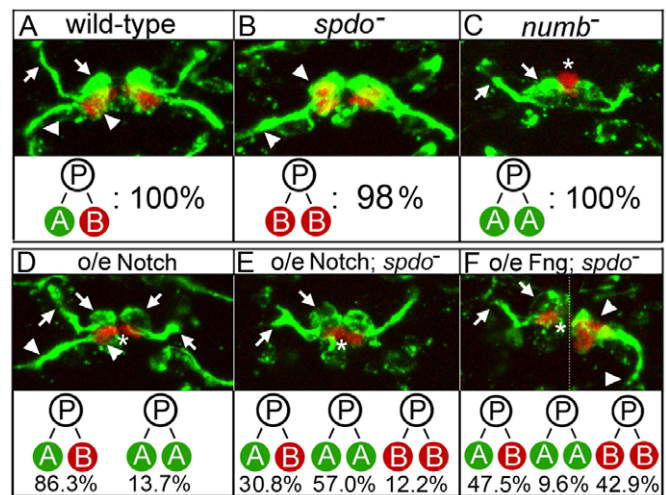


Fig. 6. *spdo* facilitates Numb-mediated inhibition of Notch signaling in the 'B' daughter cell during asymmetric divisions in the CNS. (A) In wild-type embryos vMP2 (arrows, 'A' cell) extends an axon anteriorly, while its sibling dMP2 (arrowheads, 'B' cell) expresses Odd (red) and extends an axon posteriorly. (B) In *spdo* mutant embryos both siblings adopt the Odd⁺ dMP2 fate (arrowheads) and extend axons posteriorly. (C) In *numb* mutant embryos both siblings adopt the vMP2 fate (arrows) and extend axons anteriorly. In C-F, an asterisk denotes the unrelated Odd⁺ MP1 neurons (red), which form in all genotypes shown but are obscured in some panels. (D) Single segment of wild-type embryo in which Notch has been overexpressed in the CNS. Left, daughter cells adopt normal vMP2 (arrows) and dMP2 (arrowheads) fates. Right, both daughter cells adopt the vMP2 fate and extend axons anteriorly (arrows). (E) Notch overexpression in *spdo* mutant embryos induces both daughter cells to adopt the vMP2 fate (arrows) and extend axons anteriorly in most hemisegments. (F) Fringe overexpression in *spdo* mutant embryos. Left: both daughter cells adopt the vMP2 fate (arrows) and extend axons anteriorly. Right: both daughter cells adopt the dMP2 fate (arrowheads) and extend axons posteriorly. (see Table S3 in the supplementary material). The dotted line in F indicates use of different focal planes from the same segment. Anterior is up; *N*>200 hemisegments per genotype. *prospero-GAL4* was used for gene overexpression. See also Table S3 in the supplementary material.

absence of *spdo* (arrowheads, Fig. 6B; see Fig. S1B and Tables S1 and S2 in the supplementary material). However, overexpression of either Notch or Fringe throughout the CNS of *spdo* mutant embryos restored vMP2 development ('A' fate) in 87.8 and 57.1% of hemisegments, respectively (arrows, Fig. 6E,F; see Table S1 in the supplementary material), and *Eve*⁺ U neuron development in most or all hemisegments (arrowheads, see Fig. S1F,G and Table S2 in the supplementary material). Note that a significant fraction of 'B' daughter cells also adopted the Notch-dependent 'A' fate in the MP2 lineage upon Notch or Fringe overexpression in *spdo* mutant embryos (see below).

Overexpression of Notch or Fringe can also bypass the requirement for *spdo* function during asymmetric divisions in the mesoderm. As noted, *Svp-lacZ*⁺ heart precursors divide asymmetrically to produce sibling pericardial cells ('A' cells) and cardioblasts ('B' cells), and within the EPC lineage the *Eve*⁺ EPCs represent 'A' daughter cells (Fig. 1B; arrows, Fig. 7A; Fig. 8A). In these lineages the absence of *spdo* function causes both daughter cells to adopt the 'B' fate about 75% of the time (Fig. 7B, Fig. 8B; see Table S3 in the supplementary material) (Park et al., 1998; Ward and Skeath, 2000). However,

