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Numb Independently Antagonizes Sanpodo Membrane Targeting and Notch Signaling in *Drosophila* Sensory Organ Precursor Cells

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In *Drosophila*, mitotic neural progenitor cells asymmetrically segregate the cell fate determinant Numb in order to block Notch signaling in only one of the two daughter cells. Sanpodo, a membrane protein required for Notch signaling in asymmetrically dividing cells, is sequestered from the plasma membrane to intracellular vesicles in a Numb-dependent way after neural progenitor cell mitosis. However, the significance of Numb-dependent Sanpodo regulation is unclear. In this study, we conducted a structure–function analysis to identify the determinants of Sanpodo targeting in vivo. We identified an NPAF motif in the amino-terminal cytoplasmic tail of Sanpodo, which is conserved among insect Sanpodo homologues. The Sanpodo NPAF motif is predicted to bind directly to the Numb phosphotyrosine-binding domain and is critical for Numb binding in vitro. Deletion or mutation of the NPAF motif results in accumulation of Sanpodo at the plasma membrane in Numb-positive cells in vivo. Genetic analysis of Sanpodo NPAF mutants shows that Numb-dependent Sanpodo endocytic targeting can be uncoupled from Notch signaling regulation. Our findings demonstrate that Sanpodo contains an evolutionarily conserved motif that has been linked to Numb-dependent regulation in vertebrates and further support the model that Numb regulates Notch signaling independently of Sanpodo membrane trafficking in neural progenitor cells.

INTRODUCTION

Asymmetric cell division is an important evolutionarily conserved mechanism for establishing different cell fates during development. The sensory organ precursor cells (SOPs) in *Drosophila* are a well-established system for dissecting the genetic determinants required for controlling Notch-mediated cell fate decisions (Fischer *et al.*, 2006; Knoblich, 2008). The sensory organ precursor cell divides to generate two secondary progenitor cells, the Notch-activated pIIa cell and the Notch-suppressed pIIb cell. Numb is a membrane-associated Notch-signaling inhibitor and Notch-binding protein, which contains a phosphotyrosine-binding (PTB) domain that is essential for its Notch-inhibitory function (Rhyu *et al.*, 1994; Frise *et al.*, 1996; Guo *et al.*, 1996). In sensory organ precursor cells, Numb is asymmetrically localized during mitosis and segregated exclusively to the pIIb daughter cell. Evidence points to Numb having an evolutionarily conserved role as an endocytic adaptor protein (Santolini *et al.*, 2000; Berdnik *et al.*, 2002). Numb has been shown to promote the targeting of Sanpodo, a transmembrane protein required for Notch signaling, to cytoplasmic vesicles after asymmetric cell division (O'Connor-Giles and Skeath, 2003; Hutterer and Knoblich, 2005; Roegiers *et al.*, 2005). These findings have led to the suggestion that Numb may antagonize Notch signaling by inhibiting Sanpodo membrane localization (O'Connor-Giles and Skeath, 2003), but the role of Sanpodo membrane trafficking in Notch signaling regulation is currently unclear.

In this study, we set out to determine the molecular determinants of Sanpodo membrane regulation. We generated a functional Sanpodo-green fluorescent protein (GFP) transgene that rescues the *sanpodo* mutant phenotype and recapitulates Sanpodo's localization and regulation by Numb. We demonstrate that the Sanpodo amino-terminal tail is necessary and sufficient for Numb-dependent endocytic targeting in vivo. By comparing Sanpodo homologues in insects, we identified a conserved NPAF sequence, which is a consensus motif for PTB domain binding. Using molecular modeling and biochemistry, we show that the Sanpodo NPAF motif is required for Numb PTB domain binding in vitro. On the basis of the current model of Sanpodo regulation by Numb, we hypothesized that uncoupling Sanpodo from Numb would increase Sanpodo accumulation at the plasma membrane, resulting in Notch overactivation. Surprisingly, we find that although Numb antagonizes Sanpodo plasma membrane targeting by direct interaction between the Numb PTB domain and Sanpodo NPAF motif, this interaction is dispensable for Notch inhibition, which suggests that Numb regulates Sanpodo trafficking and Notch signaling independently.

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MATERIALS AND METHODS

Generation of Wild-Type and Mutant Sanpodo-GFP Transgenes

The PfuII (Stratagene, La Jolla, CA) amplified coding region of Sanpodo was cloned into a pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA) and swapped by LR recombination into the *Drosophila* Gateway pTWG destination

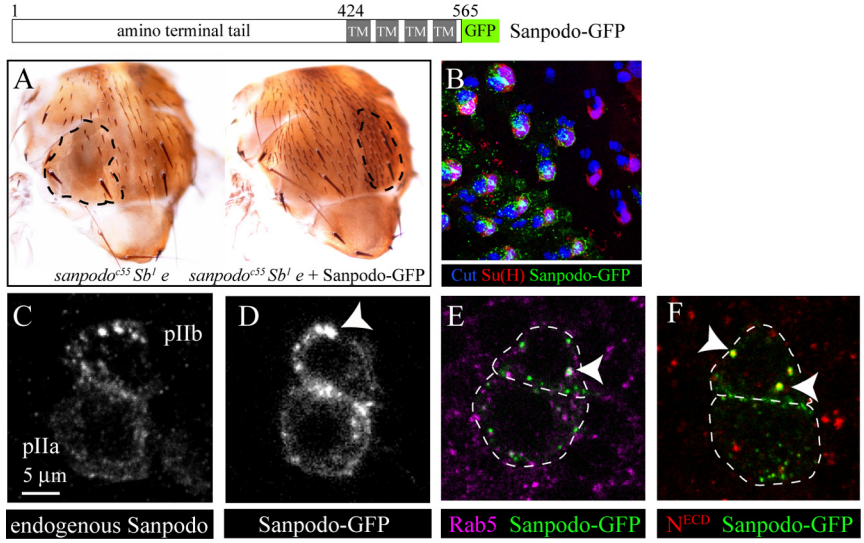


Figure 1. Analysis of Sanpodo-GFP function and localization in vivo. Schematic of wild-type Sanpodo transgene tagged with GFP at the C-terminus (TM, transmembrane). (A) Sanpodo-GFP fully restores the wild-type bristle pattern in *sanpodo*^{C55} mutant clones, which typically show extensive balding. (B) Clusters of four to five Cut-positive (blue) cells have a single Cut-positive, Su(H)-positive cell (magenta) in both wild-type and *sanpodo*^{C55} MARCM mutant clones expressing Sanpodo-GFP (green; white dashed line delineates the clone border). (C) Immunocytochemistry of endogenous Sanpodo in the pIIa and pIIb cell (here and in all subsequent figures pIIb cells are at the top, and pIIa cells are at the bottom). (D) Ten minutes after SOP cell mitotic exit, Sanpodo-GFP localizes to small puncta and to the membrane in the pIIa cell, whereas in the pIIb cell, Sanpodo-GFP localizes to large puncta (white arrowhead). (E and F) Sanpodo-GFP (green) colocalizes with Rab5 (magenta, E) and Notch^{ECD} (red, F) in pIIb cells. Genotypes: (A and B) *yw, ubx-FLP; sca-Gal4, UAS-sanpodo-GFP/+; FRT82B sanpodoC55 Sb1 e/FRT82B tub-Gal80*. Similar results were obtained with a rescue of *FRT82B sanpodo*^{G104} *e* clones. (C) *yw* (D–F) *yw; neur-Gal4/UAS-sanpodo-GFP*.

