A presenilin-1-dependent γ-secretase-like protease mediates release of Notch intracellular domain

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Signalling through the receptor protein Notch, which is involved in crucial cell-fate decisions during development, requires ligand-induced cleavage of Notch. This cleavage occurs within the predicted transmembrane domain, releasing the Notch intracellular domain (NICD), and is reminiscent of γ-secretase-mediated cleavage of β-amyloid precursor protein (APP), a critical event in the pathogenesis of Alzheimer's disease. A deficiency in presenilin-1 (PS1) inhibits processing of APP by γ-secretase in mammalian cells, and genetic interactions between Notch and PS1 homologues in *Caenorhabditis elegans* indicate that the presenilins may modulate the Notch signalling pathway[1–4]. Here we report that, in mammalian cells, PS1 deficiency also reduces the proteolytic release of NICD from a truncated Notch construct, thus identifying the specific biochemical step of the Notch signalling pathway that is affected by PS1. Moreover, several γ-secretase inhibitors block this same step in Notch processing, indicating that related protease activities are responsible for cleavage within the predicted transmembrane domains of Notch and APP. Thus the targeting of γ-secretase for the treatment of Alzheimer's disease may risk toxicity caused by reduced Notch signalling.

Notch-1 is synthesized as a type I integral-membrane protein of relative molecular mass 300,000 (M_r 300K) (Fig. 1) that is cleaved by a furin-like protease in the Golgi during trafficking of Notch to the cell surface. The two proteolytic fragments remain associated to form the functional receptor[5,6]. Following ligand binding, Notch-1 undergoes further cleavage close to or within its transmembrane domain, releasing the NICD. The NICD translocates to the nucleus and modifies transcription of target genes through its association with CSL proteins (where CSL stands for CBF1, Su(H), Lag-1)[7–9].

APP is also a type I integral-membrane protein. APP is the precursor of the amyloid-β peptide, which is deposited in the brains of Alzheimer's disease patients. Amyloid-β peptide is derived from APP as a result of cleavage of APP by β- and γ-secretase, whose molecular nature remains unclear. β-Secretase cleaves the extracellular domain of APP, producing a soluble ectodomain and a membrane-associated carboxy-terminal fragment (Fig. 1). γ-Secretase catalyses an intramembranous cleavage of this membrane-associated fragment, resulting in generation of amyloid-β peptide and the production of a C-terminal fragment of APP. Cleavage of APP by α-secretase precludes production of amyloid-β peptide (Fig. 1). The

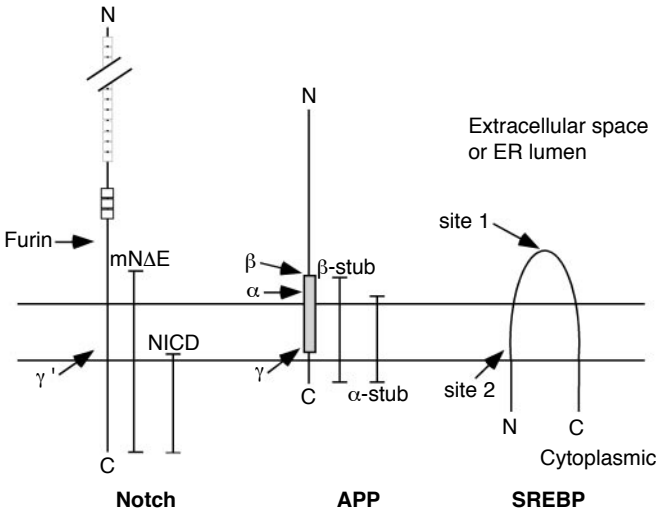

Figure 1 Proteolytic cleavage of Notch, APP and SREBP (not to scale). The proteolytic fragments (NICD and α- and β-stubs) and constructs (mNΔE) used to analyse Notch-1 and APP metabolism are indicted, as are the proteases involved in processing (furin, γ′-, α-, β- and γ-secretases, and site-1 and site-2 proteases). For a more extensive discussion on the analogies between APP, Notch-1 and SREBP processing, see ref. 30.

functional role(s) of these APP fragments is unknown. Genetic and biochemical studies have implicated the proteolytic processing events that lead to amyloid-β-peptide generation in the pathogenesis of Alzheimer's disease. Mutations in APP and the presenilins (PS1 and PS2) cause familial early-onset Alzheimer's disease, and presenilin mutations lead to increased processing of APP by γ-secretase *in vitro* and *in vivo*[10]. PS1-deficient mice show decreased γ-secretase processing of APP[4] and developmental abnormalities consistent with altered Notch signalling[2,3]. Genetic interactions between the *notch* homologues *glp-1* and *lin-12* and the *presenilin* homologues *sel-12* and *hop-1* in *C. elegans* provide indirect evidence for the involvement of the presenilins in the Notch signalling pathway[1,11]. Here we identify the specific step in Notch signalling that is controlled by PS1.

We first investigated whether the absence of PS1 interferes with the normal expression of Notch, as has been suggested[2]. We used brain extracts from PS1-deficient ($PS1^{-/-}$) mouse embryos at embryonic day (E) 14. As expected, no expression of the amino-terminal or C-terminal fragments of PS1 was found (Fig. 2a and ref. 4). Moreover, in agreement with our previous results obtained from study of neuronal cultures[4], we observed an accumulation of endogenous mouse APP C-terminal fragments in the same brain extracts, indicating deficient γ-secretase processing of APP when PS1 is absent (Fig. 2a, lower arrow). These fragments are predominantly 'stubs' produced by α-secretase, because endogenous mouse APP is not a good substrate for β-secretase (owing to the presence of amino-acid substitutions between the mouse and the human sequences at the β-secretase site)[4,12]. In the same homogenates, we detected normal steady-state levels of furin-processed Notch-1 C-terminal fragments, indicating that PS1 is not required for Notch biosynthesis or cleavage by furin within the Golgi (Fig. 2b). PS1-independent Notch maturation was also observed *in vitro*, in mouse fibroblasts derived from $PS1^{-/-}$ embryos (Fig. 2c). We conclude that $PS1^{-/-}$ mouse cells produce normal amounts of mature (furin-cleaved) Notch 1 protein.