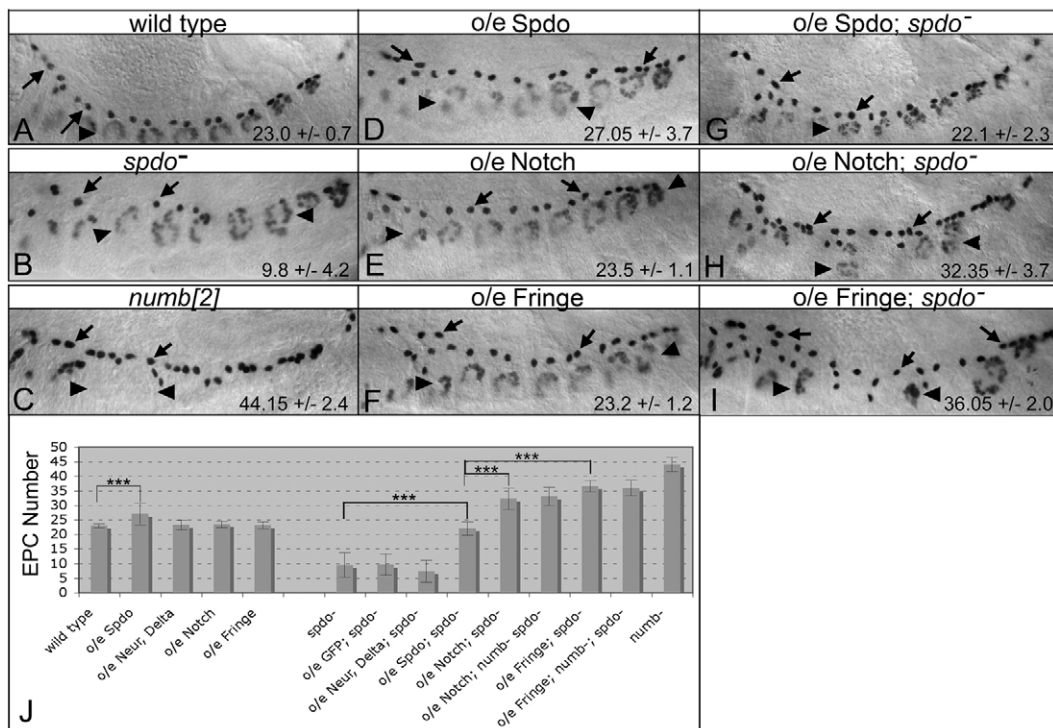


Fig. 7. Overexpression of Notch or Fringe bypasses the requirement for *spdo* function during EPC development. Each panel shows a lateral view of *eve* expression in the dorsal mesoderm. (A) Wild-type stage 14 embryo showing EPCs (arrows) and *Eve⁺* DA1 muscles (arrowheads). (B) *spdo* mutant embryos exhibit a decrease in EPC number (arrows). (C) In *numb* mutant embryos EPC numbers double (arrows) and *Eve⁺* DA1 muscles are lost (arrowheads). (D) *twist-GAL4*-mediated overexpression of Spdo (*o/e spdo*) in the mesoderm of wild-type embryos increased EPC numbers (arrows). (E,F) Identical experiments with either Notch (E) or Fringe (F) had little effect on EPCs (arrows) or DA1 muscles (arrowheads). (G-I) Mesodermal expression of *spdo* in a *spdo* mutant embryo rescued the *spdo* mutant phenotype (compare to B), whereas mesodermal overexpression of Notch (H) or Fringe (I) in *spdo* mutant embryos increased EPC numbers (arrows) and decreased DA1 muscle numbers (arrowheads), phenotypes similar to those of *numb* mutant embryos (compare with C). Numbers indicate average number of EPCs ±s.d. per bilateral side of an embryo. $n \geq 20$ embryo sides, except for *o/e spdo; spdo⁻* ($n=14$). (J) Chart shows average number of EPCs per indicated genotype.

overexpression of Notch or Fringe throughout the mesoderm of *spdo* mutant embryos restored the ‘A’ daughter cell fate in both lineages to wild-type or near wild-type levels (arrows, Fig. 7H,I; arrowheads, Fig. 8G,I; see Table S3 in the supplementary material). As observed in the CNS, ‘B’ daughter cells in both lineages also adopted the ‘A’ fate at appreciable frequencies in these backgrounds (see below). Thus, increasing the level or activity of the Notch receptor largely or completely bypassed the requirement for *spdo* to promote the ‘A’ fate, indicating that Spdo is not absolutely required for Notch signal transduction during asymmetric divisions, but rather acts to amplify pathway activity above the level required to induce the ‘A’ fate. Our prior finding that Spdo enhances Notch signaling activity during lateral inhibition in the absence of *numb* supports this conclusion. Note, that the ability of Notch or Fringe overexpression to restore ‘A’ cell development in *spdo* mutant embryos is ligand dependent, as overexpression of either transgene in the neuroectoderm/CNS or mesoderm of *Delta* mutant embryos failed to rescue Notch-dependent cell fates (see Fig. S2 in the supplementary material).

