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Sanpodo and Notch act in opposition to Numb to distinguish sibling neuron fates in the *Drosophila* CNS

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SUMMARY

In *Drosophila*, most neuronal siblings have different fates ('A/B'). Here we demonstrate that mutations in *sanpodo*, a tropomodulin actin-binding protein homologue, equalize a diverse array of sibling neuron fates ('B/B'). Loss of Notch signaling gives the same phenotype, whereas loss of *numb* gives the opposite phenotype ('A/A'). The identical effect of removing either *sanpodo* or *Notch* function on the fates of sibling CNS neurons indicates that *sanpodo* may act in the *Notch* signaling pathway. In addition, *sanpodo* and *numb* show dosage-sensitive interactions and epistasis experiments indicate that *sanpodo* acts downstream of *numb*. Taken together, these results show that interactions between *sanpodo*, the *Notch* signaling pathway and *numb* enable CNS sibling neurons to acquire different fates.

Key words: *Drosophila*, *Notch*, *Delta*, *numb*, *sanpodo*, Cell fate, Asymmetric division, Cytoskeleton

INTRODUCTION

Development of the *Drosophila* central nervous system (CNS) is characterized by the sequential production of stem-cell-like precursors called neuroblasts, intermediate precursors called ganglion mother cells (GMCs), and finally sibling postmitotic neurons and glia (reviewed in Goodman and Doe, 1993). Neuroblast formation is regulated by the balance of proneural and neurogenic gene activity in the neuroectoderm (reviewed in Campos-Ortega, 1995). Proneural genes are expressed in clusters of about five neuroectodermal cells and promote neuroblast formation, whereas cell interactions mediated by Delta (ligand) and Notch (receptor) restrict the number of neuroblasts to one per cluster. In the absence of *Delta* or *Notch* function, there is an approximate 5-fold increase in all early forming neuroblasts (Brand and Campos-Ortega, 1988; Cabrera, 1990; Martin-Bermudo et al., 1995; Skeath and Carroll, 1992). In most cases, functional Delta/Notch signaling also requires the nuclear proteins Mastermind (Mam), Supressor of Hairless (Su(H)), Neuralized and Enhancer of split (E(spl)) (reviewed in Artavanis-Tsakonas et al., 1995).

The next step of neurogenesis is the repeated asymmetric division of neuroblasts to bud off GMCs. Recent evidence indicates that the asymmetric segregation of intrinsic determinants into the GMC during neuroblast division plays a major role in distinguishing neuroblast and GMC siblings. Within neuroblasts, the Inscuteable protein coordinates mitotic spindle orientation with asymmetric protein and RNA localization so that, when a neuroblast divides, a stereotyped set of gene products asymmetrically segregate into the GMC (Kraut et al., 1996; Shen et al., 1997; Li et al., 1997). These products include Numb, Miranda, Staufen and Prospero proteins, and *prospero* RNA (Rhyu et al., 1994; Hirata et al., 1995; Spana and Doe, 1995; Knoblich et al., 1995; Ikeshima-Kataoka et al., 1997; Shen et al., 1997; Li et al., 1997; Broadus et al., 1998). *prospero* encodes a transcription factor necessary for establishing GMC-specific gene expression (Doe et al., 1991; Vaessin et al., 1991); *staufen* encodes an RNA-binding protein capable of localizing *prospero* RNA (Li et al., 1997; Broadus et al., 1998); *miranda* encodes a Prospero-binding protein that regulates localization and release of Prospero into the GMC (Ikeshima-Kataoka et al., 1997; Shen et al., 1997) and *numb* encodes a membrane-associated protein whose function in GMCs has yet to be determined (Uemura et al., 1989; Rhyu et al., 1994). The combined action of these intrinsic determinants contributes to the specification of GMC fate, but is not sufficient to explain all the differences between neuroblast and GMC cell types.

The last step of neurogenesis is the division of each GMC to produce a pair of postmitotic neurons (or glia); in most cases, the two sibling neurons differ in gene expression and/or axon projections. The mechanism by which GMCs divide to produce sibling neurons with different identities is not well understood. Proteins involved in asymmetric neuroblast/GMC division are either not localized (Prospero), or have not been examined, during sibling neuron division. A role for cell interactions is suggested from cell ablation results in the related grasshopper embryo (Kuwada and Goodman, 1985).

Cell interactions and intrinsic determinants both regulate sibling cell fate in the adult peripheral nervous system (PNS) and in the MP2 CNS lineage. In the adult PNS, the external sense organ precursor produces the SOPIIa and SOPIIb

daughter cells; SOPIIa generates a bristle and a socket cell and SOPIIb divides to make a neuron and a glial cell (Jan and Jan, 1995). Loss of *Notch* or *Delta* function at the time of sense organ precursor division duplicates SOPIIb at the expense of SOPIIa, showing that Notch signaling is required for SOPIIa cell fate (Hartenstein and Posakony, 1990; Schweisguth and Posakony, 1992; Parks and Muskavitch, 1993). Conversely, SOPIIb fate is due to the asymmetric localization of Numb into the SOPIIb cell, where it antagonizes *Notch* function (Uemura et al., 1989; Rhyu et al., 1994). In the MP2 lineage, Numb is partitioned into the dMP2 sibling neuron where it antagonizes Notch signaling to distinguish dMP2/vMP2 sibling cell fates (Spana et al., 1995; Spana and Doe, 1996).

To identify genes controlling sibling neuron cell fate (and other cell fate decisions in the CNS), we performed a saturation mutagenesis of the third chromosome. Here we describe mutations in five genes that result in the equalization of a wide variety of sibling neuron fates throughout the CNS. We find that *sanpodo* (*spdo*; Salzberg et al., 1994), *Delta*, *Notch* and *mam* are required to specify one sibling fate, whereas *numb* antagonizes the function of these genes to specify the other sibling fate. Dosage-sensitive interactions between *spdo* and *numb* indicate that these genes likely act in the same biochemical pathway. Genetic experiments show that *spdo* is downstream of *numb*. *spdo* has recently been shown to encode a homologue of tropomodulin (Dye et al., 1998), a vertebrate pointed-end F-actin-binding protein (Fowler, 1996). Our data suggest that Spdo may be a new member of the Notch signaling pathway and, together with the accompanying paper, raise the possibility of cytoskeletal regulation of Notch signaling.