The similarities between the proteolytic processing of Notch and APP are striking, especially the final proteolytic events that release the cytoplasmic domains, raising the possibility that PS1 might mediate the intramembranous proteolytic cleavage of Notch-1 to

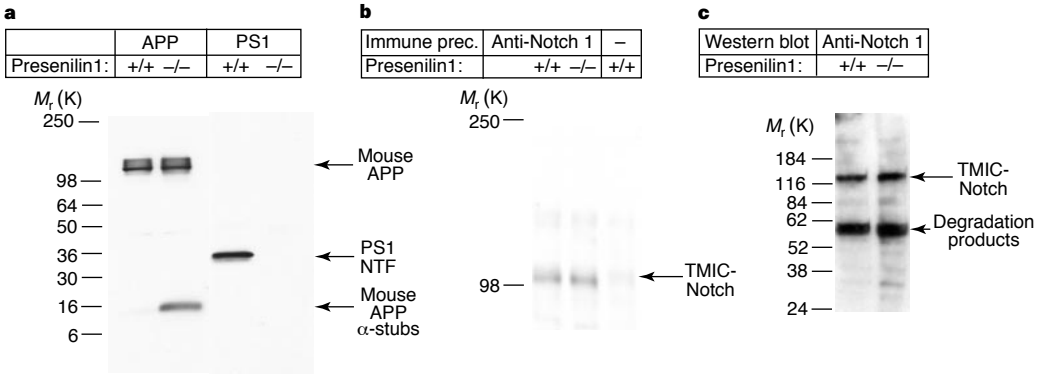

Figure 2 Processing of APP and Notch-1 in PS1$^{-/-}$ mouse brain and MEFs. Proteins were detected in western blots. **a**, Brain-membrane extracts (4–20% SDS–PAGE, 50 μg per lane) were reacted with APP675-695, an antiserum specific for the APP C terminus, or with an antiserum specific to the PS1 N terminus (PS1NTF). Arrows identify PS1NTF and a mouse APP fragment generated by α-secretase-mediated processing (mouse APP α-stubs). **b**, Notch-1 was immunoprecipitated from 100 μg brain extract using IC-antiserum, an anti-Notch-1 antiserum[5] and stained with the monoclonal antibody Tan20 (ref. 6) (6% SDS–PAGE). TMIC identifies the furin-generated mature Notch-1 fragment. **c**, MEF cell extracts were reacted with mN1A, a monoclonal antibody against Notch-1 (4–20% SDS–PAGE, 50 μg per lane).

release NICD[7–9]. As endogenous NICD levels in cells and tissues are biochemically below the level of detection[7], we took advantage of two well-characterized Notch constructs, mNotchΔE and N^{ICV}, that have been used to demonstrate the relationship between processing of and signalling through Notch-1 (refs 7, 13). The mNotchΔE construct codes for the transmembrane and intracellular domains of murine Notch-1 and undergoes a single proteolytic cleavage to release NICD. This cleavage resembles cleavage of endogenous Notch-1 in all respects[7,13]. The N^{ICV} construct encodes the intracellular domain of Notch-1 without the membrane anchor, producing a protein identical to NICD. N^{ICV} has been used as a control for the fate of NICD in PS1$^{-/-}$ cells[7]. Both constructs have part of their C-terminal domain replaced with six consecutive Myc-epitope tags[7]. We cloned these constructs into recombinant Semliki forest virus (SFV) vector, to enable their expression in primary cultures of neurons[4]. The expression and subcellular localization of mNotchΔE were similar in wild-type and PS1$^{-/-}$ cells, as was also the case for N^{ICV} (Fig. 3). However, NICD protein produced from N^{ICV} translocated to the nucleus in both PS1$^{+/+}$ and PS1$^{-/-}$ neurons (Fig. 3, lower panels), whereas nuclear staining in wild-type and PS1$^{-/-}$ neurons expressing mNotchΔE was almost undetectable, indicating that the relative amounts of NICD compared with unprocessed mNotchΔE were below the detection limit of our immunofluorescence assay (Fig. 3, upper panels). These observations are in agreement with genetic evidence from *C. elegans* that *sel-12* mutations do not interfere with *lin-12* signalling when the LIN-12 intracellular domain is expressed alone[11].

To assay directly the role of PS1 in mNotchΔE cleavage, we performed pulse-chase experiments and analysed the generation of NICD. Within 30–60 minutes after labelling with ^{35}S-methionine, NICD was first detected in PS1$^{+/+}$ cultures. NICD continued to accumulate throughout the 120-minute chase period (Fig. 4a, b; ratio of NICD to total mNotchΔE present at the start point of the chase). In contrast, NICD production was greatly reduced in PS1$^{-/-}$ neurons (Fig. 4a, b). As the PS1$^{-/-}$ embryos tend to yield fewer cells than their wild-type littermates[4], we analysed twice the amount of PS1$^{-/-}$ cell extracts. Only residual amounts of NICD were detected in PS1$^{-/-}$ neurons (Fig. 4c), even when the mNotchΔE signal was stronger than in the PS1$^{+/+}$ cell extracts. This difference could not be explained by a higher turnover rate of NICD in PS1$^{-/-}$ neurons, as NICD produced from control N^{ICV} constructs showed the same stability in both PS1$^{-/-}$ and PS1$^{+/+}$ cells (Fig. 4d).

To test the generality of this observation in another, non-neuronal, cell type, we transiently transfected PS1$^{-/-}$ fibroblasts with mNotchΔE plasmids. Although NICD was produced in PS1$^{+/+}$ cells, it was hardly detectable in PS1$^{-/-}$ cell extracts (Fig. 4e, extract). Co-immunoprecipitation of NICD with its nuclear partner, Flag-tagged CSL^{RBP3} (ref. 7), confirmed the existence of very low amounts of NICD in the PS1$^{-/-}$ cells (Fig. 4e). As residual amounts of both NICD and amyloid-β peptide can be detected in PS$^{-/-}$ cells, it is possible that the PS1 homologue PS2 (expressed in PS1$^{-/-}$ cells; K.C. and B.D.S., unpublished observations), can partially compensate for the loss of PS1 function. We conclude that NICD production is decreased markedly in PS1$^{-/-}$ cells.

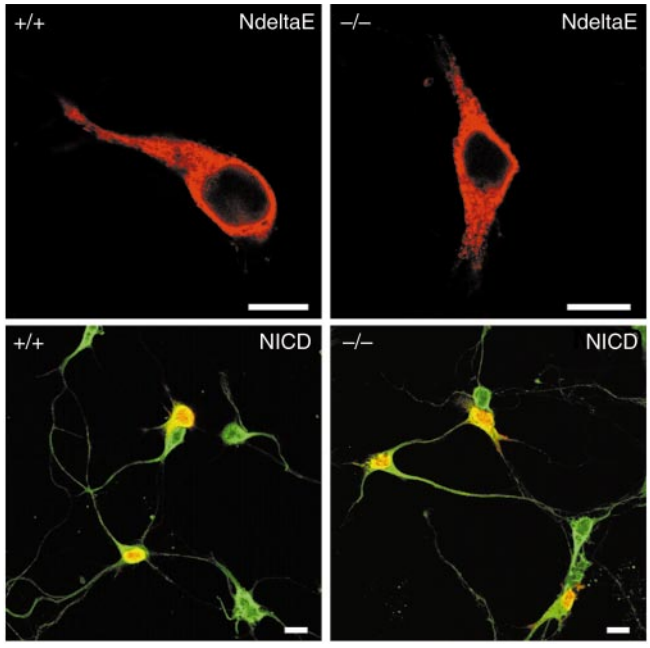

Figure 3 Nuclear transport of the intracellular domain (NICD) of Notch-1. Neurons were transfected with SFV driving expression of mNotchΔE or N^{ICV} (which is identical to the NICD), fixed and immunostained. Red fluorescence indicates Myc-tagged mNotchΔE (upper panels) or NICD fragment (lower panels). Nuclear import of NICD is not disturbed in the PS1$^{-/-}$ neurons. The green fluorescence in the lower panels indicates immunoreactivity towards calnexin, revealing the ER. The presence of uninfected neurons in the lower panels shows the specificity of the anti-Myc staining. +/+, PS1$^{+/+}$ neurons: –/–, PS1$^{-/-}$ neurons.