vector containing the UAS–carboxy-terminal GFP (T. Murphy, Carnegie). Sanpodo deletion mutant constructs were made by using primers containing targeted deletions. Site-specific mutants of Sanpodo were generated by using QuickChange II mutagenesis kit (Stratagene, Torrey Pines, CA). mCD8-Sanpodo chimeric DNA insert was generated by splicing using overlap extension PCR and then swapped into the pTWG vector. Transgenic fly lines were generated by Bestgene (Chino Hills, CA). Independent GFP-tagged transgene lines inserted in both the second and third chromosomes behaved similarly in our experiments.

Drosophila Genetics, Imaging, and Immunohistochemistry

We used the Gal4/UAS system (Brand and Perrimon, 1993) to express the Sanpodo-GFP transgenes using tissue-specific Gal4 lines. Genetic mosaics were generated using either *yw ubx-flp* (kindly provided by J. Knoblich, IMBA, Vienna, Austria) or *yw hs-flp* on the X chromosome (Justice *et al.*, 2003). MARCM stocks used were *tub-Gal80 FRT40A* and *FRT82B tub-Gal80* (Lee and Luo, 2001). Gal4 lines used for nervous system–specific expression were *neur-Gal4/TM3* and *scaGal4/CyO* as previously described in Roegiers *et al.* (2001) and Justice *et al.* (2003). Mutant fly strains used were *yw; y+ nb*^{2} *ck FRT40A/CyO, lgl*^{4} *FRT40A/CyO, ada*^{ear4} *FRT40A/CyO*, w; *FRT82B sanpodo*^{C55} *Sb*^{1} *e/TM6, y+*, w; *FRT82B sanpodo*^{G104} *e/TM6, y+, FRT82B sec15*^{1}*/TM6, FRT82B sec15*^{2}*/TM6*, and *UAS-numb-myc*. Crosses and fly stocks were maintained at 20 or 25°C. The following antibodies were used: rabbit anti-Sanpodo (1/500, kindly provided by J. Skeath, Washington University, St. Louis, MO), rabbit anti-Rab5 (1:50, kindly provided by M. Gonzalez-Gaitan, University of Geneva, Geneva, Switzerland), mouse anti-N^{ECD} (1:200, 458.2H, DSHB), mouse anti-Cut (1:100, 2B10, DSHB), and rat anti-Su(H) (1:500, kindly provided by F. Schweisguth, ENS, Paris, France). All images were acquired on a Nikon TE2000U inverted microscope equipped with a Nikon C1 confocal imaging system (488-, 543-, 633-nm lasers) or the Nikon SFC (Swept Field Confocal) live imaging system (488/514-nm laser; Melville, NY). All measurement were done using Nikon EZ-C1 software. Antibody labeling and live imaging of pupae were carried out as described in Roegiers *et al.* (2001).

Coimmunoprecipitation

Drosophila S2 cells, 5 × 10^{6}, in a 10-cm plate were transfected with 2 μg of pUAS-Numb-Myc (kindly provided by J. Knoblich) and 1.0 μg of pActin-Gal4 (kindly provided by T. Volk, Weizmann Institute, Rehovot, Israel) in addition to 2 μg of pAWF Sanpodo mutant constructs. After lysis in 1 ml of RIPA buffer 48 h after transfection, the cell lysates were incubated with 40 μl of anti-Myc agarose (Sigma, St. Louis, MO) at 4°C overnight after being precleared in 40 μl of mouse IgG agarose (Sigma). The immunoprecipitates were washed four times in 1× TBS Tween 20 buffer and run on NuPAGE gels (Invitrogen, Carlsbad, CA) along with input controls. The blots were detected with anti-Flag-HRP (Sigma) and anti-Myc (Covance, Madison, WI).

Sequence Alignments and Molecular Modeling

The alignments were produced using a multiple alignment editor Jalview (Clamp *et al.*, 2004). Multiple sequence alignments of Sanpodo were performed with ClustalW (Thompson *et al.*, 1994). The sequence of Numb was aligned to potential templates using the program MolIDE (Canutescu *et al.*, 2003), and side chain conformations of the protein and peptide were predicted with the program SCWRL (Canutescu *et al.*, 2003; Wang *et al.*, 2008), allowing all side chains to move. Structure figures were produced with the program Chimera (UCSF; for further details, see Supplementary Material).

RESULTS

Sanpodo-GFP Recapitulates Sanpodo Protein Function and Localization In Vivo

We generated several Sanpodo transgenes, including the full-length Sanpodo coding region and several truncation mutants, tagged with a carboxy-terminal GFP under the control of an upstream activating sequence (UAS; Figure 1 and see Figure 3). We introduced these transgenes into flies in order to study Sanpodo protein trafficking and function in dividing SOPs during pupal neurogenesis. First, we conducted a series of experiments including in vivo rescue, live cell imaging, and immunohistochemical labeling to determine whether the full-length Sanpodo-GFP fusion protein recapitulates the function and localization of endogenous Sanpodo protein. Here, we used the Mosaic Analysis with a Repressible Cell Marker (MARCM) system (Lee and Luo, 2001) to express Sanpodo-GFP in SOPs in *sanpodo* mutant clones in order to assess the ability of the Sanpodo-GFP transgene to restore the wild-type bristle pattern in mutant flies. Mosaic *sanpodo* mutant clones on the fly thorax exhibit significant bristle loss (~70% of mutant sensory organs) and overproduction of neurons due to a failure to induce Notch signaling to specify the pIIa progenitor cell (Hutterer and Knoblich, 2005; Jafar-Nejad *et al.*, 2005; Langevin *et al.*, 2005; Roegiers *et al.*, 2005). Sanpodo-GFP expression fully suppresses the balding and restores the missing hair and socket cells (Figure 1A). Immunohistochemical labeling confirms that rescued *sanpodo* mutant sensory organ cells (as marked by expression of Sanpodo-GFP) express the socket cell marker and Notch target gene *Suppressor of Hairless* [*Su(H)*] at wild-type frequency in clones (Figure 1B). From these data, we conclude that full-length Sanpodo-GFP is functional in promoting Notch signaling and establishing correct cell fates in progenitor cells of the adult peripheral nervous system (PNS).