To test whether increasing ligand levels was also sufficient to restore ‘A’ daughter cell development in the absence of *spdo*, we co-overexpressed Delta and Neuralized – which has been shown to increase Notch signaling (Pavlopoulos et al., 2001; Wang and Struhl, 2004) – in *spdo* mutant backgrounds. Co-expression of both factors failed to restore ‘A’ cell development in the EPC lineage (Fig. 7J), and provided only modest rescuing activity in the other three lineages (Fig. 8E; see Fig. S1D,E and Tables S1-S3 in the

supplementary material). Given this result, the ability of Notch or Fringe overexpression to restore ‘A’ cell development to near wild-type levels suggests that the levels and/or activity of the Notch receptor are limiting in the absence of *spdo* for productive Notch signaling in the ‘A’ daughter cell.

***spdo* facilitates the ability of Numb to inhibit Notch signaling in ‘B’ cells**

Next, we focused on *spdo* function in ‘B’ daughter cells, and asked whether *spdo* enables Numb to inhibit Notch signaling in this cell. Normally one cannot assess a role for *spdo* in ‘B’ daughter cells, as both daughter cells adopt the ‘B’ fate in the absence of *spdo* regardless of the presence of *numb* (Dye et al., 1998; Skeath and Doe, 1998). However, the ability of Notch or Fringe overexpression to bypass the requirement for *spdo* function in ‘A’ daughter cells allowed us to test whether *spdo* facilitates Numb-mediated inhibition of Notch signaling in ‘B’ daughter cells.

If Spdo enables Numb to inhibit Notch signaling in the ‘B’ daughter cell, then the ability of Notch or Fringe overexpression to convert ‘B’ cells to the ‘A’ fate should be greater in the absence rather than the presence of *spdo*. In agreement, Notch or Fringe overexpression was more effective at converting ‘B’ daughter cells to the ‘A’ fate in *spdo* mutant embryos than in wild-type embryos for all lineages tested. For example, in wild-type embryos the frequency of fate changes in the MP2 lineage (dMP2/‘B’ cell to vMP2/‘A’ cell) was 13.7% for Notch overexpression and 0% for Fringe overexpression (Fig. 6D; see Table