The site of cleavage of Notch-1, releasing NICD, may occur at an analogous position to the site of cleavage of APP by γ-secretase[14], N-terminal to the RKRRRQ stop-transfer signal[7]. This apparently

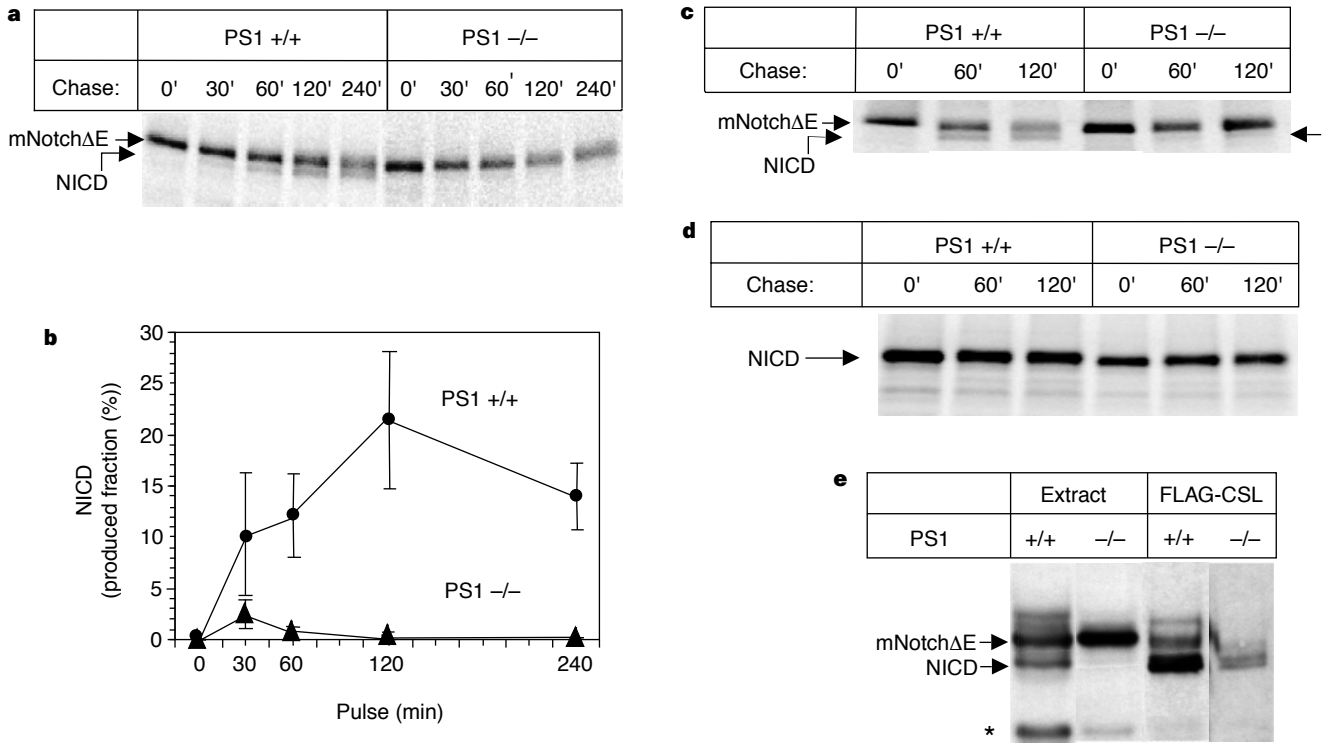

Figure 4 Generation of NICD in PS1$^{+/+}$ and PS1$^{-/-}$ neurons. **a**, Neurons transfected with SFV encoding mNotchΔE were labelled for 30 min and then chased in normal medium as indicated. Immunoprecipitated material was resolved in 4–20% SDS–PAGE. **b**, Phosphorimaging analysis of experiments performed as in **a**. The mNotchΔE signal at time zero was taken as 100% (lanes labelled 0′ in **a**). NICD produced from mNotchΔE at each time point was normalized to this signal (produced fraction). Values are means ± s.e.m. ($n = 3$). **c**, Control experiment, performed as in **a** but using twice as much material from PS1$^{-/-}$ neurons. NICD is hardly detectable at 120 min or 240 min of chase in PS1$^{-/-}$ neurons (left arrow) even when more mNotchΔE precursor is loaded. **d**, Turnover of NICD is identical in PS1$^{+/+}$ and PS1$^{-/-}$ neurons transfected with SFV encoding N^{icv}. The neurons were labelled for 30 min followed by a chase as indicated. **e**, mNotchΔE was expressed in PS1$^{+/+}$ and PS1$^{-/-}$ MEFs. We then analysed MEF extracts (left) and immunoprecipitates produced using anti-Flag–CSL antibodies (right) by western blotting. NICD is present in PS1$^{+/+}$ cell extracts and undetectable in PS1$^{-/-}$ cell extracts. Enriching for NICD using anti-CSL co-immunoprecipitation reveals residual amounts of NICD in PS1$^{-/-}$ cells (5 min exposure, compared to 20 s in the other lanes). Asterisk indicates a post-lysis degradation peptide.

intramembranous cleavage is unusual and occurs in only one other known protein, the sterol-regulatory-element-binding protein (SREBP)[15] (Fig. 1). However, mutants deficient in SREBP cleavage show no abnormality in generation of amyloid-β peptide, indicating that the enzyme that mediates SREBP cleavage may not be closely related to γ-secretase[16]. No effects on SREBP processing were observed in PS1$^{-/-}$ cells (data not shown). In contrast, our results from PS1$^{-/-}$ cells show that PS1 is important for cleavage of both Notch-1 and APP. To determine whether the enzyme responsible for NICD release has similar pharmacological properties to γ-secretase, we compared the effects of protease inhibitors on processing of Notch-1 and APP, selecting inhibitors that have been shown previously to block γ-secretase[17–20]. These inhibitors also interfere with mNotchΔE processing (Fig. 5 and refs 7, 13). MDL 28170, which is a peptide aldehyde calpain inhibitor, was the first inhibitor shown to interfere with γ-secretase[18], and MG132, another peptide aldehyde, also inhibits γ-secretase. However, MG132 (and possibly MDL 28170) are relatively broad-spectrum protease inhibitors that inhibit the proteasome. We ruled out the possibility that inhibition of the proteasome is involved in the observed effects of MDL 28170 and MG132 on Notch-1 processing by using lactacystin, a specific proteasome inhibitor, which has no effect on NICD generation (Fig. 5 and E.H.S. and R.K., unpublished observations). Likewise, lactacystin does not affect γ-secretase-mediated cleavage of APP[21].

