Sanpodo controls sensory organ precursor fate by directing Notch trafficking and binding γ-secretase

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Introduction

Asymmetric cell division (ACD) is an evolutionarily conserved mechanism for generating cell fate diversity during development (Horvitz and Hershkowitz, 1992; Knoblich, 2010). A striking example of ACD occurs during adult peripheral neurogenesis in Drosophila: the sensory organ precursor (SOP) cell, an epithelial cell–derived progenitor, undergoes four rounds of asymmetric cell division, giving rise to four mature cells that differentiate into a functional mechanosensory organ. As the SOP cell enters mitosis, the mitotic spindle aligns along the anterior–posterior axis and Numb localizes asymmetrically along the anterior membrane (Roegiers and Jan, 2004). Numb inhibits Notch signaling in the anterior SOP daughter cell (pIIb), while its absence in the adjacent pIIa cell permits Notch signaling activity (Rhyu et al., 1994).

Notch signaling in the pIIa cell is activated in trans via binding of the membrane-tethered ligand Delta, present on the surface of the pIIb cell, to the Notch receptor in the pIIa cell (Kandachar and Roegiers, 2012). Ligand binding induces sequential cleavage of the Notch receptor at the plasma membrane first by the ADAM metalloprotease (S2 cleavage) and then by the γ-secretase complex (S3 cleavage; Bray, 2006; Ilagan and Kopan, 2007). The S3 cleavage releases the Notch intracellular domain (NICD) from the membrane, and the NICD translocates to the pIIa cell nucleus and interacts with the DNA-binding protein CSL (CBF1/Suppressor of Hairless/Lag1) and the transcriptional coactivator Mastermind to regulate Notch target gene expression.

Sanpodo, a four-pass transmembrane protein, is expressed in SOP cells, and unlike Numb, is distributed to both pIIa and pIIb cells after mitosis (O’Connor-Giles and Skeath, 2003). In the pIIa cell, activation of Notch requires sanpodo, and Sanpodo protein localizes primarily at the plasma membrane (Salzberg et al., 1994; Park et al., 1998; Skeath and Doe, 1998; O’Connor-Giles and Skeath, 2003; Roegiers et al., 2005). However, the mechanism of sanpodo action in the pIIa cell has yet to be defined. In contrast, observations that Sanpodo localizes primarily to endosomes in pIIb cells in a Numb-dependent manner (Hutterer and Knoblich, 2005; Langevin et al., 2005; Roegiers et al., 2005) suggests a possible role for Sanpodo in modulating Notch activity in pIIb cells as well (O’Connor-Giles and Skeath, 2003; Babaoglan et al., 2009). In this study, we uncover the mechanistic role of Sanpodo in promoting Notch signaling in the pIIa cell, and determine the function of Sanpodo in regulating pIIb cell fate.

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Results

Sanpodo binds the Presenilin subunit of the γ-secretase complex

Sanpodo loss-of-function mutants show an incompletely penetrant phenotype of failure to induce Notch signaling in pIIa cells, resulting in areas of balding on the pupal thorax (Jafar-Nejad et al., 2005; Roegiers et al., 2005). Release of the Notch intracellular domain from the plasma membrane after ligand binding requires the proteolytic activity of the γ-secretase complex, which is composed of four distinct transmembrane protein subunits: Pen-2, Aph-1, Nicastrin, and Presenilin. Genetic analysis suggests a requirement for sanpodo at the γ-secretase cleavage step of Notch activation (O’Connor-Giles and Skeath, 2003). We therefore tested whether Sanpodo physically associates with the γ-secretase complex in a coimmunoprecipitation assay. The four components of the γ-secretase complex must be present in roughly stoichiometric levels for the ordered assembly of the complex, and subsequent processing and trafficking to the plasma membrane (Hu and Fortini, 2003; Stempfle et al., 2010). We coexpressed all four γ-secretase complex components in S2 cells from a single plasmid together with the Sanpodo amino-terminal cytoplasmic region (ATCR; amino acids 1–424). We found that the Sanpodo ATCR communoprecipitated with myc-tagged Presenilin, whereas a Sanpodo amino-terminal deletion mutant (SanpodoAN190) did not (Fig. 1 A). We failed to detect an interaction between Sanpodo and overexpressed Presenilin alone in vivo, suggesting that assembly of the γ-secretase complex may be required for the Sanpodo interaction (unpublished data). To further narrow down the region of the Sanpodo cytoplasmic domain responsible for Presenilin binding, we generated a series of amino-terminal truncations of the Sanpodo ATCR and assessed their ability to bind Presenilin. We found that the region between 100 and 125 amino acids of the Sanpodo amino terminus is necessary for binding in CoIP assays in S2 cells (Fig. 1 A).