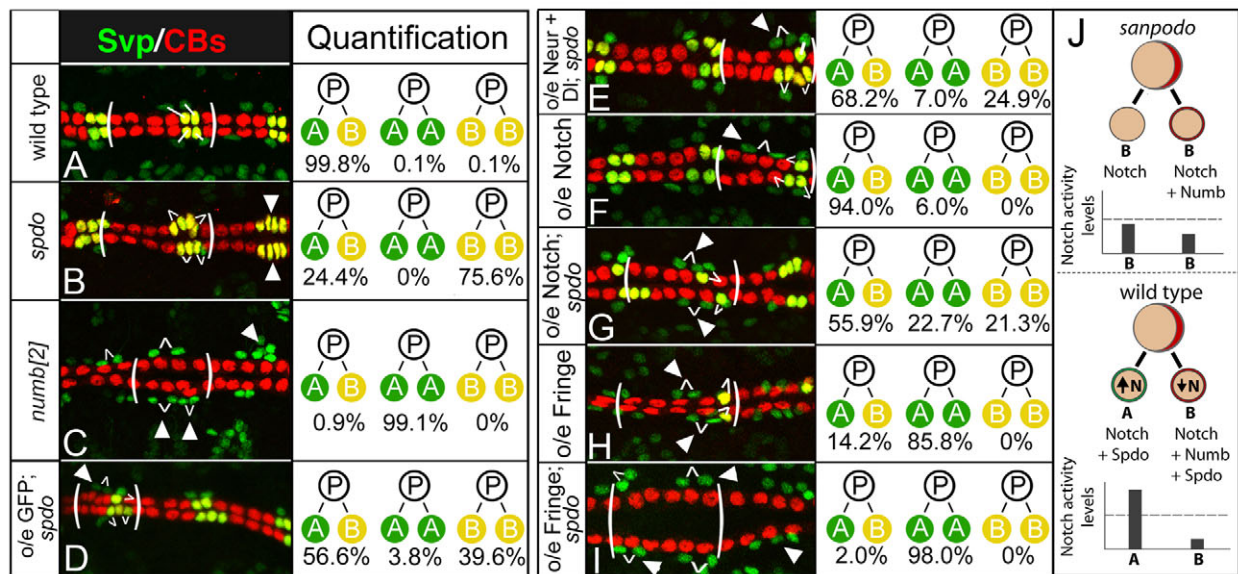


Fig. 8. *spdo* facilitates Numb-mediated inhibition of Notch signaling during asymmetric divisions in the heart. (A) Wild-type pattern of sibling Svp-lacZ⁺ pericardial cells (green, 'A' cell) and cardioblasts (CBs; yellow, 'B' cell) in the stage 16 heart. In all panels one segment is bracketed, and white lines identify sibling relationships. (B) In *spdo* mutant embryos both Svp-lacZ⁺ daughter cells normally adopt the cardioblast or 'B' fate (yellow, arrowheads). (C) In *numb* mutant embryos both daughter cells adopt the pericardial or 'A' fate (green, arrowheads). (D,E) *twist*-GAL4-mediated expression of GFP (D), or Neuralized and Delta (E), in *spdo* mutant embryos causes both daughter cells to adopt the 'A' fate at low frequency (arrowhead), and increases the frequency of both daughter cells adopting alternate fates ('A/B'). Note GFP overexpression has no effect on any other lineage tested (Fig. 3, see Tables S2 and S3 in the supplementary material). (F) Notch overexpression in wild type induces both daughter cells to adopt the 'A' fate at low frequency (arrowhead). (G) Notch overexpression in *spdo* mutant embryos increases the frequency at which both daughter cells adopt the 'A' fate (arrowheads). (H) Fringe overexpression in wild type directs both daughter cells to adopt the 'A' fate most of the time (arrowheads). (I) Fringe overexpression in *spdo* mutant embryos directs both daughter cells to adopt the 'A' fate (green, arrowheads) essentially all the time (arrowheads). Anterior, left. Quantification of sibling fates. *n*>800 sibling pairs assayed per genotype, except for *numb* (*n*=200). See also Table S3 in the supplementary material. (J) Model of *spdo* function during asymmetric divisions. Top, in *spdo* mutant embryos Notch signaling activity remains below the threshold level (dotted line) required to induce the 'A' fate, and both daughter cells adopt the 'B' fate. Bottom, in the absence of Numb, Spdo amplifies Notch signaling activity above the threshold required to induce the 'A' fate. In the presence of Numb, Spdo facilitates the ability of Numb to inhibit Notch signaling 'B' daughter cell, thereby reducing signaling activity below that observed in the absence of *spdo*.

S1 in the supplementary material). In *spdo* mutant embryos, the frequency of fate changes increased to 57.0% for Notch overexpression and 9.6% for Fringe overexpression (Fig. 6E,F, see Table S1 in the supplementary material). Similarly, the frequency of 'B' to 'A' fate changes in the Svp-lacZ⁺ heart lineage increased from 85.8% (*n*=910) in wild-type to 98.0% (*n*=895) in *spdo* mutant embryos for Fringe overexpression (compare Fig. 8H with 8I), and from 6.0% (*n*=949) in wild-type to 22.7% (*n*=942) in *spdo* mutant embryos for Notch overexpression (compare Fig. 8F with 8G; see Table S3 in the supplementary material).

The absence of *spdo* also rendered Notch or Fringe overexpression more effective at converting 'B' daughter cells to the 'A' fate in the EPC and Eve⁺ DA1 lineages. In their respective lineages, EPCs represent 'A' daughter cells, and Eve⁺ DA1 muscles represent 'B' daughter cells (see Fig. 1B). In wild-type embryos Notch or Fringe overexpression has little if any effect on EPC or DA1 development (arrows, Fig. 7E,F). By contrast, in the absence of *spdo* function the same treatment significantly increased EPC numbers ('A' cells) and decreased Eve⁺ DA1 muscle numbers ('B' cells) (arrows, Fig. 7H,I), consistent with 'B' daughter cells adopting the 'A' fate at appreciable frequencies in both lineages. In fact, the asymmetric division phenotypes observed upon Notch/Fringe overexpression in otherwise *spdo* mutant embryos often closely resembled those of *numb* mutant embryos, wherein essentially all