MATERIALS AND METHODS

Fly strains

Four independent *numb* stocks were used *numb*^{1} *pr cn Bc*/CyO; *numb*^{2} *pr cn Bc*/CyO; *numb*^{3} *pr cn Bc*/CyO and *l(2)06740* from the BDGP which we call *numb*^{4} because it harbors a mutation in the *numb* gene. For a deficiency that removed the *numb* locus we used *w; Df(2L)N22-3/CyO*. Eleven *spdo* alleles were identified in our mutagenesis (J. B. S. and C. Q. D., unpublished data); all yielded identical CNS phenotypes and one (*spdo*^{ZZ27}) does not make detectable *spdo* transcript or protein (data not shown). This suggests that all *spdo* alleles are either null or strong hypomorphs. Two independent *numb;spdo* double mutant stocks were used and both gave identical results: *numb*^{1}*/CyO, P{ftz-lacZ}; spdo*^{G104}*/TM3, P{ftz-lacZ}* and *numb*^{2}*/CyO; spdo*^{ZZ27}*/TM3, P{ftz-lacZ}*. In addition to the ten *Delta* alleles and one *mam* allele identified in our mutagenesis, we used the following fly stocks to analyze the loss-of-function phenotype of different neurogenic genes on CNS sibling cell fate: *w*^{a}*; Df(1)N*^{81K1} *rb/y w f; +/SM1 Dp(1:2)51b, Dl*^{3} *e/TM6 Tb, E(spl)*$^{Drv202.96}$ *tx/TM6 Tb, neu*^{IN94}*/TM3, cn mam*^{IL115} *bw/CyO and l(2)04615/CyO* from the BDGP P-element collection which is a hypomorphic allele of *mam*.

Antibody staining of embryos

Immunohistochemical staining was carried out as described in Skeath et al. (1992) and immunofluorescent staining was carried out as described in Spana et al. (1995). The following dilutions were used: 1:10 monoclonal antibody 2B8 (anti-Even-skipped; Patel et al., 1994); 1:500 anti-phosphohistone H3 (Upstate Biotech.); 1:1000 anti-Zfh-1 mouse polyclonal sera (Lai et al., 1991); 1:10 monoclonal antibody 22C10 (Fujita et al., 1982); 1:3000 rabbit anti-β-galactosidase (Cappel); 1:1000 rabbit anti-Odd (Ellen Ward and Doug Coulter, personal communication).

Quantitation of CNS phenotypes

We scored the number of Eve^{+} RP2 and U neurons in both thoracic and abdominal hemisegments. In wild-type embryos, there are six thoracic and ten abdominal Eve^{+} EL neurons; we only quantitated EL neurons in abdominal hemisegments. To quantitate d/vMP2 neurons, we stained appropriately staged embryos for 22C10 and scored the axonal projections from the cells in the d/vMP2 position. To quantitate mitotic Eve^{+} GMCs, we counted phosphohistone^{+}/Eve^{+} cells at stage 12-14 (for ELs) and stage 11 for medial Eve^{+} cells.

RESULTS

We performed a large scale EMS mutagenesis of the third chromosome, screening for alterations in the CNS pattern of the Even-skipped (Eve) homeodomain transcription factor. Eve is detected in a small number of identified GMCs and neurons (Patel et al., 1989; Broadus et al., 1995) and changes in Eve can be used to detect altered neuroblast, GMC, or neuronal identity (e.g. Doe et al., 1988, 1991; Chu-LaGraff and Doe, 1993; Yang et al., 1993; Bhat et al., 1995; Chu-LaGraff et al., 1995). Here we describe mutations in five genes that equalize sibling neuron fate; results from the entire screen will be described elsewhere.

Markers for identified sibling neurons

We examined six pairs of sibling neurons with unequal fates (RP2/RP2sib, aCC/pCC, dMP2/vMP2 and three pairs of U/Usibs) and five pairs of presumptive sibling neurons with indistinguishable fates (EL/EL neurons) (Fig. 1). RP2/RP2sib develop from the Eve^{+} GMC 4-2a: RP2 is Eve^{+}, expresses the Zfh-1 transcription factor and the 22C10 epitope, and extends a motor axon out the intersegmental nerve (ISN); RP2sib is smaller, downregulates Eve, does not express Zfh-1 or 22C10 (Figs 1, 2A-C, 3A-D; Broadus et al., 1995; Chu-LaGraff et al., 1995). aCC/pCC develop from the Eve^{+} GMC 1-1a: aCC is an Eve^{+}, Zfh-1^{+}, 22C10^{+} motoneuron projecting out the ISN and pCC is an Eve^{+}, Zfh-1^{-}, 22C10^{-} interneuron (Figs 1, 2A-C, 3A-D; Broadus et al., 1995). The dMP2/vMP2 interneurons develop from the Odd-skipped (Odd)-positive MP2 precursor: dMP2 is Odd^{+} with an posterior axon projection, while vMP2 downregulates Odd and has an anterior axon projection (Figs 1, 4A; Spana et al., 1995).

The U/Usib neurons develop from three Eve^{+} GMCs that divide to yield a cluster of six initially Eve^{+} neurons. The three U neurons maintain Eve, whereas the Usib neurons rapidly downregulate Eve (Figs 1, 2A-C). Although we have not used lineage techniques to confirm the U/Usib relationship, we base our sibling assignment on the proximity of these Eve^{+} neurons and their apparent similarity to the Eve expression profile of RP2/RP2sib. In older embryos, two additional Eve^{+} neurons develop near the three Eve^{+} U neurons, but we are unable to determine the identity of their siblings and will not consider them in our analysis.