The 25–amino acid region that is required for Presenilin binding is not strongly conserved among other sanpodo orthologues in insects (Fig. S1 A); however, this region contains two six–amino acid sequences beginning with RY and enriched in hydrophobic residues. Interestingly, we observed variable numbers of these RYXXXX sequences are clearly present in Sanpodo orthologues in other insect species revealed that in addition to the NPAF motif, there is a third conserved motif, A TxxLHELL (LHELL), at residues 293–301 in the amino-terminal cytoplasmic domain of Sanpodo (Fig. 2 A; Fig. S1 A). The LHELL motif contains a dileucine and an upstream acidic residue, characteristic of endocytic sorting signals (Bonifacino and Traub, 2003). To test the functional role of the LHELL motif, we generated a mutant form of Sanpodo-GFP substituting the ELL residues of the motif with alanines (LHAAA). To determine whether the ELL motif was required for controlling Notch signaling in pIIa and pIIb cells, we tested SanpodoLHAAA-GFP’s ability to rescue the sanpodoC55 mutant bristle loss phenotype in clones. We showed previously that SanpodoANRF and SanpodoANRF-GFP could restore the wild-type bristle pattern in sanpodoC55 clones when expressed using the scabrous-Gal4 driver (Fig. 2, B and C; Tong et al., 2010). Similarly, SanpodoLHAAA-GFP fully rescued sanpodoC55 clones, in contrast to GFP controls that exhibited extensive bristle loss (Fig. 2, B and D). From this result, we conclude that the ELL motif is dispensable for Sanpodo function in SOP lineage cells. We next tested whether disruption of both the NPAF and ELL motifs altered Sanpodo function. In the rescue assay, SanpodoANRF.LHAAA-GFP expression reversed the strong balding phenotype of sanpodoC55 mutant clones, indicating that the NPAF and ELL motifs together are not required for promoting Notch signaling in the pIIa cell (Fig. 2 E). However, closer examination...
revealed that while 70.1% of the rescued organs exhibited the wild-type single hair and socket cell (n = 578 organs), the remaining mutant organs contained supernumerary socket cells, a phenotype consistent with persistent Notch activation in the sensory organ lineage (Fig. 2 E, inset). We further characterized cell fate assignments of Sanpodo\textsuperscript{\textit{NPAF, LHAAA}}-GFP rescued sanpodo\textsuperscript{mutant} clones by labeling neurons in pupal sensory organ cell clusters. Expression of the Sanpodo\textsuperscript{\textit{NPAF, LHAAA}}-GFP mutant completely suppressed supernumerary neuron phenotype observed in sanpodo\textsuperscript{mutant} clones, limiting most organs to a single neuron (438/476 labeled cell clusters from 23 flies), but nearly 8% of rescued organs showed no neuronal marker expression (38/476, Fig. 2 F). These findings indicate that the Sanpodo NPAF and ELL motifs function to restrict socket cell fate and to promote neuronal cell fate in adult sensory organ cells.
detected Notch at the apical region coincident with the plasma membrane interface between the pIIa and pIIb cell, whereas Notch was not enriched at the basolateral plasma membrane interface. Mutation of NPAF and ELL motifs also decreased the number of Sanpodo-colocalizing Notch intracellular puncta in pIIa/pIIb cell pairs. From these findings we concluded that the NPAF and ELL motifs are required for controlling Sanpodo membrane targeting and may contribute to regulating membrane Notch levels in pIIb/pIIa cells.