Next, we tested whether Sanpodo-GFP exhibits a similar subcellular localization in neural progenitor cells as endog-

enous Sanpodo protein. In previous studies, Sanpodo protein has been shown to localize primarily at the plasma membrane in the pIIa daughter cell and to endocytic vesicles in the pIIb daughter cell after SOP asymmetric cell division (Hutterer and Knoblich, 2005; Jafar-Nejad *et al.,* 2005; Langevin *et al.,* 2005; Roegiers *et al.,* 2005). Similarly, Sanpodo-GFP forms large cytoplasmic puncta (>0.7 μm) in the pIIb cell, while in the pIIa cell, Sanpodo-GFP is detected at the plasma membrane and in small cytoplasmic puncta (<0.7 μm; Figure 1, C and D). As expected, in fixed samples we find that the large cytoplasmic puncta of Sanpodo-GFP in pIIb cells colocalize with the early endosome marker Rab5 and with the Notch receptor (Figure 1, E and F). To study the spatial and temporal dynamics of Sanpodo-GFP protein during and after SOP mitosis in live pupae, we used confocal imaging. The behavior of Sanpodo-GFP was remarkably consistent (n = 26 cells) and can be grouped into two phases after SOP mitosis (Figure S1 and data not shown): in the first phase (0–10 min), Sanpodo protein is localized to large vesicles in the pIIb cell, whereas in the pIIa cell, Sanpodo is targeted to the plasma membrane region adjacent to the pIIb cell. In the second phase (10–20 min), Sanpodo remains at the pIIa cell membrane and in small vesicles, whereas in the pIIb cell, Sanpodo is found in large vesicles that colocalize with early and late endosome markers. From these observations we conclude that Sanpodo-GFP localization mimics endogenous Sanpodo protein in the SOP and its daughter cells.

Sec15 Promotes and Numb Antagonizes Sanpodo Accumulation at the Plasma Membrane Interface

Regulation of Sanpodo protein membrane trafficking has been proposed as a mechanism to control Notch activation during asymmetric cell division (O'Connor-Giles and Skeath 2003). We were interested in determining how the dynamics of Sanpodo membrane trafficking would be affected by mutations in genes previously shown to regulate Sanpodo protein localization in fixed samples. Similarly to previously reported distribution of endogenous Sanpodo (O'Connor-Giles and Skeath, 2003; Hutterer and Knoblich, 2005; Langevin *et al.,* 2005; Roegiers *et al.,* 2005), loss of function of either *numb, lethal giant larvae,* or *α-adaptin* results in an increase of Sanpodo-GFP at the plasma membrane and a decrease in size and number of Sanpodo-GFP–positive intracellular vesicles in pIIb cells (Figure 2, A–D). Strikingly, we observe a strong enrichment of Sanpodo-GFP at the pIIa-pIIb cell interface in *numb, lethal giant larvae,* and *α-adaptin* mutants shortly after completion of SOP mitosis and that persists for another 5–10 min. Interestingly, we had periodically observed enrichment of the endogenous Sanpodo protein at the plasma membrane interface in fixed samples in *numb, lethal giant larvae,* and *α-adaptin* mutants (Roegiers *et al.,* 2005 and data not shown). Our live imaging suggests that *numb, lethal giant larvae,* and *α-adaptin* function to antagonize the accumulation of Sanpodo to the pIIa/pIIb cell plasma membrane interface region shortly after SOP mitosis. In contrast, overexpression of Numb results in depletion of Sanpodo-GFP from the pIIa/pIIb cell interface shortly after SOP mitosis (Figure 2E, 91% n = 35 pIIa/pIIb cell pairs), supporting the notion that Numb antagonizes the plasma membrane-associated Sanpodo protein during and after SOP cell mitosis.

We visualized Sanpodo-GFP dynamics in *sec15* mutant clones. Sec15 is a member of the exocyst complex, which delivers proteins to targeted sites in the plasma membrane and is required to promote Notch signaling in SOP lineage cells (Jafar-Nejad *et al.,* 2005). We observe that in *sec15* mutant clones, Sanpodo-GFP is largely diffuse in the cytoplasm and in a few vesicles, but fails to accumulate at the plasma membrane interface (Figure 2F, n = 12 pIIa/pIIb cell pairs) after SOP mitosis. This observation is similar to the finding reported by Jafar-Nejad *et al.* (2005), but we did not observe the large, strongly labeled Sanpodo-GFP–positive intracellular vesicles observed in their study at these early time points after asymmetric cell division, perhaps indicating that large Sanpodo vesicles form at later time points. From our live imaging study, we conclude that efficient delivery of Sanpodo to the plasma membrane interface requires the function of the exocyst in both pIIa and pIIb cells, whereas Numb antagonizes Sanpodo accumulation at the membrane interface in the pIIb cell. Taken together, these observations show that Sanpodo-GFP appears to recapitulate many aspects of Sanpodo behavior in the PNS in vivo: function in the Notch pathway, protein localization in progenitor cells,