'B' daughter cells adopt the Notch-dependent 'A' fate (compare Fig. 6C with 6E, Fig. 7C with 7I, Fig. 8C with 8I). We conclude that Spdo enables Numb to inhibit Notch signaling in the 'B' cell during asymmetric divisions. Interestingly, simultaneous removal of *numb* and *spdo* did not significantly increase the conversion of 'B' daughter cells to the 'A' fate above that observed upon Notch or Fringe overexpression in the absence of *spdo* alone (Fig. 7J; see Table S2 in the supplementary material). Thus, our data suggest that *numb* acts primarily through *spdo* to block Notch signaling in the 'B' daughter cell. Together with the finding that superimposing *spdo* expression during lateral inhibition enables Numb to inhibit Notch signaling, these results explain why *numb* can only inhibit Notch signaling during asymmetric divisions – this is the only context in which Spdo is present.

DISCUSSION

Work from many labs indicates that the state of Notch signaling determines daughter cell fate during asymmetric divisions – high-level Notch signaling induces the 'A' fate; low-level Notch signaling permits the 'B' fate (Gonczy, 2008; Matsuzaki, 2000; Schweisguth, 2004). In this context, our work demonstrates that *spdo* acts in both daughter cells to accentuate the difference between Notch signaling levels in the two cells – amplifying Notch signaling in the absence of Numb in the 'A' cell, and enabling Numb to inhibit Notch

signaling in the 'B' cell (Fig. 8J). By exerting opposite effects on Notch signaling in a Numb-dependent manner, Spdo simultaneously ensures that Notch signaling exceeds threshold levels in the 'A' cell, yet remains well below such levels in the 'B' cell, thus enabling the faithful execution of asymmetric divisions.

Why Numb can inhibit Notch signaling during asymmetric divisions but no other Notch-dependent event has long remained unclear. Our genetic data demonstrate that *numb* acts through *spdo* to inhibit Notch signaling. As *spdo* is expressed exclusively in asymmetrically dividing cells, and Numb segregates exclusively into the 'B' daughter cell during asymmetric divisions, these results account for the specific ability of Numb to inhibit Notch signaling in 'B' daughter cells – the only cell type in *Drosophila* that co-expresses *spdo* and *numb*. *spdo* does not appear to enable Numb to inhibit Notch signaling by regulating the localization of Numb, as Numb localization is grossly normal in *spdo* mutant embryos (see Fig. S3 in the supplementary material).

Why does productive Notch signaling require *spdo* function in 'A' daughter cells during asymmetric divisions, but not during any other Notch-dependent event in *Drosophila*? We find that in the absence of Numb, Spdo amplifies but is not obligately required for transduction of Notch signaling. Thus, while Notch signaling can occur in 'A' daughter cells in the absence of *spdo*, *spdo* function is normally required to enable signaling levels to exceed the threshold required to induce the 'A' fate.

Our results indicate that limiting levels or activity of the Notch receptor probably underlies the sub-threshold nature of Notch signaling in 'A' daughter cells in the absence of *spdo*. Notch levels or activity may be limiting in 'A' daughter cells owing to the downregulation of proteins that localize to adherens junctions in asymmetrically dividing cells. Notch has been shown to localize preferentially to adherens junctions in epithelial cells, and asymmetrically dividing cells display reduced levels of Notch as well as other proteins that normally localize to adherens junctions (Fehon et al., 1991; Tepass et al., 1996; Uemura et al., 1996). Some of these other proteins, such as Echinoid, are known to facilitate Notch signaling during lateral inhibition and other Notch-dependent events (Ahmed et al., 2003; Fehon et al., 1991; Rawlins et al., 2003; Spencer and Cagan, 2003). Thus, reduced levels of Notch and facilitators of Notch signaling in asymmetrically dividing cells may account for the specific requirement for Spdo to amplify Notch signaling levels during asymmetric divisions.