Sanpodo binds the Notch receptor directly

Although Sanpodo has been shown to associate with Notch in vivo and is required for robust Notch signaling activity in the pIIa cell (Roegiers et al., 2001, 2005; O’Connor-Giles and Skeath, 2003), it is unclear if the physical association between Sanpodo and Notch is direct, or whether it depends on other proteins such as Numb. We assessed the nature of the Sanpodo–Notch interaction using in vitro protein interaction assays and coimmunoprecipitation studies. We generated tagged versions of the Notch intracellular domain (NICD) and Sanpodo amino-terminal cytoplasmic domain in vitro and found that in binding assays, Notch and Sanpodo intracellular domains coprecipitated (Fig. 4 A). We next verified that Sanpodo’s interaction with Notch requires the amino-terminal portion of the Sanpodo...
Figure 3. The NPAF and ELL motifs control Sanpodo trafficking in pIIa and pIib cells. (A–D) Live-cell imaging of neuralized-Gal4–driven expression of GFP-tagged Sanpodo transgenes in pIIa/pIib cell pairs; XY equatorial view. Bar, 2 µm. Sanpodo^wt–GFP (A) and Sanpodo^NPAF–GFP (C) localize to the plasma membrane and in intracellular puncta, whereas Sanpodo^ΔNPAF–GFP (B) and Sanpodo^ΔNPAF, ΔELLS–GFP (D) accumulate at the plasma membrane in both daughter cells, particularly at the membrane interface between pIIa/pIib (B and D, white arrowheads). (E) Live-cell analysis of Sanpodo mutant transgenes (green) in pIIa and pIib cells expressing His-RFP (red) 7 min after SOP mitotic exit; YZ view, apical at top, anterior to the left. Bar, 2 µm. Sanpodo^ΔNPAF–GFP and Sanpodo^ΔNPAF, ΔELLS–GFP accumulate strongly at the basolateral interface (white arrowhead), whereas Sanpodo^ΔELLS–GFP accumulates at the apical plasma membrane in pIIa and pIib cells, compared with Sanpodo^wt–GFP. Notch (Notch^ECD, red) is depleted from apical (white arrowheads) pIIa/pIib membrane interface in Sanpodo-GFP–expressing cells. (F) Apical membrane Notch (white arrowheads) is detected at low levels in cell rescued with Sanpodo^ΔNPAF, ΔELLS–GFP, whereas basolateral Notch levels are similar to wild type [YZ planes, apical is at the top, approximate cell outlines represented by dashed lines]. Bar, 2 µm. (G) Quantification of colocalization of Sanpodo and NECD in intracellular vesicles in pIIa and pIib cells. Wild-type Sanpodo-GFP accumulates in large intracellular (>0.5 µm) puncta, which partially colocalize with Notch, in higher numbers in pIib cells. Sanpodo^ΔNPAF, ΔELLS–GFP puncta are less abundant and are equally distributed in pIIa and pIib cells (standard deviation of 2.213–3.599 total Sanpodo + puncta per cell, n = 7 WT; n = 22 NPAF, ΔELLS).
depleted Notch from the apical junctions of epithelial cells, and targeted Notch to intracellular puncta (Fig. 5, B–D and H), consistent with recently reported findings (Couturier et al., 2012). Mutation of ELL motif had at best a modest effect on Sanpodo’s ability to deplete Notch from the apical junction of epithelial cells (Fig. 5 E; Fig. S1 A). However, disruption of both the NPAF and ELL motifs from Sanpodo-GFP abrogated Notch depletion from the apical cell junctions (Fig. 5, F and H), suggesting that either motif is sufficient to regulate Notch levels at the membrane, but Notch trafficking regulation is lost in the absence of both. In contrast, the Sanpodo \(^{100-125}\) mutant, which does not bind Presenilin, depleted apical Notch similarly to wild-type Sanpodo-GFP (unpublished data), whereas the amino-terminal truncated Sanpodo cytoplasmic domain [190–424] does not (asterisk indicates Notch uninduced). We conclude from this analysis that Sanpodo promotes Notch endocytosis from the apical junctions of epithelial cells through binding to the Notch receptor and driving Notch internalization through the redundant activity of the NPAF and ELL motifs.