Each abdominal hemisegment has 9-10 Eve^{+} lateral (EL) neurons (Figs 1, 2C; Patel et al., 1989). Using anti-phosphohistone as a mitotic marker, we have never observed Eve^{+} mitotic GMCs at the EL position (0/290 hemisegments; stages 12-14). In contrast, it is common to observe Eve^{+} mitotic

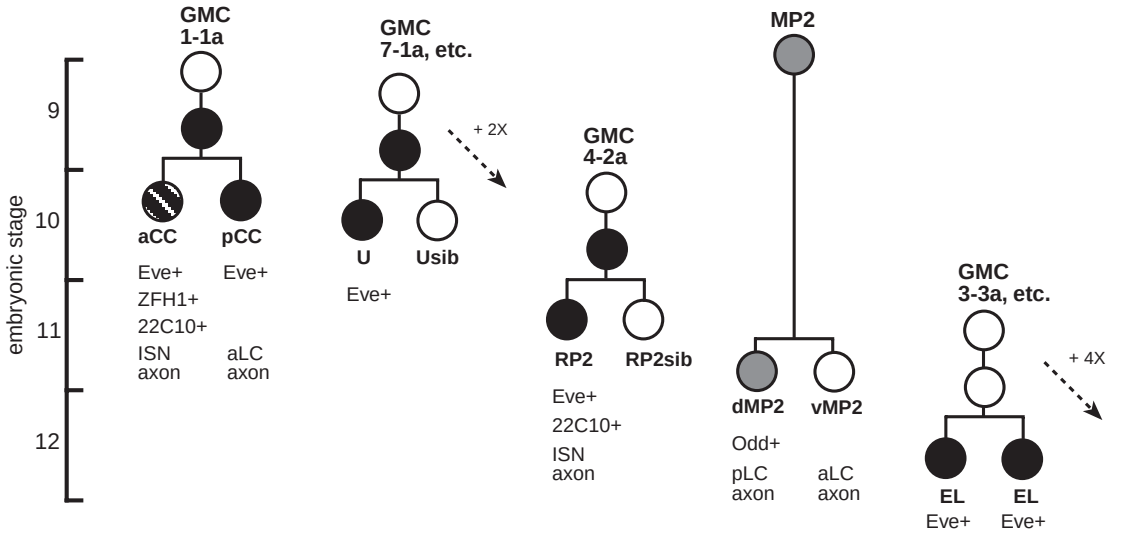

Fig. 1. Cell lineage and molecular markers for the sibling neurons described in this paper. Stages of embryonic development are shown at left (Campos-Ortega and Hartenstein, 1985), molecular markers and axon projections used to distinguish sibling neurons are listed below each neuron. There are two later-born pairs of U/Usib neurons and four later-born pairs of EL neurons (dashed arrows). Eve, even-skipped (black); Odd, odd-skipped (gray); Zfh1 (white cross hatch); pLC, posterior-directed in the longitudinal connective; aLC, anterior-directed in the longitudinal connective; ISN, intersegmental nerve.

GMCs among the medial Eve$^+$ cells (6/36 hemisegments; stage 11). Thus, the EL neurons develop from Eve$^-$ GMCs. The ELs are derived from a neuroblast lineage containing only 10-13 interneurons (Schmidt et al., 1997; A. Schmid and C. Q. D., data not shown), suggesting that the EL neurons are siblings. Thus, it appears that five Eve$^-$ GMCs produce five pairs of Eve$^+$ EL sibling neurons (Fig. 1).

sanpodo is required to establish asymmetric sibling neuron identity

spdo was first identified as a gene controlling neuronal number during embryonic PNS development (Salzberg et al., 1994). We isolated 11 alleles of *spdo* based on dramatic alterations in *eve* expression in the CNS. Embryos homozygous for the null *spdo*^{ZZ27} allele, subsequently called '*spdo* embryos,' show normal Eve$^+$ GMCs (Fig. 2D) but an equalization of sibling neuron identity as detected by Eve and other markers. The RP2 motoneuron is duplicated at the expense of the RP2sib, as shown by staining for Eve (Figs 2D,E, 3E-H), Zfh-1 (Fig. 3E-G) and 22C10 (Fig. 3H). The aCC motoneuron is duplicated at the expense of the pCC interneuron, as shown by staining for Zfh-1, 22C10 and by following axonal projections (Fig. 3E-H). The Usib fates are duplicated at the expense of the U neurons, as shown by Eve staining (Fig. 2D,F). Finally, dMP2 is duplicated at the expense of vMP2, as shown by Odd and 22C10 staining (Fig. 4B). We see no change in the EL neurons Eve$^+$ expression (Fig. 2F); this is not surprising, because each sibling neuron is Eve$^+$ (Fig. 1; see above). We have no markers to differentiate the siblings in this lineage.

Although the *spdo* sibling neuron phenotype is identical to the *Notch* sibling neuron phenotype (see below), none of the 11 *spdo* alleles show the excess neuroblast formation characteristic of *Notch* mutations. Furthermore, *spdo* germline clones yield an Eve CNS phenotype identical to embryos that lack only zygotic *spdo* function (data not shown). Thus, *spdo* does not appear to function during Notch-mediated lateral inhibition in the neuroectoderm.

Notch, *Delta* and *mastermind* are required to establish asymmetric sibling neuron identity

We identified mutations in two other genes, *Delta* (10 alleles) and *mam* (1 allele), that yield similar equalization of sibling neuron fates. Because both genes are in the well-characterized Notch signaling pathway (Artavanis-Tsakonas et al., 1995), we tested null and hypomorphic alleles of several 'Notch pathway' genes: *Delta*, *Notch*, *mam*, *neuralized* and *E(spl).* As expected, mutations in all genes result in an excess of neuroblasts due to failure of lateral inhibition within the neuroectoderm (data not shown); however, we could still score sibling neuron fates due to the specificity of our markers.

Mutations in *neuralized* and *E(spl)* have no effect on the identity of the sibling neurons that we assayed, despite strong defects in the earlier process of neuroblast formation. In contrast, *Delta*, *Notch* and *mam* mutations all yield similar sibling neuron phenotypes in addition to excessive neuroblast formation; we illustrate these results using embryos homozygous for a hypomorphic *mam* allele in which neuroblast formation is essentially normal but sibling neuron fates are equalized. Loss of *mam* does not affect *eve* expression in GMCs (Fig. 2G), but leads to the duplication of RP2, Usib (Figs 2G-I, 3I-L), aCC (Fig. 3I-L) and dMP2 (Fig. 4D) fates at the expense of the RP2sib, U, pCC and vMP2 fates, respectively. We observe no change in the number of Eve$^+$ EL neurons in embryos that lack *Notch*, *Delta* or *mam* function (Fig. 2I; data not shown); this is not surprising, because each sibling neuron appears to be Eve$^+$. Thus, mutations in three genes (*Delta*, *Notch* and *mam*) have precisely the same sibling neuron phenotype as *spdo* mutations, suggesting that *spdo*, *Delta*, *Notch* and *mam* act together to specify asymmetric sibling neuron fate.