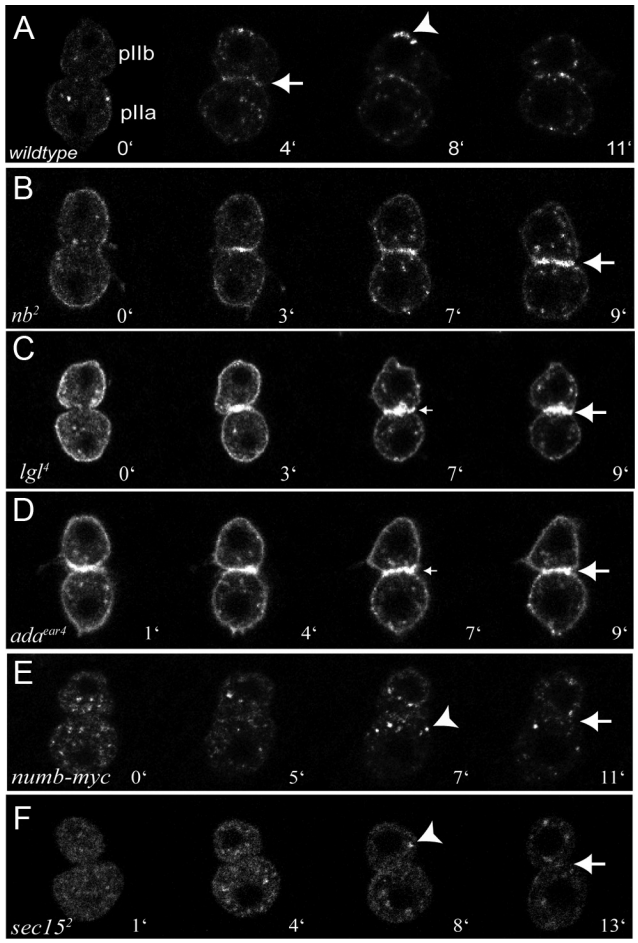
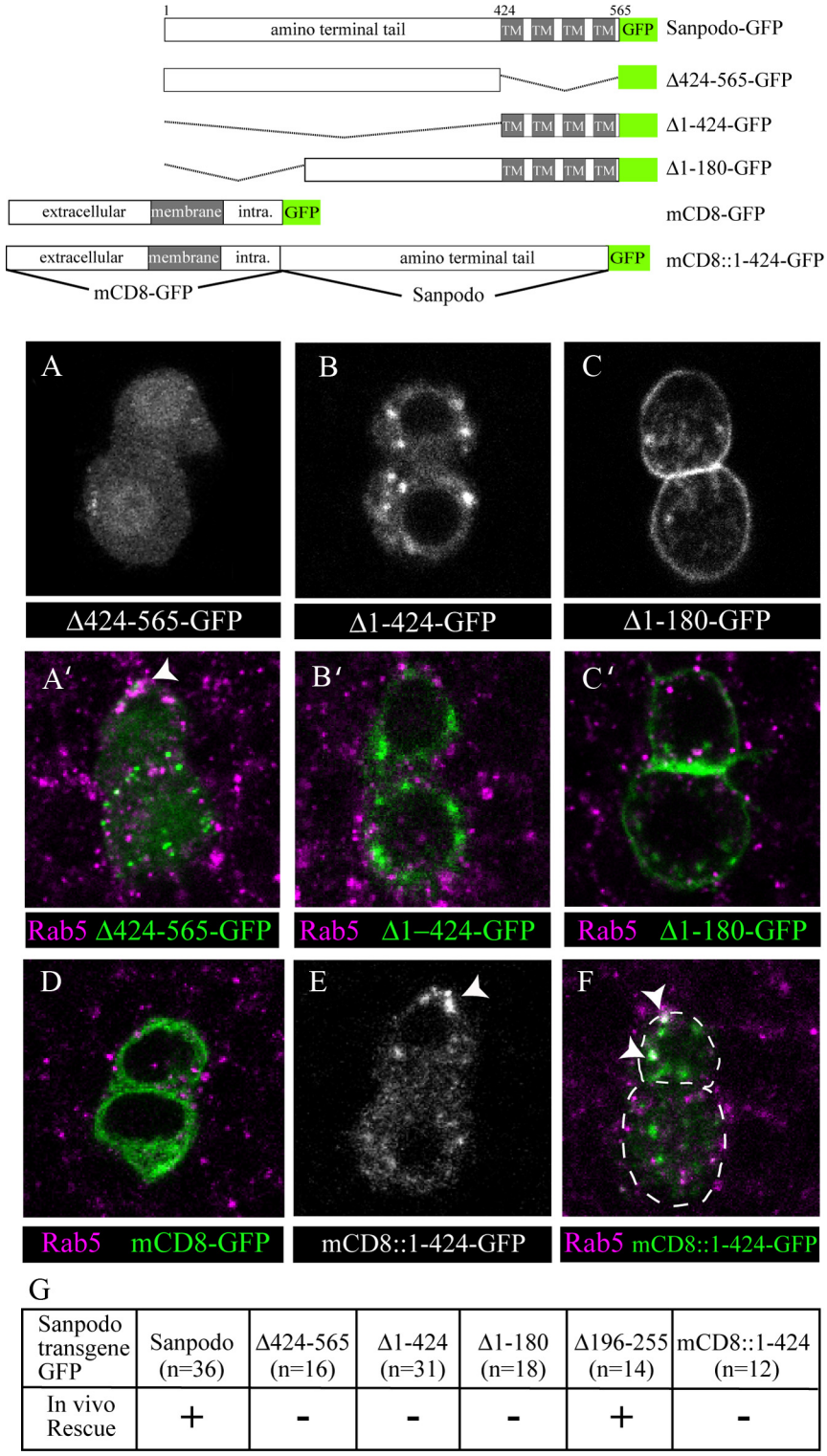


Figure 2. Dynamic regulation of Sanpodo-GFP membrane targeting during asymmetric cell division. (A) Sanpodo-GFP localizes to the pIIa/pIIb cell interface within ten minutes of completion of cytokinesis and is targeted to anterior vesicles in the pIIb cells. (B and D) In nb^2, lgl^4, and α-ada^{ear4} mutants, Sanpodo-GFP is enriched at the membrane during mitosis and accumulates strongly at the pIIa/pIIb cell membrane after cytokinesis. (E and F) Under conditions of *numb-myc* overexpression, or in $sec15^2$ mutants, Sanpodo is depleted from the plasma membrane of the mitotic SOP and the newly formed pIIa and pIIb cells. Genotypes: (A) *yw/+; neur-Gal4/ UAS-sanpodo-GFP, yw, ubx-FLP/+*; (B) *y+* nb^2 *ck FRT40A*, or (C) lgl^4 *FRT40A* or (D) ada^{ear4} *FRT40A/tub-Gal80 FRT40A; neur-Gal4, UAS-sanpodo-GFP/+*, (E) *yw/+; neur-Gal4, UAS-sanpodo-GFP/UAS-numb-myc*, and (F) *yw, ubx-FLP/+; sca-Gal4, UAS-sanpodo-GFP/+; FRT82B* $sec15^2$*/FRT82B tub-Gal80*.



G

Sanpodo transgene GFP	Sanpodo (n=36)	Δ424-565 (n=16)	Δ1-424 (n=31)	Δ1-180 (n=18)	Δ196-255 (n=14)	mCD8::1-424 (n=12)
In vivo Rescue	+	-	-	-	+	-

Figure 3. The Sanpodo amino-terminal tail is sufficient for endocytic targeting in vivo. Schematic of Sanpodo truncation mutants and mCD8-GFP, and the mCD8-Sanpodo amino-terminal tail-GFP chimera (mCD8::1-424-GFP). (A–C) Deletion of the Sanpodo transmembrane domains (Δ424-565, A and A′), N-terminal tail (Δ1-424, B and B′), or the N-terminus (Δ1-180, C and C′), result in failure to target the transgene to endosomes in pIIb cells. (D) mCD8-GFP (green) is localized to the plasma membrane and intracellular membrane compartments in pIIa and pIIb cells, but is excluded from the cell nucleus and Rab5 early endosomes. (E) mCD8::1-424-GFP localizes to large puncta in pIIb cells following asymmetric cell division (white arrowhead). (F′) mCD8::1-424-GFP puncta colocalize with Rab5 in pIIb cells (white arrowheads). *(A–C and E) Live cells; (A′–C′, D and F) fixed cells; GFP-tagged transgenes in green and Rab5 in magenta. (G) Rescue experiments testing the ability of mutant transgenes to restore external cell fates in *sanpodo*^{C55} mutant clones (n = number of flies of each genotype scored for rescue).

and membrane trafficking regulation by *numb, lethal giant larvae*, *α-adaptin,* and *sec15.*

The Sanpodo Amino Terminal Tail Is Necessary and Sufficient for Endocytic Targeting in pIIb Cells