To further explore the apparent similarity between γ-secretase and the Notch-1 protease, we used the γ-secretase inhibitor MW167, a difluoro ketone peptide analogue designed on the basis of the primary sequence of the site of cleavage by γ-secretase in APP[17]. This compound inhibited, in a concentration-dependent manner, γ-secretase-mediated cleavage of human APP expressed in neurons using recombinant SFV (Fig. 6), leading to the accumulation of APP C-terminal stubs and the inhibition of amyloid-β-peptide secretion into the medium. As both α- and β-secretase-processed fragments appeared in the cells (α- and β-stubs, Fig. 6) and as secretion of the APP ectodomain into the medium was unaffected (data not shown), neither α- nor β-secretase enzymatic activity is inhibited by MW167 (see also ref. 17). Processing of mNotchΔE was inhibited by MW167 at concentrations similar to those that inhibit APP processing (Fig. 6). The estimated half-maximal inhibitory concentration (IC_{50}) for MW167 (10 μM) in mNotchΔE processing is nearly identical to its IC_{50} for APP[17]. MW167 is also a potent inhibitor of cathepsin D[22]; however, using a series of related difluoro ketone analogues, we found that the effect of MW167 on APP processing is not a consequence of cathepsin-D inhibition[22]. The three inhibitors (MG132, MDL28170 and the chemically different MW167) all inhibit mNotchΔE processing and γ-secretase-mediated cleavage of APP to similar extents. Although neither γ-secretase nor the enzyme that cleaves mNotchΔE has been purified yet, our results are consistent with the hypothesis that these two proteolytic activities are closely related.

We have shown that efficient processing of Notch-1 to produce NICD requires PS1 and is inhibited by γ-secretase inhibitors. We showed this by using the mNotchΔE construct, a reliable model for intracellular processing of Notch-1 (ref. 7). Furthermore, the expression of endogenous Notch-1, its maturation by a furin-like enzyme, and the translocation of NICD to the nucleus are not affected by PS1 deficiency. Notch-1 and APP both exhibit a genetic

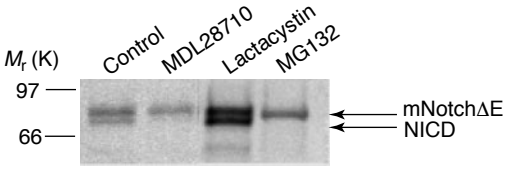

Figure 5 Effect of γ-secretase inhibitors on mNotchΔE processing. Neurons were transfected with SFV encoding mNotchΔE as in Fig. 4a, c. Label was chased for 4 h in the presence of 1% DMSO (control), MDL 28170 (100 μM), lactacystin (100 μM) or MG132 (20 μM). The stronger signals in the lactacystin lane probably reflect stabilization of mNotchΔE when the proteasome is inhibited.

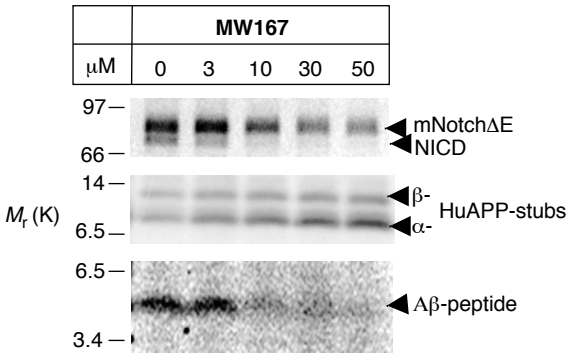

Figure 6 Effect of γ-secretase inhibitor MW167 on processing of mNotchΔE (upper panel) and APP (lower panels). SFV-infected neurons were pulse-labelled for 30 min and label was chased for 4 h in the presence of DMSO vehicle or the indicated concentrations of MW167. mNotchΔE and NICD were immunoprecipitated using anti-Myc antibodies. APP stubs (reflecting α-secretase- and β-secretase-mediated processing of human APP) and amyloid-β peptide (Aβ peptide; 4 h labelling) were immunoprecipitated as described[4,12].

interaction with presenilins[1–3,11] and are cleaved by a γ-secretase-like activity that requires PS1 protein to perform an apparently intramembranous cleavage. These results provide a molecular explanation for the widely reported genetic interaction between Notch-1 and PS1 (refs 1–3, 11) in different species.

There is also a direct physical interaction between Notch and PS1 (ref. 23), as there is between APP and PS1/2 (refs 24, 25). The function of PS1 in Notch/APP processing could be that of a 'facilitator', analogous to the role of the endoplasmic-reticulum (ER)-resident, multimembrane-spanning protein SCAP in SREBP[15] processing. Such a facilitator could directly expose APP and Notch-1 to the γ-secretase. Alternatively, PS1 could indirectly regulate the correct trafficking of APP, Notch-1 and γ-secretase to an as-yet-undefined subcellular (micro) domain where cleavage occurs[26]. However, our results are also consistent with a more direct role for PS1 as a regulatory or catalytic component of the protease(s) that cleave(s) Notch-1 and APP. In this case, PS1 would have a role similar to that of the ER-resident multimembrane-spanning site-2 protease[27] in SREBP processing (Fig. 1).

Finally, our results have significant implications for the potential use of γ-secretase inhibitors as drugs for treatment of Alzheimer's disease. The resulting inhibition of Notch-1 processing in the adult would be predicted to lead to immunodeficiency and anaemia because of the important function of Notch-1 in haematopoiesis throughout life[28,29]. Thus, it may be necessary to develop inhibitors specific for γ-secretase-mediated cleavage of APP or to target other steps in the generation and deposition of amyloid-β peptide. □

Methods

The generation and characterization of $PS1^{-/-}$ mice, the generation of neuronal cell cultures, the use of recombinant SFV, metabolic labelling, cell extraction and double immunoprecipitation were done as described[4,12]. For pulse-chase experiments, cells from one embryo were seeded in a 12-well or 4-well cell-culture plate (Nunc) pretreated with polylysine and serum. After infection with the appropriate recombinant SFV[4,12], neurons were pulse-labelled for 30 min with 200 μCi ^{35}S-methionine per ml, and chased for the indicated time in normal medium. Cells were processed as before[12]. Every lane (Figs 4a, c, d, 5, 6) shows the material obtained from one well, and signals were compared between littermate embryos. MG132 (Calbiochem), MDL 28170 (provided by B. Cordell) and MW167, each dissolved in dimethylsulphoxide (DMSO), were added at the end of the pulse labelling to the indicated final concentrations. DMSO in the cell cultures was 1%.

Mouse brain was homogenized in 5 mM Tris-HCl, pH 7.4, 250 mM sucrose, 1 mM EGTA, 5 mm EDTA, 1 μg ml^{-1} pepstatin A and 100 U ml^{-1} aprotinin, and centrifuged at 500*g* for 10 min. The resulting supernatant was centrifuged at 100,000*g* for 60 min. The pellet was resuspended in PBS and protein concentration was measured (Biorad protein assay).