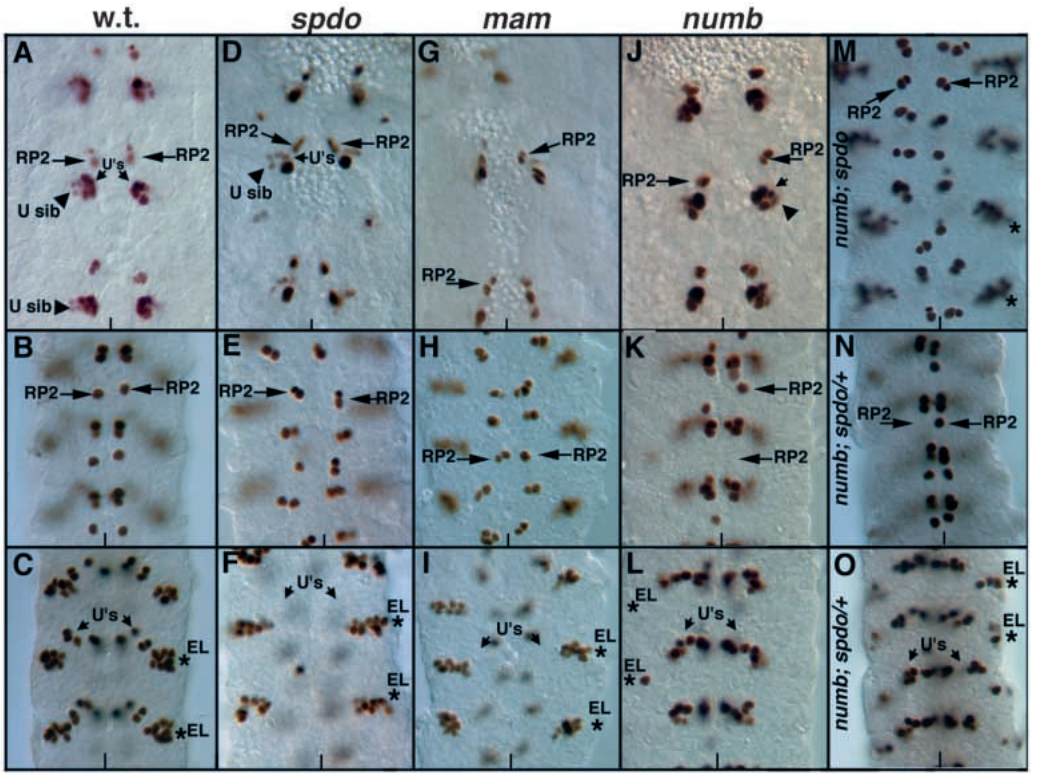

Fig. 2. Mutations in the *Notch/spdo* pathway and *numb* exhibit widespread and opposite effects on CNS sibling cell fates. Three consecutive segments of wild-type (A-C), *spdo* (D-F), *mam* (G-I), *numb* (J-L), *numb; spdo* (M), *numb; spdo/+* (N-O) embryos labeled for Eve protein expression. (A,D,G,J) Ventral views of stage 11 embryos. Dorsal (B,E,H,K,M,N) and ventral (C,F,I,L,O) views of stage 16 nerve cords. Anterior is up; ventral midline, small line. (A) In wild-type embryos, RP2/RP2sib and U/Usib neurons all initially express *eve*, although both RP2sib and the Usibs quickly turn *eve* off. (B,C) At stage 16 only RP2 and the U neurons express *eve*. (D-F) Lack of *spdo* transforms the RP2sib and U neurons into RP2 and the Usib neurons, respectively. (D) RP2/RP2sib and the U/Usib neurons all form and initially express *eve*. (E,F) At stage 16, both RP2 and RP2sib express *eve*; conversely, neither the U nor Usib neurons express *eve*. (G-I) Loss of Notch signaling as illustrated in *mam* embryos duplicates the RP2 and Usib neurons at the expense of RP2sib and the U neurons. (G) RP2/RP2sib and U/Usib neurons all develop and initially express *eve*. (H,I) At stage 16, both RP2 and RP2sib express *eve*, but neither the U nor Usib neurons express *eve*. (J-L) Loss of *numb* yields the opposite phenotype to loss of Spdo/Notch signaling. (J) In *numb* embryos, the RP2/RP2sib and the U/Usib neurons form normally and express *eve*. (K,L) By stage 16 roughly half of all RP2s have extinguished *eve* expression and the Usib neurons still express *eve* resulting in on average three additional Eve^+ neurons within the U-CQ cluster. (M) Embryos doubly mutant for *numb* and *spdo* exhibit an Eve CNS phenotype indistinguishable from embryos that lack only *spdo*. (N,O) Halving the normal copy number of *spdo* in a *numb* mutant background rescues Eve^+ RP2 (Q) and EL neurons (N,O) relative to *numb* embryos (K,L).

Numb antagonizes Notch to specify asymmetric sibling neuron identity

Numb is known to bind to the intracellular domain of Notch and antagonize Notch signaling but, with the exception of the dMP2/vMP2 neurons, it has not been reported to play a role in sibling neuron development in the CNS (Guo et al., 1996; Spana et al., 1995; Spana and Doe, 1996). However, due to the widespread role of *Notch* in specifying asymmetric sibling neuron identity, we re-investigated the CNS function of *numb*. We assayed sibling neuron development using four different *numb* alleles and a deficiency that uncovers the *numb* locus (Table 1). We find that two of these alleles, nb^2 and nb^4, are stronger alleles than those used in previous studies. The CNS phenotypes indicate that the four alleles fall into an allelic series ($nb^2 > nb^4 > nb^1/nb^3$; Table 1). In embryos homozygous for the strongest *numb* allele (nb^2), we observe an equalization of sibling neuron phenotype for all siblings tested, with the exception of aCC/pCC (Fig. 3M-P). RP2 is transformed into RP2sib approximately 50% of the time (Fig. 2J-L; Table 1); three Usibs are transformed into three U neurons (Fig. 2L; Table 1); and dMP2 is transformed into vMP2 (Fig. 4C; Spana et al., 1995). The *numb* phenotypes for RP2, Usib and dMP2 neurons are reciprocal to those observed in *spdo*, *Delta*, *Notch* or *mam* embryos. This is consistent with studies showing that Numb antagonizes Notch function (Guo et al., 1996; Spana and Doe, 1996) and extends this interaction to a diverse array of CNS sibling neurons. In addition, we observe a strong decrease in the number of Eve^+ EL neurons in *numb* mutant embryos (Fig. 2L; Table 1).