We generated three Sanpodo mutant transgenes: two amino-terminal deletions and one carboxy-terminal deletion (Figure 3) in order to identify critical regions of the protein for Sanpodo localization and function. We assessed the localization of these mutant Sanpodo proteins by driving their expression using *neuralized*-Gal4 in SOP, pIIa, and pIIb cells and visualizing the cells live by confocal microscopy. SanpodoΔ424-565-GFP, which deletes the four transmembrane domains, is uniformly distributed in the cytoplasm, with a

few small puncta, throughout pIIa and pIIb cells (Figure 3, A and A′). In contrast, deletion of the amino-terminal Sanpodo tail results in protein targeting to perinuclear puncta and decreased Rab5 colocalization in pIIb cells (Figure 3, B and B′). Numb, via its PTB domain, binds the amino-terminal intracellular tail of Sanpodo in vitro (O'Connor-Giles and Skeath, 2003; Hutterer and Knoblich, 2005). We therefore assayed two targeted deletions within the Sanpodo tail to identify the Numb-binding region of Sanpodo: one that deletes a previously identified conserved region (Δ196-255 from O'Connor-Giles and Skeath, 2003) and a truncation of the first 180 amino-terminal amino acids (Δ1-180). In contrast to Δ196-255 Sanpodo deletion, which is targeted to endosomes in pIIb cells (not shown), the Δ1-180 Sanpodo-GFP localizes to the periphery in both pIIa and pIIb cells (Figure 3C), and its colocalization with Rab5 endosomes is strongly decreased in pIIb cells (Figures 3C′ and see also 5D).

We next asked whether the amino-terminal tail of Sanpodo is sufficient to target an unrelated membrane protein to endosomes in vivo by generating a chimeric protein fusing the Sanpodo amino-terminal tail (amino acids 1-424) to the cytoplasmic domain of the mouse CD8 protein (mCD8). We show that although the chimeric protein colocalizes with the early endosome marker Rab5 in pIIb cells, mCD8 alone is excluded from early endosomes (Figure 3, D–F). Furthermore, in *numb* mutants the chimeric protein is depleted from vesicles and accumulates at the pIIa/pIIb cell interface, whereas no change in mCD8-GFP distribution is observed in *numb* mutant clones (data not shown). Taken together with the data from our Sanpodo deletion mutants, we conclude that Sanpodo amino-terminal tail is both necessary and sufficient for Numb-dependent early endocytic targeting in vivo. We further assayed our mutant and chimeric transgenes for their ability to restore the hair and socket cell fates in *sanpodo* mutants clones using the MARCM system and found that both the carboxy-terminal transmembrane domains as well as the first 180 amino acids of amino-terminal region are required for Sanpodo function in vivo (Figure 3G).

The Sanpodo Amino Terminal Tail Contains a Conserved NPAF Motif

Sanpodo is a rapidly evolving gene in insects (O'Connor-Giles and Skeath, 2003). We reasoned that sequence comparison of Sanpodo orthologues in other insect species might reveal conserved motifs within the amino-terminal tail that contribute to Sanpodo function and regulation. We identified Sanpodo orthologues in seven insects of the Superorder *Endopterygota* (Figure 4A): three mosquitoes, (*Aedes aegypti*, *Anopheles gambiae*, *Culex pipiens*); red flour beetle, *Tribolium castaneum*; honeybee, *Apis mellifera*; wasp, *Nasonia vitripennis*; and silkworm, *Bombyx mori* and searched for conserved motifs. In our alignments, we found a fully conserved NPAF amino acid sequence within the amino-terminus of the Sanpodo orthologues in all seven non-*Drosophila* species (Figure 4A). In *Drosophila*, the Sanpodo NPAF motif is at the extreme amino-terminus (amino acids 8-11), and our deletion analysis indicates that determinants of endocytic targeting and Numb-binding reside within amino acids 1-180 of Sanpodo. The Numb PTB domain is required for the Sanpodo/Numb interaction in vitro (Hutterer and Knoblich, 2005), and NPxY/F motifs have been previously shown to mediate intermolecular interactions with both PTB and FERM (4.1 protein/ezrin/radixin/moesin) domains (Borg *et al.*, 1996; Calderwood *et al.*, 2003; Uhlik *et al.*, 2005). Thus, we hypothesized that the NPAF motif mediates direct binding to Numb.

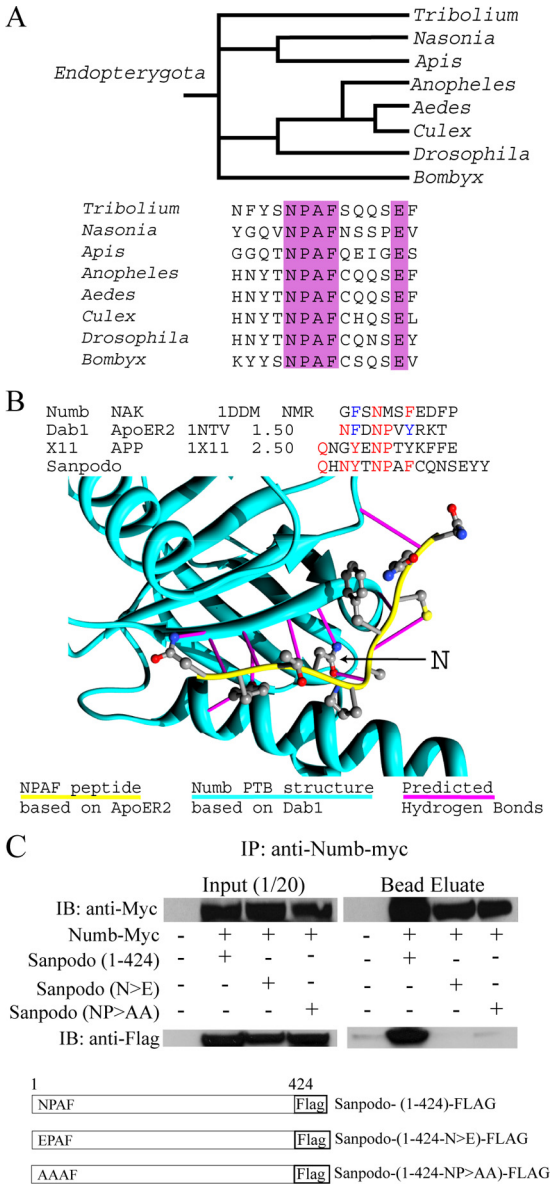


Figure 4. The conserved Sanpodo NPAF motif regulates Numb binding. (A) The evolutionary relationship of species of the Superorder *Endopterygota* and the predicted amino acid sequence of the region containing the NPAF within the amino-terminus (perfectly conserved amino acids labeled with magenta blocks) and the alignment of the Sanpodo peptide with the NAK, ApoER2, and APP peptides. (B) A structural model of the cleft formed by the Numb PTB domain (cyan ribbon) binding to the Sanpodo NPAF motif (yellow backbone) based on the crystal structure of Disabled1 bound to ApoER2. Predicted hydrogen bonds are indicated (magenta lines), and the conserved asparagine residue (N) forms hydrogen bonds with the Numb PTB domain and the Sanpodo peptide backbone. (C) Amino acid substitution mutations (NP>AA and N>E) in the NPAF motif abrogate the Sanpodo amino-terminal tail binding to Numb-Myc in an immunoprecipitation assay.