We plated 5×10^5 mouse embryonic fibroblasts (MEFs) immortalized with SV40 large T antigen in 100-mM plates and transfected them by the Ca_2PO_4/BBS method[13] with both Flag–CSL^{RBP3} and mNotchΔE. After lysis in co-immunoprecipitation buffer[7] containing 200 mM KCl, 10% of the sample was used for crude extract analysis. The remainder was co-immunoprecipitated[7] using anti-Flag antibody. The washed pellet was resuspended in 50 μl Laemmli lysis buffer and resolved by 6% SDS–PAGE, transferred to nitrocellulose membrane and visualized using 9E10 anti-Myc antibody[7,13]. For immunocytochemistry, neurons cultured on glass coverslips were fixed in 4% paraformaldehyde and permeabilized in ice-cold methanol and acetone. Antibodies were diluted in blocking buffer and cover slips were mounted in Mowiol (Calbiochem). Cells were analysed using a MRC1024 confocal microscope (Biorad).

The complementary DNAs coding for mNotchΔE and N^{ICV}[7,13], antiserum APF675-695 (ref. 12) used to immunoprecipitate the APP C-terminal stubs, and antiserum APF597-612 (ref. 12) used to immunoprecipitate amyloid-β peptide have been described. We used mNotchΔE in which Met 1727 was mutated to Val to prevent alternative translation initiation. NICD production from mNotchΔE is unaffected by this mutation, as shown previously[7,13]. B19/2 was raised against amino acids 30–44 of PS1. IC-antiserum against the cytoplasmic domain of murine Notch-1 (ref. 15) was provided by A. Israël and used to immunoprecipitate endogenous Notch-1 from brain extracts. Rat monoclonal antibody Tan20 (provided by S. Artavanis-Tsakonas) was used for immunodetection of precipitated Notch-1 (ref. 6). mN1A, a monoclonal antibody specific to the cytoplasmic domain of Notch-1 (provided by L. Milner), was used for detection of mouse Notch-1 from MEF SDS extracts. Calnexin antiserum and the anti-Myc monoclonal antibody 9E10 were provided by A. Helenius and J. Cremers.

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Presenilin is required for activity and nuclear access of Notch in *Drosophila*

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Presenilins are membrane proteins with multiple transmembrane domains that are thought to contribute to the development of Alzheimer's disease by affecting the processing of β-amyloid precursor protein[1]. Presenilins also facilitate the activity of transmembrane receptors of the LIN-12/Notch family[2–5]. After ligand-induced processing, the intracellular domain of LIN-12/Notch can enter the nucleus and participate in the transcriptional control of downstream target genes[6–9]. Here we show that null mutations in the *Drosophila Presenilin* gene abolish Notch signal transduction and prevent its intracellular domain from entering the nucleus. Furthermore, we provide evidence that presenilin is required for the proteolytic release of the intracellular domain from the membrane following activation of Notch by ligand.

β-Amyloid precursor protein (β-APP) is a transmembrane protein that travels by way of the endoplasmic reticulum and Golgi to the cell surface and undergoes proteolytic processing (reviewed in ref. 1). A set of β-amyloid (Aβ) peptides are generated from β-APP by proteases known as the β- and γ-secretases. The β-secretase cleavage occurs in the extracellular domain and the heterogeneous γ-secretase cleavages in the transmembrane domain. Dominant mutations in either of two human *Presenilin* genes appear to cause Alzheimer's disease by increasing the amount of the Aβ42(43) fragment that is produced. A null allele of mouse *Presenilin1* appears selectively to reduce γ-secretase activity[10]. These observations indicate that presenilin either stimulates the activity of γ-secretase, or is itself a component of γ-secretase[10].

LIN-12/Notch proteins act as transmembrane cell-surface receptors for intercellular signals during development. It has been proposed that signal transduction involves cleavage and transport of the intracellular domain to the nucleus (reviewed in ref. 11), and results from experiments in *Drosophila*[6,8,9] and mammalian cells[7] strongly support this idea, indicating that cleavage occurs in or near the transmembrane domain. In mammalian cells, at least one

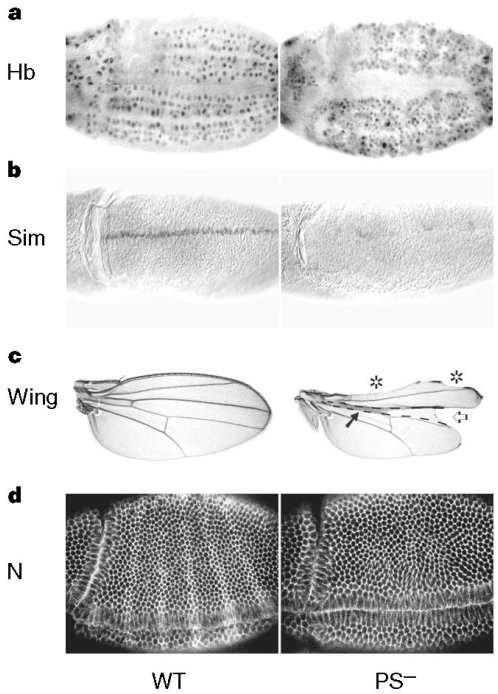

Figure 1 Presenilin activity is required for Notch activity. **a**, Hunchback (Hb) expression marks neuroblasts. Notch activity normally prevents more than one cell in a proneural cluster from segregating as a neuroblast. In *PS*⁻ embryos, clusters of neuroblasts form instead of single neuroblasts, generating longitudinal bands, rather than lines, of neuroblasts. Hb expression is shown just after the completion of germ-band extension [the dorsal (top) and ventral (bottom) aspects of each embryo are shown in different focal planes; anterior is to the left]. **b**, Single-minded (Sim) expression marks ventral midline cells[19]. *Notch* activity normally promotes differentiation of midline cells. In *PS*⁻ (and *N*⁻) embryos, relatively few of these cells arise, in contrast to wild-type embryos where they form a continuous two-cell wide column along the midline. Sim expression is shown dorsally, just after completion of germ-band extension. **c**, Right: a wing containing clones of *PS*⁻ cells, marked with the *mwh* mutation (the *mwh* marker is not visible at this magnification). *Notch* activity is normally required to transduce signals between the dorsal and ventral compartments at the wing margin which are necessary for the survival and growth of wing blade cells[20]. *PS*⁻ clones that arise near the wing margin (asterisks) cause scalloping of the wing, as do *N*⁻ clones in this region[26]. Clones of mutant cells at a distance from the boundary can survive and populate internal portions of the wing blade (one such clone is indicated by an open arrow and outlined with dashed lines). However, such clones are associated with vein thickening (filled arrow), also attributable to a failure in signal transduction by Notch[21]. *PS*⁻/*PS*⁻ embryos derived from *PS*⁻/+ females can survive until the onset of pupation; however, the imaginal discs are abnormally small. **d**, Notch protein (N) accumulates to similar levels and is mainly associated with the plasma membrane in *PS*⁺ and *PS*⁻ embryos. Both embryos are derived from *PS*⁻ female germ cells and are shown ventro-laterally at the same stage of gastrulation with the presumptive Sim-expressing midline cells forming single cell columns flanking the ventral midline. Right: *PS*⁻/*PS*⁻ zygote; left: *PS*⁻/+ zygote, marked by the striped expression of β-galactosidase from a paternally derived third chromosome carrying both a *PS*⁺ allele and a *ftz-lacZ* reporter transgene (seven stripes of β-galactosidase expression can be seen superimposed on the otherwise normal pattern of N-protein expression). Such paternally rescued embryos can develop into normal first-instar larvae and give rise to adults that appear phenotypically wild-type.

proteolytic event occurs in the extracellular domain during Notch transit to the cell surface[12,13], and it has been suggested that ligand-binding might trigger additional extracellular proteolytic processing[13]. Thus, LIN-12/Notch proteins undergo proteolytic processing events that resemble the β- and γ-secretase cleavages of β-APP. These parallels, as well as genetic studies of presenilin in *Caenorhabditis elegans*, indicate that the presenilins may promote proteolytic cleavage during receptor maturation or activation[6,14].