There is clear evidence of maternal *numb* function during CNS development (Table 1), which may account for the lack of a fully penetrant *numb* sibling neuron phenotype. For example, when females heterozygous for the weak nb^1 allele are crosses to males heterozygous for the strong nb^2 allele, the nb^2/nb^1 embryos have an intermediate CNS phenotype; however, when females heterozygous for the strong nb^2 allele are crossed to males heterozygous for the weak nb^1 allele, the nb^2/nb^1 embryos have a more severe CNS phenotype (Table 1). Thus, changing the dose of maternal *numb* product directly affects CNS development and suggests that *numb* may have earlier CNS functions in addition to sibling neuron specification.

Genetic interactions and epistasis between *sanpodo* and *numb*

spdo and *numb* have opposite sibling neuron phenotypes and so we determined the epistatic relationship between the two genes by examining the phenotype of a *numb;spdo* double

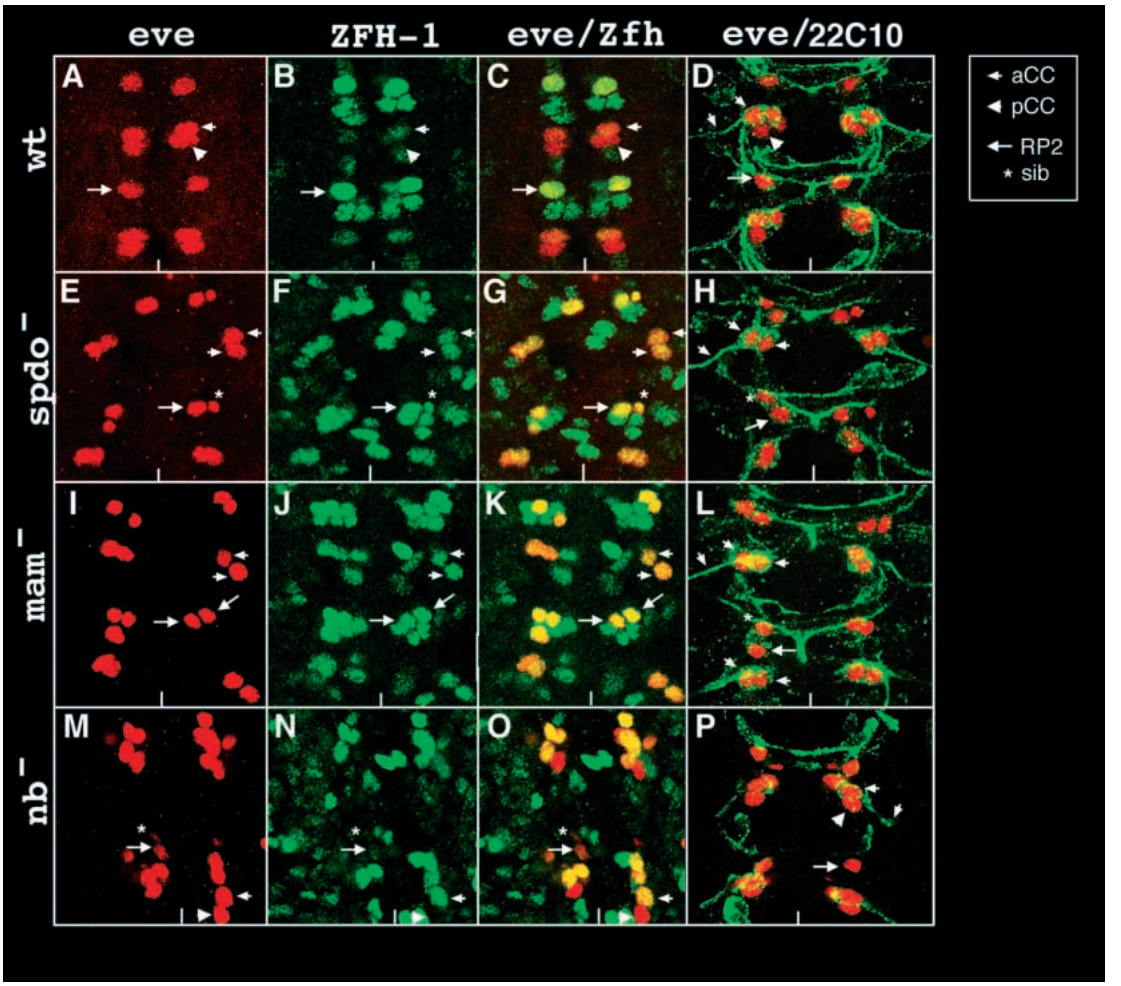

Fig. 3. The *Notch/spdo* pathway and *numb* distinguish RP2/RP2sib and aCC/pCC sibling cell fates. Two consecutive segments of wild-type (A-D), *spdo* (E-H), *mam* (I-L) and *numb* (M-P) mutant embryos labeled for eve (A,E,I,M), Zfh-1 (B,F,J,N), eve and Zfh-1 (C,G,K,O), or eve (red) and 22C10 (green; D,H,L,P) expression. Anterior, top; ventral midline, small line. (A-D) In wild-type embryos, aCC and RP2 express both eve (A,C,D), Zfh-1 (B,C) and 22C10 (D). aCC also extends an 22C10^{+} axon out the intersegmental nerve (small arrow, D). pCC expresses eve (A,C,D) but not Zfh-1 (B,C) or 22C10 (D). (E-H) Loss of *spdo* duplicates the aCC and RP2 fates. Both corner cells express eve (E,G,H), Zfh-1 (F,G) and 22C10 (H) and extend axons out the intersegmental nerve (H) just like aCC. In addition, RP2sib (asterisk) retains eve (E,G,H) expression and now expresses Zfh-1 (F,G) and 22C10 (H) like the endogenous RP2 neuron. (I-L) Removal of Notch signaling as illustrated in *mam* embryos yields the identical phenotype as loss of *spdo*. Both corner cells express Zfh-1 (J,K) and 22C10 (L) in addition to eve (I,K,L) and extend axons out the intersegmental nerve (L). RP2sib now retains eve expression (I,K,L) and expresses Zfh-1 (J,K) and 22C10 (L). (M-P) Loss of *numb* does not alter the pCC fate but duplicates the RP2sib fate. pCC expresses eve (M,O,P) but not Zfh-1 (N,O) or 22C10 (P), while neither RP2sib (asterisk) nor RP2 (arrow) express Zfh-1 (N,O).

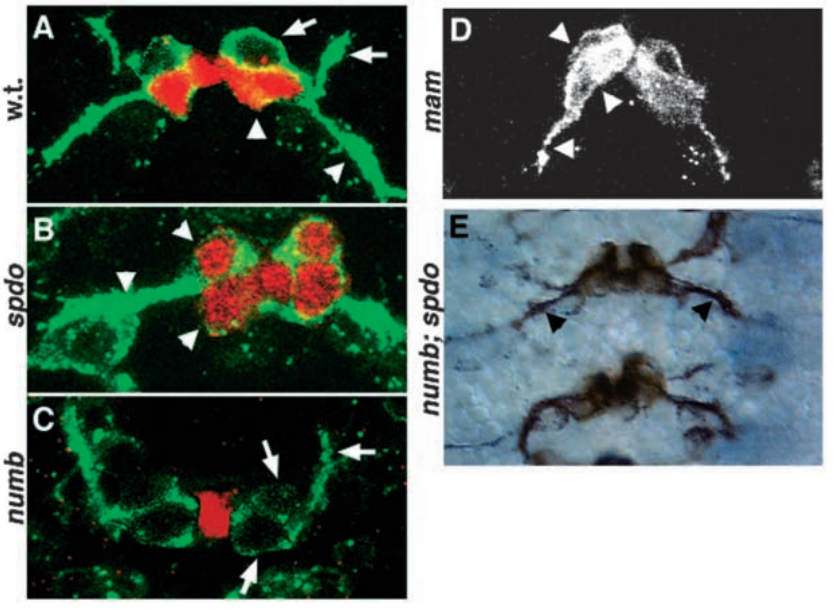