The Sanpodo NPAF Motif Mediates Direct Binding to Numb

To explore the potential molecular interaction between Numb and Sanpodo we produced three different three-dimensional models of the Sanpodo amino-terminal peptide containing the NPAF motif binding to the Numb PTB domain. As templates, we used three structures of PTB domains binding peptide ligands: the *Drosophila* Numb PTB

domain interacting with an NxxF motif of Numb-Associated Kinase (Zwahlen *et al.*, 2000); Disabled1 with a bound peptide from ApoER2 (Stolt *et al.*, 2003); the X11 protein with a peptide of amyloid precursor protein (APP; Borg *et al.*, 1996; Figure 4B). Using the published structures and our models, we identified common points of contact between the NPxF/Y peptides and PTB domain residues. In the templates as well as the models, each of the liganded peptides serves to add a fifth strand to the β-sheet of the PTB domain (Figure 4B). In all three models, the SCWRL program reproduced a hydrogen bond present in all three templates between the conserved asparagine side chain in the Sanpodo peptide (position −3) and the backbone carbonyl of Numb residues Ile144 and Val147 (Figure 4B; for further details, see Supplementary Materials, modeling section). Some side chains of the Sanpodo peptides are in different conformations in the different models, which indicate some uncertainty about their positions relative to Numb. Nevertheless, the models demonstrate that the Sanpodo sequence is likely to make favorable interactions with Numb at several positions along the length of the peptide motif with no obvious unfavorable ones.

To specifically test whether our model accurately predicts the direct Numb-Sanpodo interaction, we generated two mutant versions of the Sanpodo NPAF motif that specifically target residues critical to the NPAF-PTB domain interaction based on our model. In the first mutant, we changed the conserved NP sequence at positions −3 and −2 to alanines (NP>AA, the conserved phenylalanine is arbitrarily assigned position 0), and in the second mutant, we substituted the conserved asparagine at position −3 with glutamic acid (N>E). From our model, the asparagine-to-glutamic acid change in particular is predicted to maintain the overall backbone of the Sanpodo motif, but creates unfavorable side chain interactions with the PTB domain. Both mutant conditions strongly reduce the interaction of the Sanpodo tail with Numb in the coimmunoprecipitation assay (Figure 4C), whereas a nearby tyrosine- (−5)-to-alanine substitution has no effect (not shown). These data led us to conclude that the evolutionarily conserved Sanpodo NPAF motif is required for binding Numb, likely by hydrogen bonding between the asparagine amino group of the Sanpodo NPAF and two backbone carbonyls of the Numb PTB domain.

The Sanpodo NPAF Motif Is Required for Endocytic Targeting In Vivo

Our finding that the Sanpodo NPAF motif mediates the in vitro interaction with Numb prompted us to test whether the NPAF motif region controls Sanpodo's localization in pIIb cells in vivo using the GAL4/UAS system. We assayed two Sanpodo-GFP mutant transgenes, one mutant removing the first of 18 amino acids of the amino-terminus, including the entire NPAF motif (Sanpodo-ΔN18-GFP) and an amino acid substitution of the conserved NP amino acids to AA (Sanpodo-NP>AA-GFP), both of which abrogate Sanpodo binding to Numb in vitro (Figure 4C and data not shown). In contrast to wild-type Sanpodo-GFP in pIIb cells, targeting to Rab5-positive endosomes is strongly reduced in Sanpodo mutants with the NPAF motif deleted or mutated, whereas membrane accumulation enhanced (Figure 5, A–D). Although we observed an increase in membrane targeting of the Sanpodo NPAF mutants, cytoplasmic puncta were also present in both cells. To determine whether the presence of puncta in Sanpodo NPAF mutants were dependent on Numb, we expressed the Sanpodo-ΔN18-GFP under conditions of Numb overexpression. Overexpression of Numb-myc with *neuralized*-Gal4 results in a strong balding phenotype on the pupal thorax (Figure S2) and inhibition of Sanpodo-GFP targeting at the plasma membrane (Figure 2E). In contrast, Sanpodo-ΔN18-GFP remains localized strongly to the plasma