Sanpodo controls Notch trafficking via conserved NPAF and ELL motifs

Based on our observations in SOP cells expressing wild-type and mutant Sanpodo transgenes, we hypothesized that if Sanpodo is being actively endocytosed and recycled, and binding to the Notch receptor directly, we would expect to see alterations in Notch receptor localization in the presence of Sanpodo. We tested this in wing disc epithelial cells in flies, which endogenously express Notch and Numb but not Sanpodo (Fig. 5 A). Ectopic expression of Sanpodo\(^{ws}\)-GFP, or a Sanpodo mutant lacking the region containing the Numb-binding amino-terminal NPAF motif (Sanpodo\(^{\Delta NPAF}\)-GFP) with the \(\text{apterous-Gal4}\) driver, which drives expression in a subset of wing-disc epithelial cells, depleted Notch from the apical junctions of epithelial cells, and targeted Notch to intracellular puncta (Fig. 5, B–D and H), consistent with recently reported findings (Couturier et al., 2012). Mutation of ELL motif had at best a modest effect on Sanpodo’s ability to deplete Notch from the apical junction of epithelial cells (Fig. 5 E; Fig. S1 A). However, disruption of both the NPAF and ELL motifs from Sanpodo-GFP abrogated Notch depletion from the apical cell junctions (Fig. 5, F and H), suggesting that either motif is sufficient to regulate Notch levels at the membrane, but Notch trafficking regulation is lost in the absence of both. In contrast, the Sanpodo\(^{100-125}\) mutant, which does not bind Presenilin, depleted apical Notch similarly to wild-type Sanpodo-GFP (unpublished data), whereas the amino-terminal truncated Sanpodo\(^{\Delta NPAF}\)-GFP, which does not bind Notch in vitro, failed to deplete Notch from the apical membrane (Fig. 5 G). We conclude from this analysis that Sanpodo promotes Notch endocytosis from the apical junctions of epithelial cells through binding to the Notch receptor and driving Notch internalization through the redundant activity of the NPAF and ELL motifs.
Figure 5. Sanpodo promotes Notch endocytosis. (A) opterous-Gal4–driven ectopic expression of GFP-tagged Sanpodo transgenes (green) in wing disc epithelial cells labeled with the NotchECD antibody (red). Bar, 100 µm. White box is the approximate region of disc shown in B–G. Merged apical XY confocal sections through wing disc epithelial cells (1.5-µm thickness, the border between GFP+ and GFP− regions are marked by a dashed yellow line). Expression of GFP alone has no effect on NECD localization to apical cell junctions in wing disc epithelial cells (B), whereas expression of Sanpodo−Δ-GFP depletes Notch from the apical membrane in epithelial cells and causes Notch to accumulate in large apical vesicles, which in some cases colocalize with Sanpodo-GFP (C, white arrowheads). Both the ΔNPAF mutant (D) and the LHAAA mutant (E) retain the ability to deplete apical Notch, whereas the ΔNPAF, LHAAA mutant (F) or ΔN180 mutant of Sanpodo (G, ΔN180) abrogate Notch apical depletion. Quantification of apical NECD staining represented as a ratio of the area occupied by apical NECD staining in the GFP+/GFP− regions of merged apical XY planes from opterous-Gal4–driven ectopic expression of GFP-tagged Sanpodo transgenes and a GFP control (H). Sanpodo-GFP and SanpodoΔNPAF-GFP significantly deplete membrane Notch levels, whereas Notch levels are decreased in the LHAAA; they do not attain statistical significance. In contrast, Notch levels in the SanpodoΔNPAF, LHAAA–GFP expression region were indistinguishable from GFP control expression, and significantly increased relative to Sanpodo-GFP. Bars, 25 µm.
and in large cytoplasmic vesicles whereas Sanpodo\textsuperscript{LHAAA}\_GFP, and Sanpodo\textsuperscript{LHAAA} mutant shows that the NPAF-dependent control of membrane trafficking of transmembrane proteins relies on the interaction between tyrosine-based and dileucine-based motifs within the cytoplasmic domain of the membrane protein and specific endocytic adaptor–protein complexes. In a previous study, our laboratory established that the Numb phosphotyrosine-binding domain binds to a tyrosine-based YxxN-PAF motif at the Sanpodo amino terminus. Although disruption of this interaction clearly altered Numb-dependent bulk trafficking of Sanpodo from the basolateral plasma membrane to endosomes, the Sanpodo\textsuperscript{LHAAA} mutant was fully able to restore \textit{sanpodo} function in SOP lineage cells in vivo (Tong et al., 2010). Here, we show that the ELL motif limits Sanpodo accumulation in the apical membrane, both in epithelial cells and in SOP lineage cells. Dileucine signals regulate both internalization and lysosomal targeting of membrane proteins (Bonifacino and Traub, 2003). In Sanpodo, a dileucine signal could either function to promote endocytosis of Sanpodo from apical membrane, or to shunt endocytosed Sanpodo from the recycling pathway to the lysosome, or both. Our analysis of the trafficking of the Sanpodo\textsuperscript{LHAAA} mutant shows that the NPAF-dependent sorting of Sanpodo from the basolateral membrane to endosomes is required for apical accumulation of the Sanpodo\textsuperscript{LHAAA} mutant. Therefore, the most likely scenario is that Sanpodo enters the endocytic system primarily from the basolateral membrane via the NPAF motif and the dileucine signal diverts most of the internalized Sanpodo to the lysosome. The dileucine may