Fig. 4. The *Notch/spdo* pathway and *numb* distinguish dMP2/vMP2 sibling cell fates. Single segments of wild-type (A), *spdo* (B) and *numb* (C) embryos labeled for Odd protein (red) and 22C10 epitope (green) expression, and a single segment of a *mam* (D) embryo and two segments of a *numb; spdo* (E) embryo labeled for 22C10. Anterior, top; ventral midline, small line; dMP2 and its projection, arrowhead; vMP2 and its projection, arrow. (A) In wild-type embryos, dMP2 expresses Odd and projects its axon posteriorly while vMP2 does not express Odd and extends its axon anteriorly. (B) In *spdo* embryos, both d/vMP2 acquire the dMP2 fate and express Odd protein and extend their axons posteriorly. (C) In *numb* mutant embryos, d/vMP2 acquire the vMP2 fate and extend axons anteriorly but do not express Odd protein. (D) In *mam* embryos, d/vMP2 extend axons posteriorly. (E) In *numb; spdo* double mutant embryos, both d/vMP2 acquire the dMP2 fate and extend axons posteriorly.

Table 1. *numb* allelic series reveals maternal *numb* contribution to CNS development

Males		Females				
		Df(2L)N22-3	*numb²*	*numb⁴*	*numb¹*	*numb³*
Df(2L)N22-3	RP2	41%* (160)	32% (302)	74% (200)	95% (297)	99% (204)
	Us	115% (44)†	155% (91)	139% (60)	127% (72)	134% (85)
	ELs	3% (70)†	5% (220)	8% (100)	11% (194)	12% (100)
numb²	RP2	48% (238)	64% (643)	56% (200)	98% (200)	98% (209)
	Us	158% (54)	152% (36)	142% (60)	133% (60)	134% (90)
	ELs	5% (99)	4% (294)	6% (100)	11% (120)	12% (112)
numb⁴	RP2	64% (212)	50% (172)	48% (212)	96% (202)	99% (206)
	Us	140% (66)	139% (48)	146% (60)	130% (60)	135% (60)
	ELs	6% (110)	4% (108)	9% (100)	16% (100)	18% (100)
numb¹	RP2	91% (172)	77% (224)	86% (211)	99% (268)	100% (208)
	Us	139% (54)	135% (60)	132% (60)	126% (132)	128% (84)
	ELs	7% (108)	6% (118)	13% (100)	21% (134)	24% (100)
numb³	RP2	83% (228)	68% (216)	87% (200)	99% (220)	97% (222)
	Us	135% (69)	135% (66)	130% (60)	124% (66)	132% (68)
	ELs	8% (118)	10% (132)	9% (100)	22% (132)	24% (105)

*Percentages give percent formation relative to wild-type embryos; number of hemisegments scored indicated in parentheses. Wild-type embryos have 1.0 RP2s (233/234), 4.94 Us (494/100) and 9.17 ELs (917/100).

†Homozygous Df(2L)N22-5 embryos exhibit apparently multinucleate U and EL neurons. This apparent cytokinetic defect likely arises due to the loss of one or more genes in addition to numb.

mutant. We find that the *numb;spdo* double mutant phenotype is identical to embryos lacking *spdo* alone (Figs 2M, 4E). Thus, *spdo* is genetically downstream of numb, just as has been observed for Notch pathway mutations in other lineages (Spana and Doe, 1996; Guo et al., 1996).