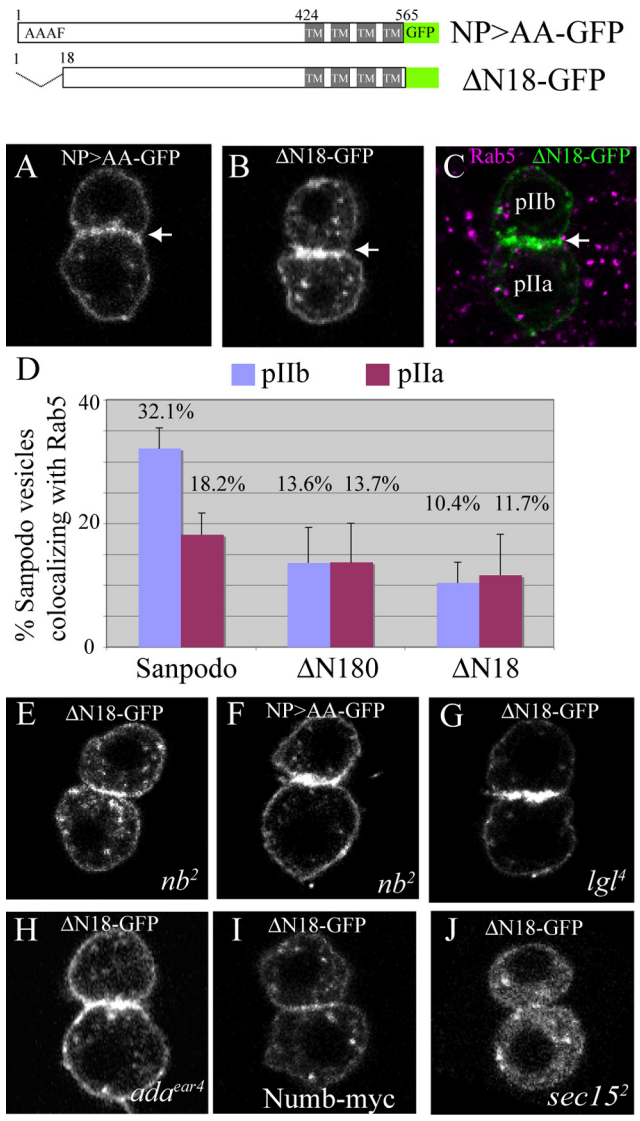


Figure 5. The Sanpodo NPAF is required for endocytic targeting in vivo. (A–C) Sanpodo mutants with either point mutations in the NPAF motif (NP>AA) or the NPAF motif removed (ΔN18) localize to the plasma membrane (white arrow) and small puncta in pIIa and pIIb cells and do not colocalize with early endosomes (labeled by Rab5, magenta) in pIIb cells. (D) Graph representing the decrease in percentage of puncta colocalizing with the early endosome marker Rab5 in Sanpodo-Δ1-180-GFP and Sanpodo ΔN18-GFP mutants in the pIIb cell compared with wild-type Sanpodo. Colocalized puncta were visually identified from individual planes of apical-basal z stacks through pIIa/pIIb cell pairs at a0.5-μm interval. Error bars, SEM. (E–I) Sanpodo NPAF mutant (NP>AA or ΔN18) localization in nb^2, lgl^4, and α-ada^{ear4} mutants or under conditions of *numb-myc* overexpression (I) is similar to Sanpodo-NPAF mutant localization in wild-type cells shown in A and B. (J) In $sec15^2$ mutants, Sanpodo-NPAF mutant is depleted from the plasma membrane of the mitotic SOP and the newly formed pIIa and pIIb cells. Genotypes: *yw*; *neur-Gal4/UAS-sanpodo-NP>AA-GFP* (A), *yw*; *neur-Gal4/UAS-sanpodo-ΔN18-GFP* (B and C), *yw, ubx-FLP/+*; *y+ nb^2 ck FRT40A/CyO* (E and F), or *lgl^4 FRT40A/CyO* (G), or *ada^{ear4} FRT40A/CyO* (H) were crossed to *yw; tub-Gal80 FRT40A*; *neur-Gal4, UAS-sanpodo-NP>AA-GFP/TM6* (F) or *yw; tub-Gal80 FRT40A*; *neur-Gal4,UAS-sanpodo-ΔN18-GFP* (E, G, and H), *yw*; *neur-Gal4, UAS-sanpodo-ΔN18-GFP/UAS-numb-myc* (I), and *yw, ubx-FLP/+; sca-Gal4, UAS-sanpodo-ΔN18-GFP/+; FRT82B $sec15^2$/FRT82B tub-Gal80* (J).

membrane when Numb-myc is overexpressed (Figure 5I). We next asked whether Sanpodo-ΔN18-GFP localization is altered in *numb, lethal giant larvae,* and *α-adaptin* mutant clones. Using live cell imaging, we find that under these mutant conditions, Sanpodo-ΔN18-GFP maintains an increase in membrane targeting and small cytoplasmic puncta, similar to Sanpodo NPAF mutant protein localization in wild-type cells (Figure 5, E–I). However, both wild-type Sanpodo-GFP and Sanpodo-ΔN18-GFP mutant fails to target to the plasma membrane in *sec15* mutant clones (Figure 5J). From these results we conclude that Sanpodo NPAF motif is dispensable for plasma membrane targeting but is required for Numb-dependent targeting to Rab5 endosomes in vivo.

Accumulation of Sanpodo at the plasma membrane in the pIIb cell correlates with overactivation of Notch signaling in *numb, α-adaptin,* and *lethal giant larvae* mutant SOP cells (Roegiers *et al.*, 2005).We therefore asked whether the NPAF mutant Sanpodo, which is no longer regulated by Numb, could bypass Numb inhibition and activate Notch signaling when overexpressed in SOP lineage cells. Overexpression of Sanpodo-GFP with either *neuralized*-Gal4 or *scaberous*-Gal4 does not show evidence of Notch activation in pIIb cells (extra hair or socket cells, Figure 6A and Figure S2). Similarly, overexpression of the Sanpodo-ΔN18-GFP and Sanpodo-NP>AA-GFP mutants (two independent lines of each mutant), showed only modest increases in extra hair and socket cells (<5%), even under conditions of lowered *numb* and/or *lethal giant larvae* gene dosage (Figure 6A), and overexpression of either Sanpodo-GFP or Sanpodo-ΔN18-GFP with *neuralized*-Gal4 does not strongly reduce the balding phenotype induced by Numb-myc overexpression (Figure S2). We conclude from these findings that NPAF mutant Sanpodo cannot robustly activate Notch in the presence of Numb in SOP lineage cells.

Lack of ectopic Notch activation in pIIb cells under the conditions described above could be due to presence of endogenous Sanpodo protein and/or that disruption of the NPAF motif abrogates Sanpodo's ability to promote Notch signaling in vivo. To ascertain whether the Sanpodo NPAF motif is required for Notch activation in SOP lineage cells in vivo, we performed a rescue assay by expressing the Sanpodo-ΔN18-GFP protein in *sanpodo* clones using the MARCM system. Surprisingly, expression of Sanpodo-ΔN18-GFP in SOP lineage cells driven by *scaberous*-Gal4 restores the wild-type bristle pattern and extra sensory (ES) organ cell fates in *sanpodo* mutant clones in a manner indistinguishable from wild-type Sanpodo-GFP (Figure 6, B, B′, C, and C′). Restoration of the bristle pattern was not transgene-, temperature-, or allele-dependent: Rescue of the *sanpodo* mutant phenotype was equally effective with two independent Sanpodo-ΔN18-GFP transgenic lines, in flies maintained at 18 or 25°C, and in clones of both a nonsense and a missense allele of *sanpodo* (*sanpodo*^{C55} and *sanpodo*^{G104}, respectively, Figure 6 and data not shown). The amino acid substitution mutant, Sanpodo-NP>AA-GFP, also restores missing bristles in *sanpodo*^{C55} mutant clones on the thorax similarly to wild-type Sanpodo-GFP (Figure 6, D and D′). From these findings we conclude the regulation of Sanpodo membrane targeting by Numb binding to the NPAF motif is dispensable for correct cell fate specification in the external sensory organ precursor cells.