Giagtzoglou et al., 2012). Similarly, genetic screens had identified \textit{sanpodo} as an essential gene in activation of the Notch pathway for pIIa cell fate, but it has remained unclear how Sanpodo fulfills this function. The Sanpodo–Presenilin interaction we describe may provide a mechanism to ensure that low levels of ligand-bound Notch receptor in the pIIa cell are linked to the \(\gamma\)-secretase complex for prompt proteolytic processing upon ligand binding. Although our analysis suggests that Sanpodo could act as a linker between Notch and the \(\gamma\)-secretase complex, Sanpodo could also control either the activity of the enzyme or regulate its subcellular localization.

Control of membrane trafficking of transmembrane proteins relies on the interaction between tyrosine-based and dileucine-based motifs within the cytoplasmic domain of the membrane protein and specific endocytic adaptor–protein complexes. In a previous study, our laboratory established that the Numb phosphotyrosine-binding domain binds to a tyrosine-based YxxN-PAF motif at the Sanpodo amino terminus. Although disruption of this interaction clearly altered Numb-dependent bulk trafficking of Sanpodo from the basolateral plasma membrane to endosomes, the Sanpodo\textsuperscript{LHAAA} mutant was fully able to restore \textit{sanpodo} function in SOP lineage cells in vivo (Tong et al., 2010). Here, we show that the ELL motif limits Sanpodo accumulation in the apical membrane, both in epithelial cells and in SOP lineage cells. Dileucine signals regulate both internalization and lysosomal targeting of membrane proteins (Bonifacino and Traub, 2003). In Sanpodo, a dileucine signal could either function to promote endocytosis of Sanpodo from apical membrane, or to shunt endocytosed Sanpodo from the recycling pathway to the lysosome, or both. Our analysis of the trafficking of the Sanpodo\textsuperscript{LHAAA} mutant shows that the NPAF-dependent sorting of Sanpodo from the basolateral membrane to endosomes is required for apical accumulation of the Sanpodo\textsuperscript{LHAAA} mutant. Therefore, the most likely scenario is that Sanpodo enters the endocytic system primarily from the basolateral membrane via the NPAF motif and the dileucine signal diverts most of the internalized Sanpodo to the lysosome. The dileucine may

Discussion

Notch is a central and conserved regulator of neuronal cell fate. In asymmetrically dividing neural progenitors, activation of Notch in one daughter cell and suppression of the pathway in the other determines whether a cell will proliferate, differentiate, or die; or decide between neural versus glial cell fates. The mechanisms by which Notch is activated or inhibited after mitosis have been extensively studied, but our understanding remains incomplete (Kandachar and Roegiers, 2012). In contrast to well-studied proteins like Numb, we show that the four-pass transmembrane protein Sanpodo acts both to promote Notch signaling on lateral inhibition, and that deletion of the Sanpodo amino terminus (Sanpodo\textsuperscript{N180}) or mutation of the ELL motif abrogates the lateral inhibition phenotype (Fig. S3), suggesting that these regions are important for reducing Notch activity in this assay.

Figure 6. A potential model for Sanpodo trafficking in SOP lineage cells. Sanpodo expression is induced in SOP cells and the bulk of Sanpodo (red) enters the endocytic system, along with Numb, from the basolateral membrane via an interaction of Numb (green) and the Sanpodo NPAF motif. An alternative route for Sanpodo and Notch endocytosis requires the ELL motif. Endocytosed Sanpodo is directed to the lysosome and shunted away from the recycling to the endocytic system, along with Notch, from the basolateral membrane via an interaction of Numb (green) and the Sanpodo NPAF motif. An alternative

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also play a role in endocytosis of Sanpodo–Notch complexes, as it is redundant with the NPAF motif for depletion of Notch from apical cell junctions in epithelial cells. This would be consistent with our observation that in the sanpodo clones rescued with the Sanpodo-NPAF, LHAAA mutant, Notch accumulates at the apical membrane in the pIIb cells, resulting in a cell fate change from pIIb to pIla.