Consistent with a role for *spdo* in simply amplifying Notch signaling levels in the absence of Numb, the Notch-dependent 'A' fate develops at low frequency in some lineages in the absence of *spdo* (Fig. 7B,J and Fig. 8B, see Table S3 in the supplementary material) (Jafar-Nejad et al., 2005; Park et al., 1998; Ward and Skeath, 2000). Thus, in the absence of *spdo*, Notch signaling levels appear close to, but usually below, the threshold required to induce the 'A' fate (Fig. 8J). Surprisingly, we also observe rare instances where Numb-dependent 'B' daughter cells adopt the 'A' fate in *spdo* mutant embryos, specifically in the development of Svp⁺ heart cells at 18°C (see Table S3 in the supplementary material). Such events have not been observed in wild type, and indicate that Numb requires Spdo in the 'B' cell to maintain Notch signaling levels reliably below the threshold required for the 'A' fate. Thus, the dual and opposing roles of *spdo* in the regulation of Notch signaling levels during asymmetric divisions are crucial for the unerring ability of the two daughter cells to adopt distinct fates.

What is the molecular mechanism through which *spdo* regulates Notch signaling during asymmetric divisions? Our results indicate that any mechanistic model for *spdo* function must account for the ability of *spdo* to boost Notch signaling in the absence of Numb and to reduce Notch signaling in the presence of Numb. Present models of *spdo* function, such as a postulated role for Spdo in promoting recycling of Delta in the 'B' cell (Emery et al., 2005; Jafar-Nejad et al., 2005), do not fully address the duality of *spdo* function in the two daughter cells. Rather our genetic data, together with prior work on Spdo physical interactions and Numb-dependent localization, lead us to postulate that in the absence of Numb, Spdo localizes to the cell membrane of the 'A' cell, where it increases Notch association with effectors, and in so doing boosts Notch signaling levels.

How could Numb convert Spdo from an activator to an inhibitor of Notch signaling? Numb binds directly to Spdo and regulates its subcellular localization, preventing Spdo from localizing to the cell membrane (Hutterer and Knoblich, 2005; Langevin et al., 2005; O'Connor-Giles and Skeath, 2003). If either Notch or an effector is internalized with Spdo by Numb, a quantitative decrease in Notch signaling would result. However, the levels of Notch at the cell membrane appear roughly equivalent between the two daughter cells (Berdnik et al., 2002), suggesting that if *numb* functions in this manner it may do so by targeting a Notch effector rather than Notch itself along with Spdo. Alternatively, small changes in Notch receptor levels may be sufficient to decrease signaling levels below the threshold required to induce the 'A' fate. The elucidation of the precise mechanism through which Spdo exerts opposite effects on Notch pathway activity in the two daughter cells probably awaits the systematic identification of the factors that physically interact with Spdo during asymmetric divisions.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/136/24/4089/DC1>

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Table S1. Overexpression of Notch or Fringe bypasses *spdo* function during the asymmetric division of MP2 to form vMP2 ('A' cell) and dMP2 ('B' cell)

Genotype	Asymmetric divisions (vMP2, dMP2) %	Symmetric divisions (dMP2, dMP2) %	Symmetric divisions (vMP2, vMP2) %	<i>n</i>
Wild type	100	0	0	200
<i>spdo</i> ^{G104}	1.3	98.7	0	234
<i>o/e GFP; spdo</i> ^{G104}	2.3	97.7	0	214
<i>numb</i> ²	0	0	100	200
<i>o/e Spdo</i>	100	0	0	230
<i>o/e Spdo; spdo</i> ^{G104}	82.1	17.9	0	246
<i>o/e Delta</i>	100	0	0	246
<i>o/e Delta; spdo</i> ^{G104}	1.8	98.2	0	224
<i>o/e Delta, o/e Neur</i>	100	0	0	232
<i>o/e Delta, o/e Neur;</i> <i>spdo</i> ^{G104}	11.4	88.1	0.4	236
<i>o/e Notch</i>	85.1	1.1	13.7	450
<i>o/e Notch; spdo</i> ^{G104}	30.8	12.2	57.0	906
<i>o/e Fringe</i>	99.7	0.3	0	308
<i>o/e Fringe; spdo</i> ^{G104}	47.5	42.9	9.6	774

ene overexpression mediated by *prospero-GAL4* via the GAL4/UAS system.

n, number of hemisegments scored.