We next assayed whether *sanpodo* and *numb* exhibit dosage-sensitive interactions, as gene products that act in the same biochemical pathway often do. We find that the sibling neuron phenotype in *numb* embryos is sensitive to the level of *spdo*. For example, homozygous *nb*^{2} embryos show a loss of EL and RP2 neurons, but reducing the dosage of *spdo* by one-half in *nb*^{2} embryos leads to a recovery of Eve$^+$ EL and RP2 neurons (Fig. 2N,O; Table 2). We observed similar results using independently isolated alleles of both *numb* and *spdo* (Table 2). Thus, halving the dosage of *spdo* strongly suppresses the *numb* CNS phenotype. These results show that the *numb* phenotype is extremely sensitive to the dosage of *spdo*, consistent with the two proteins acting in the same biochemical pathway.

Table 2. Epistatic relationship between *sanpodo* and *numb*

numb¹		*numb²*		wild-type
RP2	99%* (268)	RP2	64% (643)	1.00† (233/234)‡
Us	126% (132)	Us	152% (36)	4.94 (494/100)
ELs	21% (134)	ELs	4% (294)	9.17 (917/100)

numb¹ ; spdo^{G104}/+		*numb² ; spdo^{zz27}/+*	
RP2	99.6% (284)	RP2	86% (573)
Us	113% (101)	Us	133% (90)
ELs	51% (151)	ELs	18% (290)

numb¹ ; spdo^{G104}		*numb² ; spdo^{zz27}*	
RP2	198% (244)	RP2	200% (112)
Us	0% (242)	Us	0% (112)
ELs	102% (100)	ELs	99% (52)

*Percentages give percent formation relative to wild-type embryos; number of hemisegments scored indicated in parentheses.

†Indicates average number of Eve-expressing RP2, U or EL neurons found per hemisegment.

‡Indicates total number of eve-Expressing RP2s, Us or ELs counted over the total number of hemisegments scored.

The function of *numb* and *sanpodo* in the EL lineage

In *numb* embryos, there is a striking decrease in the number of Eve$^+$ EL neurons (Fig. 2L; Table 2). *Notch*, *Delta*, *mam* and *spdo* single mutants do not alter the number of Eve$^+$ EL neurons (Fig. 2; data not shown for *Notch* and *Delta*). Importantly, *numb;spdo* double mutant embryos show a complete rescue of Eve$^+$ EL neurons (Fig. 2M; Table 2), suggesting that Numb acts to prevent Spdo-mediated downregulation of *eve* expression (i.e. in the absence of Spdo, the loss of Numb is irrelevant). These data are consistent with a model in which Numb blocks Notch/Spdo-mediated downregulation of *eve* in the neurons of the EL lineage (see Discussion).

DISCUSSION

The Notch/Numb/Sanpodo pathway and asymmetric sibling neuron fate

We have shown that the Notch/Numb/Spdo pathway regulates asymmetric cell fate between many sibling neurons in the CNS. Our model (Fig. 5) is that Numb protein is asymmetrically segregated during GMC mitosis into one neuronal sibling where it blocks Notch/Spdo signaling, resulting in ‘B’ cell fate. In contrast, the neuronal sibling lacking Numb protein has an active Notch/Spdo signaling pathway, resulting in the ‘A’ cell fate. In addition, the Notch/Numb/Spdo pathway regulates binary sibling cell fate in the embryonic PNS (Dye et al., 1998), suggesting that it plays a fundamental and widespread role in establishing asymmetric sibling cell fates in multiple *Drosophila* tissues. Because *Notch* controls such a diverse array of cell fates, its activity is unlikely to specify directly a particular cell fate, but rather to allow sibling cells to respond differently to a shared environment of intrinsic or extrinsic cues. A current model is that Notch activity delays cell

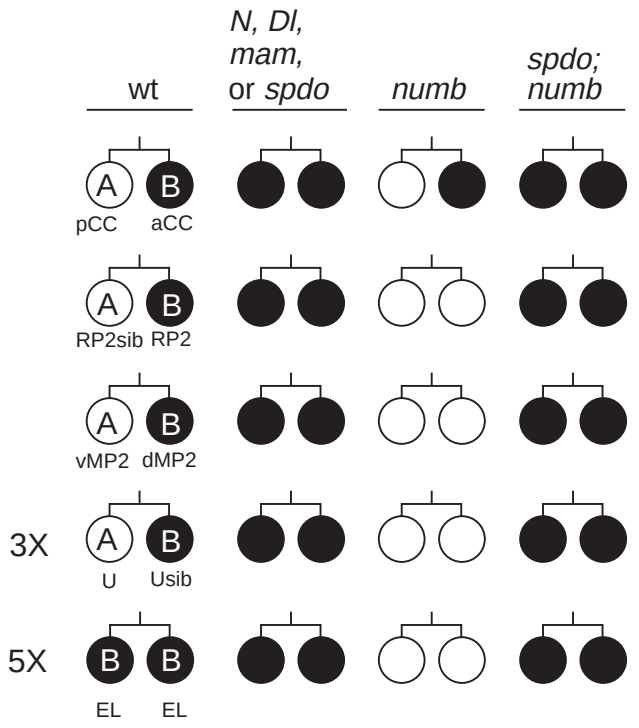

Fig. 5. Summary of *spdo/Notch* and *numb* CNS phenotypes. Each pair of sibling neurons has a Spdo/Notch-dependent 'A' cell fate (white) and a Numb-dependent 'B' cell fate (black). Numb is asymmetrically localized into the 'B' cell in the MP2 lineage (Spana et al., 1995); for the other lineages, we propose that Numb is inherited asymmetrically (aCC, RP2, Usib lineages) or equally (EL lineage) into the 'B' cell (black). *spdo*, *Notch*, *Delta* and *mam* mutations have an identical transformation of 'A' into 'B' cell fate (black). *numb* mutations transform 'B' into 'A' cell fate; the aCC/pCC neurons are unaffected, and the U/Usib and RP2/RP2sib lineages show partially penetrant phenotypes, probably due to persistent maternal Numb protein (see text).

determination, allowing two adjacent cells to respond differently in an environment of extrinsic signals that are spatially uniform but temporally distinct (e.g. Fortini et al., 1993; Artavanis-Tsakonas et al., 1995; Dorsky et al., 1997). It is likely that sibling neurons use the Notch/Numb/Spdo pathway in a similar manner, but to generate two distinct cell fates in combination with intrinsic factors present in each GMC (and different in each GMC). This could occur by two mechanisms: (1) Notch signaling might actively specify cell fate in conjunction with existing intrinsic factors, or (2) Notch signaling might delay differentiation of one sibling until the intrinsic factors have changed (due to cell intrinsic or extrinsic events). In either case, the result is two different sibling cell fates.