Regulation of membrane Notch levels in pIIa and pIIb cells correlate with changes in cell fate (Benhra et al., 2011; Couturier et al., 2012). For example, asymmetric segregation of Numb into the pIIb cell acts to inhibit Notch membrane localization in pIIb cells and promote pIIb cell fate. However, endogenous Numb has virtually no inhibitory effect on Notch signaling in cells where Notch membrane levels are high, such as epithelial cells undergoing Notch-mediated lateral inhibition. In contrast, Numb overexpression strongly enhances Sanpodo’s dominant-negative effect on lateral inhibition (Fig. S3). Numb interacts with endocytic proteins such as α-adaptin and EPS15 (Santolini et al., 2000; Berdnik et al., 2002; Tang et al., 2005), and a recent live-cell imaging study supports the notion that Numb suppresses Notch receptor accumulation at the membrane interface between pIIb and pIIa cells through endocytosis, thereby inhibiting Notch activation in the pIIb cell (Couturier et al., 2012). Our findings support the notion that Sanpodo-mediated endocytosis of apical Notch is essential for robust inhibition of Notch signaling in the pIIb cell. Sanpodo may therefore amplify the difference in Notch signaling in the pIIa cell and pIIb cell by first depleting membrane Notch levels before asymmetric division, then further reducing Notch levels together with Numb in the pIIb cell after S0P mitosis. The mutation of the NPAF and ELL motifs results in a fairly weak Notch activation phenotype in SOP lineage cells, which suggests either that Sanpodo activity is partially redundant with Numb in controlling membrane Notch levels, or that other sorting signals, such as putative tyrosine-based motifs (RYXXXX, which are conserved in sanpodo insect orthologues; Fig. S1 A), contribute to further Notch internalization.

Although sanpodo orthologues have yet to be identified in vertebrates, it is likely that similar mechanisms evolved to control Notch activation and Notch membrane levels, perhaps by more than one protein. Little is currently known about the regulation of membrane trafficking of the Notch receptor in mammalian cells, but both Numb and the ubiquitin ligase Ich/Su(dx) have been implicated in the control of membrane levels of vertebrate Notch1 (Chastagner et al., 2008; McGill et al., 2009). Future studies may therefore identify regulators of Notch trafficking and activation that act like Sanpodo in the vertebrate context and further enhance our understanding of the regulation of Notch signaling during development.

Materials and methods

Generation of mutant Sanpodo-GFP transgenes

The PfuII (Agilent Technologies) amplified coding region of Sanpodo was cloned into a pENTR/D-TOPO vector (Invitrogen), and swapped by LR recombination into the Drosophila Gateway pTWG destination vector containing the UAS–carboxy-terminal GFP (P. Murphy, Carnegie Institute of Washington, Washington, DC; Tang et al., 2010). Sanpodo deletion mutant constructs were made using primers containing targeted deletions. Site-specific mutants of Sanpodo were generated using the QuickChange II mutagenesis kit (Agilent Technologies). Transgenic fly lines were generated by Bestgene. Independent GFP-tagged transgene lines inserted in both the second and third chromosomes behaved similarly in our experiments.