To investigate the involvement of presenilin in proteolysis of LIN-12/Notch proteins, we wanted to use an *in vivo* assay for ligand-dependent cleavage and nuclear access of the intracellular domain that we developed in *Drosophila*[6]. We therefore identified null alleles of the single *Drosophila Presenilin* gene *PS* and examined their effects on the nuclear access and activity of Notch.

The *PS* gene has been identified by sequence analysis and mapped to a centromere proximal portion of the left arm of chromosome 3 (refs 15–17). To identify *PS* mutants, we examined a collection of recessive-lethal mutations that map to this location and cause a neurogenic phenotype in genetic mosaics (J. Jiang, C.-M. Chen and G.S., unpublished observations) for DNA sequence alterations in *PS*. Two independent mutations, PS^{C1} and PS^{C2}, contained lesions in *PS*. Both alleles are predicted to cause premature termination and appear to be null alleles: the predicted product of PS^{C1} lacks the last two transmembrane domains and other highly conserved sequences, and PS^{C2} contains a stop codon which truncates the coding sequence before the first transmembrane domain. PS^{C1} and PS^{C2} have indistinguishable phenotypes, and both alleles are fully complemented by the presence of a transgene expressing the PS^{+} coding sequence (see Methods), confirming that the phenotype is due to the alterations in the *PS* gene.

To generate embryos with no *PS* activity, we removed both maternal and zygotic *PS* activity by generating PS^{-} embryos derived from PS^{-} female germ cells. These embryos, referred to here as PS^{-} embryos, appear identical to embryos without maternal and zygotic *Notch* activity (N^{-} embryos). For example, in both PS^{-} and N^{-} embryos[18], clusters of neuroblasts segregate at the positions normally occupied by single neuroblasts, as visualized by hunchback (Hb) staining (Fig. 1a). Both PS^{-} and N^{-} embryos also show extensive neural hyperplasia during subsequent development and die as pharate first-instar larvae lacking both dorsal and ventral cuticle (data not shown). In addition, the number of midline cells, as defined by the expression of *Single-minded* (*Sim*), is greatly reduced (ref. 19; Fig. 1b). Notch protein is found predominantly at the plasma membrane and at similar levels in both wild-type and PS^{-} embryos (Fig. 1d, and data not shown). Hence, the profound developmental defects in PS^{-} embryos appear to result from the absence of Notch signal-transducing activity, rather than from a marked decrease in Notch protein at the plasma membrane.

We have also generated clones of PS^{-} cells in the imaginal discs, which give rise to the adult appendages, and find that these cells exhibit phenotypes similar to those of N^{-} cells. For example, PS^{-} clones in the wing cause scalloping and vein thickening (Fig. 1c), reflecting failures in well defined signalling events that depend on *Notch*, such as the specification of Wingless-secreting margin cells along the dorso–ventral compartment boundary[20] and the lateral inhibition of vein differentiation[21].

The PS^{-} phenotypes indicate that presenilin is essential for Notch function (see also ref. 3). *PS* null mutations do not significantly affect other aspects of *Drosophila* development, consistent with indirect evidence from *C. elegans* that the presenilins are not required for transport or processing of membrane proteins in general[14].

We investigated the effect of *PS* null mutations on nuclear access by the Notch intracellular domain by using three Notch proteins in which the chimaeric transcription factor Gal4–VP16 (GV) is inserted in-frame into Notch (N) just after the transmembrane domain (at site 3; ref. 6). Nuclear access is assayed by *UAS–lacZ* expression[6]. N^{+}–GV3 functions like the wild-type Notch protein and the intracellular domain gains nuclear access and has signal transducing activity only in the presence of the ligand, Delta (ref. 6; Fig. 2). N^{ECN}–GV3 contains a deletion that removes most of the extracellular domain and causes constitutive signal transducing activity and nuclear access in the absence of Delta (ref. 6; Fig. 2).

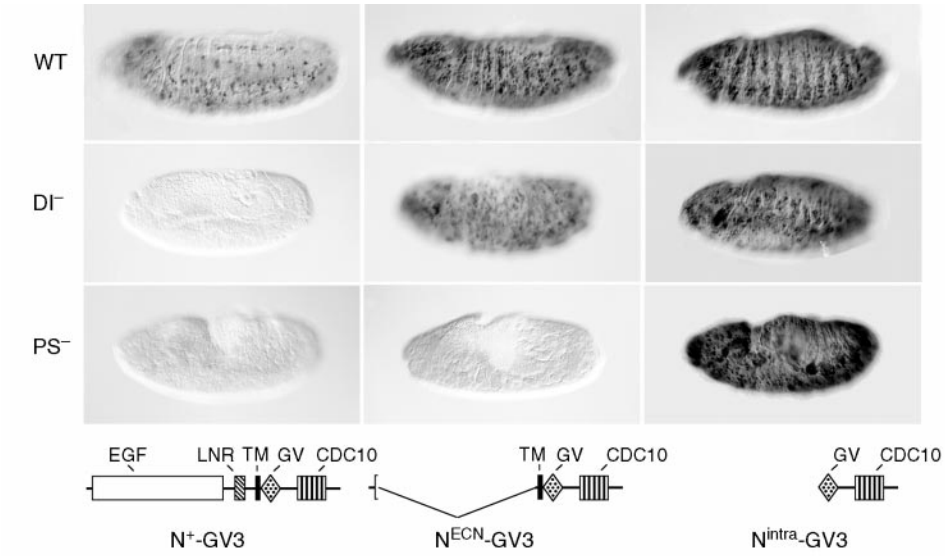

Figure 2 Presenilin is required for nuclear access of Notch. β-Gal expression indicates nuclear access of the Notch intracellular domain[6]. The N–GV3 constructs assayed are shown schematically at the bottom of each column. Wild-type Notch protein contains a region of 36 tandem EGF repeats (EGF), a region of three tandem LIN-12/Notch repeats (LNR), a single transmembrane domain (TM) and a region of six CDC10/SWI6/ankyrin repeats (CDC10). In all cases, the Gal4–VP16 domain (GV) is inserted at the beginning of the intracellular domain (site 3 in ref. 6). All embryos are shown in lateral aspect around the end of germ-band shortening (left: anterior). The same results were obtained using either the PS^{C1} or PS^{C2} allele. N^{+}–GV3 (left column): β-Gal expression is observed in wild-type (WT) but not in embryos lacking Delta (D^{-}), indicating that nuclear access of the intracellular domain is ligand-dependent. β-Gal expression is not seen in PS^{-} embryos (bottom), indicating that nuclear access requires presenilin. N^{ECN}–GV3 (middle column): Nuclear access is ligand-independent, as β-Gal is widely expressed in WT and Dl^{-} embryos. However, no β-Gal expression is observed in PS^{-} embryos, indicating that nuclear access requires presenilin. N^{intra}–GV3 (right column): There is widespread β-Gal expression in WT, Dl^{-} and PS^{-} embryos, indicating that nuclear access does not require presenilin.