Loss of *numb* affects all sibling neurons assayed except aCC/pCC. However, maternal *numb* product may control aCC/pCC fate as well, because there is a clear maternal *numb* contribution to CNS development (Table 1) and aCC/pCC are the earliest sibling neurons to form among those assayed here (see Fig. 1). Alternatively, *numb* might have no function in this lineage and binary fates could be determined by restricted activation of Notch in the future pCC due to restricted distribution of a Notch ligand, or to competitive 'lateral inhibition' between the aCC/pCC siblings (as ablation experiments suggest for the aCC/pCC siblings in the grasshopper embryo; Kuwada and Goodman, 1985).

During mammalian neurogenesis, proliferating cells within the ventricular zone of the brain can produce siblings with different fates: one cell remains in the ventricular zone as a proliferating precursor, while the sibling migrates away and may differentiate as a neuron (Chenn and McConnell, 1995). Vertebrate homologues of *Notch*, *numb* and *spdo* are known (Zhong et al., 1996; Fowler, 1996; Chenn and McConnell, 1995), raising the possibility that the Notch/Numb/Spdo signaling pathway may be an evolutionarily conserved mechanism for establishing asymmetric sibling cell fates in the CNS.

The Notch/Numb/Sanpodo pathway and EL sibling neuron fate

The ten Eve$^+$ EL neurons appear to be five pair of siblings that develop from five Eve$^-$ GMCs: we never detect Eve$^+$ mitotic GMCs within this lineage, the EL lineage contains only 10-13 neurons and there is no evidence for apoptosis in this lineage (Schmidt et al., 1997; A. Schmid and C. Q. D., data not shown). *numb* is required for *eve* expression in the EL neurons, but loss of *spdo*, *Notch*, *Delta* or *mam* does not affect the number of Eve$^+$ EL neurons. Importantly, the concomitant removal of both *spdo* and *numb* completely restores the normal number of Eve$^+$ EL neurons. These data are consistent with a model in which Notch/Spdo signaling represses *eve* expression in the EL neurons but, during wild-type development, Numb is equally distributed to both siblings at mitosis and thereby blocks Notch signaling in both EL sibling neurons (Fig. 5). Determining the distribution of Numb in the mitotic GMCs of the EL lineage awaits the development of a marker for these GMCs.

Sanpodo: a new member of the Notch pathway?

Mutations in *spdo*, *Notch*, *Delta* and *mam* all yield the identical sibling neuron phenotypes, suggesting that Spdo may be involved in the Notch signaling pathway. *spdo* encodes a homologue of vertebrate tropomodulin (Dye et al., 1998). Tropomodulin caps the pointed ends of microfilaments and is thought to regulate their length (Fowler, 1996). Spdo could regulate the distribution of either Delta ligand or Notch receptor in the membrane, for example, to facilitate 'capping' observed when these two proteins interact in cell culture (Fortini and Artavanis-Tsakonas, 1994). Alternatively, Spdo could regulate the intracellular trafficking or processing of Notch protein from the endoplasmic reticulum to the plasma membrane required to produce a functional receptor (Pan and Rubin, 1997; Blaumueller et al., 1997).

Another interesting possibility is that Spdo may play a role in Notch signaling from the membrane to the nucleus. Notch signal transduction is not fully understood. The Notch intracellular domain is necessary and sufficient for Notch signaling, it can be detected in the nucleus in cell culture or when overexpressed, its nuclear localization is essential for its function and it can be released from a membrane tether via proteolytic cleavage (Coffman et al., 1993; Rebay et al., 1993; Struhl et al., 1993; Lieber et al., 1993; Kopan et al., 1994, 1996). The Su(H) transcription factor (Furukawa et al., 1991; Schweisguth and Posakony, 1992) can bind to the intracellular domain of Notch and is translocated into the nucleus upon

activation of Notch in tissue culture assays (Fortini and Artavanis-Tsakonas, 1994). Taken together, these results suggest that Notch signal transmission may occur via ligand-induced cleavage of the intracellular domain of Notch followed by its nuclear translocation, where it functions with Su(H) to activate transcription. The endogenous Notch intracellular domain has never been observed in the nucleus, however, and thus the exact mechanism of Notch signal transduction remains unresolved. To elucidate the role, if any, Spdo plays during Notch signaling, it will be important to determine where it acts within the Notch pathway and whether it physically associates with any members of the Notch pathway.

Are Sanpodo and Numb tissue or cell-type specific members of the Notch pathway?

Notch is known to function in many different tissues to control cell fate. Notch signaling mediates 'lateral inhibition' in the ectoderm and mesoderm which controls the number of neural and muscle precursors (Cabrera, 1990; Corbin et al., 1991; Skeath and Carroll, 1992; Martin-Bermudo et al., 1995), as well as cell interactions during oogenesis, eye development and limb patterning (e.g. Cagan and Ready, 1989; Ruohola et al., 1991; Kim et al., 1996; for review see Artavanis-Tsakonas et al., 1995). It is possible that the Notch pathway utilizes different components in each tissue. *E(spl)* and *neuralized* have no role in sibling neuron specification (data not shown), although they are essential for the earlier process of Notch-mediated lateral inhibition (Martin-Bermudo et al., 1995; Skeath and Carroll, 1992). Conversely, we have shown that *spdo* regulates *Notch*-mediated sibling cell fate decisions but is not involved in *Notch*-mediated lateral inhibition. In addition, cell clones homozygous for *numb*^{3} do not affect lateral inhibition in imaginal discs (Rhyu et al., 1994), but this is not a null allele of *numb*. To test whether *numb* is an obligate member of the *Notch* signaling pathway, it will be necessary to make germline and imaginal disc clones using null alleles of *numb*. Nonetheless, one interesting possibility is that *spdo* and *numb* regulate *Notch* signaling in non-epithelial cells (e.g. sibling neurons) but do not affect *Notch*-based signaling in epithelia (e.g. neuroectoderm or imaginal discs).

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