Drosophila genetics, imaging, and immunohistochemistry

Crosses and fly stocks were maintained at 20 or 25°C. Stocks used for these studies were as follows: laboratory stocks yw; apterous-Gal4, yw; neuralized-Gal4/TM3, Sb, yw; scabrous-Gal4/Cyo, yw; 109–68-Gal4 FRT20a/ CyO, yw; UAS-EGFP, yw; UAS-Sanpodo-GFP, yw; UAS-SanpodoNH-175–275-GFP, yw; UAS-SanpodoNH-275–325-GFP, yw; UAS-SanpodoNH-325–375–645-GFP, yw; UAS-SanpodoNH-325–375–645-LHAAA-GFP, yw; UAS-SanpodoNH-325–375–645-Sb1, yw; tm6B, ubx. Stocks derived from Bloomington Drosophila Stock Center stocks: w; UAS-Sanpodo, yw; ubx-FP; FRT20a ksbulinGal80. Pupae were removed from the pupal case, dissected in PBS and fixed for immunohistochemistry, and mounted in Vectashield (Vector Laboratories; Roegiers et al., 2001; Zipserman and Roegiers, 2011). Antibodies used were as follows: mouse anti-NECD (1:100, 485.2H, Developmental Studies Hybridoma Bank [DSHB], mouse anti-Cut (1:100, 2B10, DSBH); Rat anti-ELAV 7E8A10 (1:200, DSDB), and mouse anti-22C10/Futch (1:200, DSDB); secondary antibodies were conjugated to Alexa Fluor 555 or Alexa Fluor 633 Fluorochromes (Invitrogen). Pupae were dissected out of the pupal case and mounted between slide and coverslip for live-cell imaging (Roegiers et al., 2001; Zipserman and Roegiers, 2011). All images were acquired at room temperature (23°C) on an inverted microscope (model TE2000U; Nikon) equipped with a C1 confocal imaging system [488-, 543-, and 633-nm lasers; Nikon] or the SFC (swept field confocal) live-imaging system (488/514-nm temperature (23°C) on an inverted microscope (model TE2000U; Nikon) equipped with a C1 confocal imaging system [488-, 543-, and 633-nm lasers; Nikon] or the SFC (swept field confocal) live-imaging system (488/514-nm lasers; Nikon), using either 60 or 100x 1.49 NA objective lenses. All measurements were done using EZ-C1 software (Nikon). Wing disc apical Notch staining was quantified using Meta Morph imaging software (Molecular Devices) by acquiring XY images of the apical region of epithelial cells, merging the XY planes, and applying a threshold to both the NECD and the GFP images. The GFP threshold was used to establish GFP+ and GFP– regions. The NECD staining threshold was set to include the apical Notch staining in the GFP– (control) region. We then calculated the percentage of the total area of the region (GFP+ or GFP–) that contained apical NECD. These analyses were conducted at multiple regions of at least three wing discs for each genotype.

Protein biochemistry and comunnoprecipitation

For γ-secretase comunnoprecipitation assays, 7 × 10^6 Drosophila S2 cells seeded in a 10-cm plate and were cotransfected with 3 µg of pGF-PSN-Myc (kindly provided by M. Fortini, Thomas Jefferson University, Philadelphia, PA) and 3 µg of pAWF Sanpodo wild-type or mutant constructs. After 48 h of transfection, cells were harvested and lysed in 0.5 ml lysis buffer and the cell lysates were incubated with 40 µl of anti-Myc agarose [Sigma-Aldrich] at 4°C overnight after being preincubated in 40 µl of mouse IgG agarose [Sigma-Aldrich]. The immunoprecipitates were washed five times in 1x phosphate-buffered saline (PBS) and resolved on NuPAGE gels (Invitrogen) or the SFC (swept field confocal) live-imaging system (488/514-nm lasers; Nikon), using either 60 or 100x 1.49 NA objective lenses. All measurements were done using EZ-C1 software (Nikon). Wing disc apical Notch staining was quantified using Meta Morph imaging software (Molecular Devices) by acquiring XY images of the apical region of epithelial cells, merging the XY planes, and applying a threshold to both the NECD and the GFP images. The GFP threshold was used to establish GFP+ and GFP– regions. The NECD staining threshold was set to include the apical Notch staining in the GFP– (control) region. We then calculated the percentage of the total area of the region (GFP+ or GFP–) that contained apical NECD. These analyses were conducted at multiple regions of at least three wing discs for each genotype.

Online supplemental material

Fig. S1 shows the manual alignment of the Presenilin-binding region with other sanpodo homologues, and sequences of the amino-terminal region of sanpodo, su(dx), and other homologues, and sequences of the amino-terminal region of sanpodo, uheil, and rxx/y motifs highlighted. Fig. S2 shows that deletion of aa 100–125 of the Sanpodo ATCR abrogates Presenilin binding, but has no effect Notch ICD binding, and that mutation of the RY residues to alanine has no effect on Presenilin binding in vitro. Fig. S3 shows that Sanpodo misexpression in wing disc cells results in wing Notching and lateral inhibition defects on the scutellum. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201209023/DC1

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References