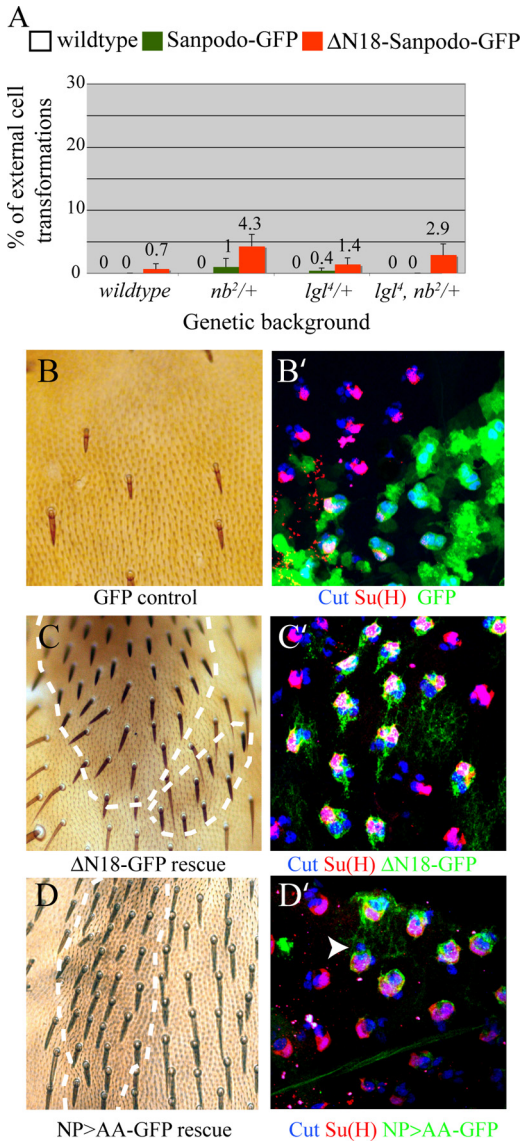


Figure 6. The Sanpodo NPAF motif is not required Sanpodo function in external sensory organ lineage cells. (A) Overexpression of either wild-type or ΔN18 Sanpodo mutant transgenes show low frequencies of external cell fate transformations (i.e., extra sockets) in sensory organs of the head, even in backgrounds where *numb* and/or *lgl* dose is lowered. (B–D) Both Sanpodo ΔN18-GFP and Sanpodo NP>AA-GFP completely restore wild-type cell fates to all external sensory organs in *sanpodo*^{C55} *Sb e* MARCM clones, whereas GFP alone does not. *sanpodo* mutant ES organs are marked by *Sb/Sb* bristles and approximate clone boundaries are marked by dashed lines in C and D. GFP, Sanpodo ΔN18-GFP, and Sanpodo NP>AA-GFP (green) mark the *sanpodo* mutant ES organ cells in B′, C′, and D′. External organ lineage cells are labeled with Cut (blue) and socket cells with Su(H) (red) antibodies. Cell fate transformations, such as twinned bristles, are observed in rescued flies at a low frequency (<2%, not shown). Genotypes: *sca-Gal4,* or *sca-Gal4, UAS-sanpodo-GFP,* or *sca-Gal4, UAS-sanpodo-ΔN18-GFP* were crossed to the following: (A) *yw, nb*^{2}*/CyO* or *lgl*^{4}*/CyO,* or *lgl*^{4}*, nb*^{2}*/CyO*; (B and B′) *yw, ubx-FLP/+; sca-Gal4/UAS-EGFP*; (C and C′) *UAS-sanpodo-ΔN18-GFP*; or (D and D′) *UAS-sanpodo-NP>AA-GFP* or *FRT82B sanpodo*^{C55} *Sb*^{1} *e/FRT82B tub-Gal80*; similar results were obtained with a rescue of *FRT82B sanpodo*^{G104} *e* clones.

DISCUSSION

How Numb regulates Notch signaling is not well understood (Cayouette and Raff, 2002; Roegiers and Jan, 2004; Petersen *et al.*, 2006). Studies in a variety of organisms has implicated Numb as a regulator of protein membrane trafficking (Santolini *et al.*, 2000; Berdnik *et al.*, 2002; Smith *et al.*, 2004; Nilsson *et al.*, 2008; McGill *et al.*, 2009). Our study

demonstrates that direct binding of the Numb PTB domain to the NPAF motif in the Sanpodo amino-terminus controls inhibition of Sanpodo targeting to the plasma membrane. We hypothesize that Numb binding to the Sanpodo NPAF motif at the plasma membrane results in Sanpodo endocytosis and targeting to endosomes, although we cannot exclude the possibly that Numb functions in endosomes to prevent Sanpodo recycling to the plasma membrane. Nonetheless, we find that uncoupling Sanpodo from Numb-mediated endocytic targeting by deletion or mutation of the NPAF motif results in accumulation of Sanpodo at the plasma membrane, but defective trafficking of Sanpodo NPAF mutants does not appear to impact Numb-dependant cell fate determination in the adult sensory organ lineage. Both Numb and Notch have been shown to physically interact with Sanpodo, suggesting that these three proteins may form a complex (O'Connor-Giles and Skeath, 2003; Hutterer and Knoblich, 2005). Our findings support the model that the formation of a Numb/Sanpodo/Notch complex results in Numb-mediated Notch cleavage inhibition, perhaps through posttranslational modification and degradation or endocytosis of the Notch receptor (McGill and McGlade, 2003). In parallel, Numb likely promotes targeting of Sanpodo from the plasma membrane to endosomes, perhaps as consequence of Numb-mediated Notch inhibition. This result is puzzling, as the NPAF is conserved in Sanpodo homologues spanning divergent insect species, implying that it serves a function. We speculate that the Sanpodo NPAF motif may have an essential function in other Sanpodo-dependent developmental contexts, such as in CNS or muscle progenitor cells. Alternatively, Numb-dependant Sanpodo trafficking may contribute to Notch regulation through a redundant mechanism not revealed in our assays. Our experiments demonstrate however, that Numb-dependant depletion of Sanpodo from the plasma membrane in pIIb cells is unlikely to be the primary mechanism by which Numb inhibits Notch following asymmetric cell division.

At present, no Sanpodo orthologues have been identified in vertebrates (O'Connor-Giles and Skeath, 2003). Nonetheless, our study reveals that Sanpodo contains a functional NPAF motif. In vertebrates, the NPxF/Y family of motifs is present in diverse classes of membrane proteins such as β-integrins, the APP protein, LDL, and IGF-I receptors, where these motifs are important for controlling internalization and/or signaling activation (Chen *et al.*, 1990; Hsu *et al.*, 1994; Borg *et al.*, 1996; Calderwood *et al.*, 2003; Garcia-Alvarez *et al.*, 2003). Interestingly, Numb regulates β-integrin endocytosis during cell migration and has been shown to regulate APP processing (Nishimura and Kaibuchi, 2007; Kyriazis *et al.*, 2008), suggesting that the molecular interaction we have uncovered between Numb and an NPxF/Y motif in Sanpodo may be evolutionarily conserved. In the case of Sanpodo, the NPAF motif appears to be an endocytic targeting signal.

Our findings are consistent with Sanpodo functioning primarily as a permissive factor for Notch-dependent cell fates in the adult PNS. Wild-type Sanpodo and Sanpodo mutants that are defective for endocytic targeting are fully functional in rescue assays, but overexpression of wild-type or mutant Sanpodo does not cause strong Notch overactivation phenotypes in SOP wild-type cells. Two models have been proposed for how Sanpodo might function to promote Notch signaling in the pIIa cell after asymmetric cell division. In the first model, Sanpodo functions at the plasma membrane in the signal-receiving cell (the pIIa cell) to induce γ-secretase–mediated cleavage of Notch through an interaction with the Notch receptor (O'Connor-Giles and Skeath, 2003). In the second model, Sanpodo functions to promote Delta trafficking in the signal-sending cell (Jafar-Nejad *et al.*, 2005). Our results do not exclude either model; however, it appears unlikely that Sanpodo is required in endosomes in the signal-sending cell (the pIIb cell), as our NPAF mutants, that fail to target to endosomes, restore Notch-dependent fates in the PNS. Our live imaging studies demonstrate that the Sanpodo protein accumulates within minutes after SOP cell mitosis at the membrane interface between the pIIa and pIIb cell. This accumulation is strongly enhanced when Sanpodo is blocked from endosomal targeting and fully suppressed in *sec15* mutants. These data lead us to hypothesize that the exocyst complex, mediated by Sec15, controls rapid delivery of Sanpodo to the membrane interface after SOP mitosis. Delivery of Sanpodo to the plasma membrane interface may contribute to the establishment of a membrane domain that promotes Notch signaling.

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