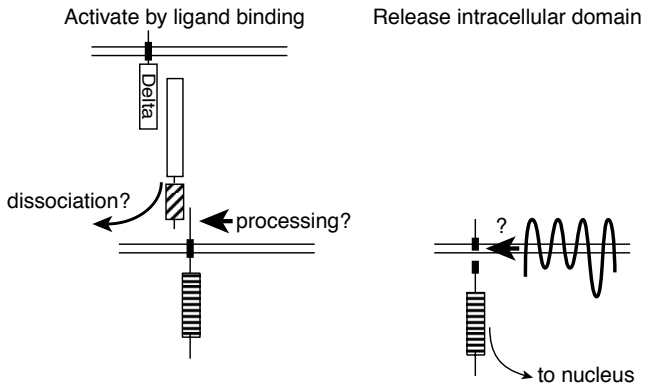

Figure 3 Model for LIN-12/Notch signal transduction. LIN-12/Notch proteins are represented as in Fig. 2, and presenilin is represented as an eight-transmembrane domain protein[23]. Transmembrane protein ligands such as Delta, present on neighbouring cells, bind to LIN-12/Notch and activate signal transduction. We show the consequences of ligand-binding as two distinct phases, each of which may involve many events. The first phase is activation by ligand-binding, which may have one or more consequences. Ligand-binding may displace the amino-terminal portion of the heterodimeric receptor or otherwise lead to a conformational change. After ligand has bound, the receptor may dimerize[27,28] or undergo processing of the extracellular domain[13]. The second phase is release of the intracellular domain, which involves proteolytic cleavage in or near the transmembrane domain. In principle, presenilin might be required for any event triggered by binding of ligand. However, presenilin is required both for ligand-independent access of N^{ECN} and ligand-dependent access of N^{+}. We therefore favour the view that presenilin is required for the cleavage in the second phase. Presenilin might directly mediate cleavage within or near the transmembrane domain, perhaps by functioning as, or activating, a protease. Alternatively, presenilin may act indirectly, for example in the endoplasmic reticulum or Golgi, to facilitate the transport of other components needed for this cleavage.

N^{intra}–GV3 lacks the extracellular and transmembrane domains and also displays ligand-independent nuclear access (Fig. 2). The key difference between the two constitutively active forms is that N^{ECN}–GV3 retains the transmembrane and extracellular juxtamembrane domains whereas N^{intra}–GV3 is a cytosolic protein.

In *PS*$^{-}$ embryos, neither N^{+}–GV3 nor N^{ECN}–GV3 has access to the nucleus, as indicated by the complete absence of β-galactosidase (β-Gal) expression (Fig. 2). In contrast, the nuclear access of N^{intra}–GV3 is unaffected by the absence of presenilin activity (Fig. 2). The N^{+}–GV3 observation indicates that presenilin activity is normally required for nuclear access of the Notch intracellular domain. Furthermore, the observation that presenilin is needed for nuclear access of N^{ECN}–GV3, a constitutively active transmembrane form, but not for N^{intra}–GV3, a constitutively active cytosolic form, suggests that presenilin participates in the release of the intracellular domain from the plasma membrane. Only about 35 amino acids of the Notch extracellular juxtamembrane region remain in the N^{ECN}–GV3 protein. Thus, if there are specific signals required for presenilin-dependent cleavage, they are likely to be somewhere in this region or within the transmembrane domain.

Although we have demonstrated that presenilin is necessary for the ligand-dependent nuclear access of the intracellular domain, we do not know whether presenilin directly mediates proteolytic release of the intracellular domain or if it acts more indirectly, for example by activating a protease or mediating its transit to the plasma membrane. Presenilin is found in a variety of cellular membranes, and β-APP may be processed at multiple sites, including the plasma membrane[1]. Furthermore, in *Drosophila*, endogenous presenilin has been detected in the plasma membrane[22], which is a likely site of ligand-dependent proteolysis.

Our findings can be incorporated into a model of events involved in LIN-12/Notch signal transduction, in which ligand-binding activates LIN-12/Notch, thereby creating a substrate for presenilin-dependent release of the intracellular domain from the membrane (Fig. 3). Although there are other possibilities (Fig. 3 legend), release could require the direct participation of presenilin in the proteolytic cleavage of LIN-12/Notch proteins in or near the transmembrane domain. Presenilin may play an analogous role in the processing of β-APP[10]. □

Methods

Identification of *PS* mutations. Genomic DNAs were obtained from wild-type larvae and from *PS*$^{-}$/*PS*$^{-}$ larvae, and the *PS* genes were recovered by PCR amplification and sequenced. The *PS*^{C1} allele is associated with the insertion of 8 base pairs (bp) (GATATATA) in place of 2 bp (CG) immediately downstream of the codon Glu 438 (GAA) (numbered as in ref. 16). This insertion is predicted to destroy a splice donor and to truncate the protein before the last two transmembrane domains (according to the eight-transmembrane-domain topology model[23]). The *PS*^{C2} allele is associated with a CAA → TAA mutation in the codon Gln52.

The *Tubulinα1-PS*$^{+}$ transgene was generated by placing the coding sequence of *PS* downstream of the *Tuba1* promoter[18]. Several independent transformants rescued both *PS* mutant alleles.

Generation of clones for phenotypic analysis. *PS*$^{-}$ germline clones were obtained by the FLP/FRT/ovoD method[24] and females carrying these clones were fertilized by males carrying the same *PS*$^{-}$ mutation in trans to a *TM3*, *ftz-lacZ* balancer chromosome, which allows paternally rescued zygotes to be distinguished from mutant zygotes. For the analysis of *PS*$^{-}$ mosaics in the adult, clones of *PS*$^{-}$ cells marked with *yellow* (*y*) and *multiple wing hairs* (*mwh*) were generated using the FLP/FRT method[25] by heat-shocking first-instar larvae of the genotype *y hs-FLP;mwh PS*^{C1} *FRT2A/Dpsc*^{J4},*y*$^{+}$ *FRT2A*.