Table S2. Fringe or Notch overexpression bypasses *spdo* function in the U motoneuron lineage

Genotype	Average number of Eve ⁺ U neurons	Maximum number of Eve ⁺ U neurons	<i>n</i>
Wild type	4.94±0.1	6	7/109
<i>o/e Spdo</i>	5.07±0.12	6	12/222
<i>o/e Delta</i>	4.82±0.12	6	12/212
<i>o/e Neur</i>	4.78±0.14	6	11/202
<i>o/e Neur, Delta</i>	4.92±0.07	6	12/214
<i>o/e Notch</i>	4.90±0.16	6	12/206
<i>o/e Fringe</i>	4.95±0.05	6	12/208
<i>spdo</i> ⁻	0	–	10/200
<i>o/e GFP; spdo</i> ⁻	0	–	11/238
<i>o/e Spdo; spdo</i> ⁻	2.64±0.2	4	10/198
<i>o/e Delta; spdo</i> ⁻	0.15±0.2	3	12/222
<i>o/e Neur; spdo</i> ⁻	0	–	6/108
<i>o/e Neur, Dl; spdo</i> ⁻	0.68±0.43	5	12/216
<i>o/e Notch; spdo</i> ⁻	2.82±1.0	8	17/332
<i>o/e Notch; numb</i> ² ; <i>spdo</i> ⁻	2.90±0.64	7	13/245
<i>o/e Fringe; spdo</i> ⁻	0.68±0.45	6	17/295

Values indicate the average number of Eve⁺ neurons per hemisegment ± s.d.

n, number of embryos scored/number of hemisegments scored.

Spdo⁻ refers to embryos homozygous mutant for *spdo*^{G104}.

Gene overexpression achieved through the use of *prospero-GAL4*.

Table S3. Overexpression of Notch or Fringe bypasses *spdo* function during the asymmetric division of *Svp-lacZ*⁺ precursors to produce sibling *Svp-lacZ*⁺ pericardial cells ('A') and cardioblasts ('B')

Genotype	Temp.	Asymmetric divisions (A, B) %	Symmetric divisions (B, B) %	Symmetric divisions (A, A) %	<i>N</i>	<i>n</i>
Wild type*	25	100	0	0	47	1001
	18	100	0	0	49	945
<i>spdo</i> ^{G104/ZZZ7}	25	13.5	86.5	0	27	459
	18	57.4	41.9	0.8	26	488
<i>spdo</i> ^{AC81/ZZZ7}	25	25.6	74.4	0	29	548
	18	64.0	30.5	5.6	42	827
<i>numb</i> ²	25	0.9	0	99.1	20	229
<i>o/e</i> [†] GFP	25	99.8	0.1	0.1	53	848
<i>o/e</i> GFP; <i>spdo</i> ^{-‡}	25	56.6	39.6	3.8	44	875
<i>o/e</i> Spdo	25	99.8	0.1	0.1	53	829
<i>o/e</i> Spdo; <i>spdo</i> ⁻	25	98.8	0.8	0.4	50	843
<i>o/e</i> Notch	25	94.0	0	6.0	51	949
	18	89.4	0	10.6	49	881
<i>o/e</i> Notch; <i>spdo</i> ⁻	25	55.9	21.3	22.7	52	942
	18	34.9	1.6	63.6	56	835
<i>o/e</i> Fringe	25	14.2	0	85.8	47	910
	18	25.4	0	74.6	65	843
<i>o/e</i> Fringe; <i>spdo</i> ⁻	25	2.0	0	98.0	47	895
	18	2.7	0	97.3	63	803
<i>o/e</i> Delta	25	99.4	0.36	0.24	54	838
<i>o/e</i> Delta; <i>spdo</i> ⁻	25	40.9	58.6	0.5	50	856
	18	66.1	26.4	7.5	28	570
<i>o/e</i> Neur	25	98.7	1.3	0	55	875
<i>o/e</i> Neur; <i>spdo</i> ⁻	25	62.5	31.9	5.6	49	834
	18	68.0	9.0	23.1	20	490
<i>o/e</i> Neur, Delta	25	99.0	0.6	0.4	46	977
<i>o/e</i> Neur, DI; <i>spdo</i> ⁻	25	68.2	24.9	7.0	44	848
<i>o/e</i> Sec15	25	99.7	0	0.3	28	291
<i>o/e</i> Sec15; <i>spdo</i> ⁻	25	32.1	67.6	0.3	63	1279

*Wild type refers to *twist-Gal4/+; UAS-GFP/+; Svp-lacZ/+*.

[†]*o/e* refers to *twist-GAL4*-mediated overexpression of the indicated gene.

[‡]*spdo*⁻ refers to *spdo*^{G104/Svp-lacZ spdo}^{ZZZ7} mutant embryos.

N, number of embryos scored; *n*, number of precursor divisions scored.