Transgenes and assay for nuclear access. The *UAS–lacZ* expression assay, the *hs-N*$^{+}$*–GV3* and *hs-N*^{ECN}*–GV3* transgenes, and the results of expressing these transgenes in wild-type and *Dl*$^{-}$ embryos have been described[6]. The *hs-N*^{intra}*–GV3* transgene is similar to the other two transgenes, except for the deletion of the upstream Notch coding sequence and the introduction of a start codon in front of the Gal4–VP16 coding sequence. To assay Notch nuclear access in *PS*$^{-}$ embryos, females carrying *PS*$^{-}$ germline clones were crossed to *UAS–lacZ/UAS–lacZ;PS*$^{-}$*hs-N–GV3/TM3,ftz–lacZ* males.

Protein localization Hunchback, Sim and β-galactosidase proteins were detected immunohistochemically[6,18,19]. Notch was detected by immunofluorescence using a rabbit polyclonal antiserum directed against the intracellular domain[9]; predominant localization of Notch at the plasma membrane was confirmed by counterstaining with concanavalinA–Alexa594 (Molecular Probes) (data not shown).

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Neurogenic phenotypes and altered Notch processing in *Drosophila Presenilin* mutants

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Presenilin proteins have been implicated both in developmental signalling by the cell-surface protein Notch and in the pathogenesis of Alzheimer's disease. Loss of presenilin function leads to *Notch/lin-12*-like mutant phenotypes in *Caenorhabditis elegans*[1,2] and to reduced *Notch1* expression in the mouse paraxial mesoderm[3]. In humans, presenilins that are associated with Alzheimer's disease stimulate overproduction of the neurotoxic 42-amino-acid β-amyloid derivative (Aβ42) of the amyloid-precursor protein APP[4]. Here we describe loss-of-function mutations in the *Drosophila Presenilin* gene that cause lethal *Notch*-like phenotypes such as maternal neurogenic effects during embryogenesis, loss of lateral inhibition within proneural cell clusters, and absence of wing margin formation. We show that presenilin is required for the normal proteolytic production of carboxy-terminal Notch fragments that are needed for receptor maturation and signalling, and that genetically it acts upstream of both the membrane-bound form and the activated nuclear form of Notch. Our findings provide evidence for the existence of distinct processing sites or modifications in the extracellular domain of Notch. They also link the role of presenilin in Notch signalling to its effect on amyloid production in Alzheimer's disease.

To test directly the involvement of presenilin in Notch-receptor processing and signalling, we isolated lesions in the *Drosophila Presenilin* (*Psn*) gene by performing a lethal mutagenesis of the third chromosome region 77A-C uncovered by the deficiency *Df(3L)rdgC-co2*, an interval that harbours the *Psn* gene (see Methods). We recovered five lethal alleles of a single complementation group that is phenotypically rescued by genomic DNA fragments containing a functional *Psn* gene and which fails to be rescued by a DNA fragment from which the *Psn* coding region has been deleted (Fig. 1). All five *Psn* mutants have indistinguishable lethal phases and mutant phenotypes at the end of the larval period when assayed in any heteroallelic combination or *in trans* to *Df(3L)rdgC-co2*, and so are likely to be strong or complete loss-of-function alleles. Mutant larvae secrete a pupal case and complete the last stages of larval development but do not form any adult structures; instead, they collapse into a homogeneous oily mass within the pupal case. This zygotic lethal null phenotype closely resembles that observed for *Suppressor of Hairless* (*Su(H)*), which encodes an effector protein of the Notch signalling pathway[5,6].

Formation of the dorsal/ventral (D/V) boundary along the presumptive wing margin requires Notch activity, and may be visualized by the expression of specific reporter genes in the margin zone of the late larval wing disc[7–10]. Expression of *cut–lacZ* along the wing margin is abolished in *Psn* mutant wing discs (Fig. 2a, d). Similarly, margin zone expression of a *wingless–lacZ* construct and a *vestigial* D/V enhancer–*lacZ* transgene is absent in the *Psn* mutants, whereas *lacZ* expression outside the wing pouch persists in the mutants (Fig. 2b, c, e, f). Expression of a *vestigial* quadrant enhancer–*lacZ* transgene, which is specific to the non-margin zones of the wing pouch, is also completely absent in the *Psn*-mutant wing discs (Fig. 2g, j). Expression of these *wingless* and *vestigial* gene reporters along the wing margin and in the pouch region is directly dependent upon Notch signalling activity, and the *vestigial* D/V enhancer contains a critical binding site for the Su(H) protein[11]. The absence of margin structures in the *Psn*-mutant wing discs, together with the overall reduction in size of the mutant wing pouch region, is reminiscent of conditional *Notch* mutant and *Su(H)* null mutant phenotypes[5,7–10].

A second feature of wing development that is mediated by *Notch* is the emergence of individual sensory organ precursor (SOP) cells at defined locations of the wing disc, a process that depends upon lateral inhibition among proneural cell clusters at each location to ensure that a single cell of the cluster adopts the proper SOP fate[12,13]. Histochemical characterization of *Psn*-mutant wing discs using the early SOP markers *achaete–lacZ* and *scabrous–lacZ* reveals clusters of supernumerary SOP cells arising at certain normal SOP locations, as seen in typical neurogenic mutants (Fig. 2h, i, k. l). Lateral inhibition within these proneural cell clusters is severely impaired in *Psn*-mutant discs, resulting in enlarged SOP territories and increased proneural *achaete–lacZ* and *scabrous–lacZ* activity. To determine whether a similar requirement for presenilin in lateral signalling within the embryonic nervous system is masked by maternal deposition of the protein, we generated females with *Psn*-mutant germline clones (see Methods). Embryos derived from these females that lack both maternal and zygotic *Psn* activity

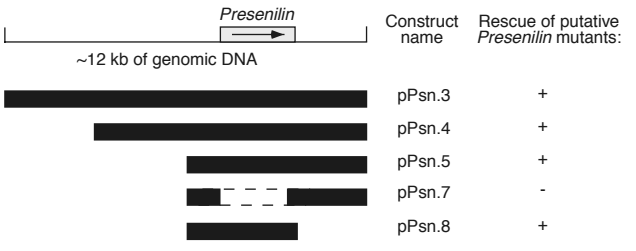

Figure 1 Identification of *Psn* mutations by genomic DNA rescue experiments. Transgenic flies were generated bearing different segments of wild-type genomic DNA from the *Psn* gene region and used to test different lethal complementation groups in the cytological interval 77A-C for phenotypic rescue to full viability. Construct pPsn.3 contains ~12 kb of genomic DNA, including the ~2.5 kb *Psn* transcribed region and ~7 kb and ~3 kb of 5′ and 3′ flanking sequences, respectively. Constructs pPsn.4 and pPsn.5 are similar to pPsn.3, except that they contain ~4 kb and ~1.2 kb of 5′ flanking sequences, respectively. Construct pPsn.7 is identical to pPsn.5 except that it bears an internal deletion of most of the *Psn* gene extending from –23 bp (within the putative TATA box) to +2,331 bp (51 bp upstream of the stop codon). Construct pPsn.8, which extends from ~1.2 kb upstream of the *Psn* coding region to ~100 bp downstream of the *Psn* polyadenylation site, rescues only one of sixteen lethal complementation groups uncovered by *Df(3L)rdgC-co2*.