Identification and Characterization of Presenilin-Independent Notch Signaling


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SUMMARY

Presenilin (PS) proteins control the proteolytic cleavage that precedes nuclear access of the Notch intracellular domain (NICD). Here we observe that a partial activation of the HES1 promoter can be detected in PS1/PS2 (PS1/2) double null cells using Notch1ΔE constructs or following Delta1 stimulation, despite an apparent abolition of the production and nuclear accumulation of NICD. PS1/2-independent Notch activation is sensitive to Numblike (Nbl), a physiological inhibitor of Notch. PS1/2-independent Notch signaling is also inhibited by an active γ-secretase inhibitor in the low micromolar range and not inhibited by an inactive analog, similar to PS-dependent Notch signaling. However, experiments using a Notch1-Gal4-VP16 fusion protein indicate that the PS1/2-independent activity does not release Gal4-VP16 and is therefore unlikely to proceed via an intramembranous cleavage. These data reveal that a novel PS1/2-independent mechanism plays a partial role in Notch signal transduction.
INTRODUCTION

Mutations in the two human presenilin (PS) genes account for the majority of familial Alzheimer’s disease (FAD) cases (reviewed in 1). PS genes also have a critical role in embryonic development. In mice, PS1 mutant embryos die with axial truncations and brain hemorrhages (2, 3), while PS2 knockout mice have a very mild phenotype (4, 5). Reducing levels of PS2 in a PS1-null background greatly enhances the embryonic lethality of these mice and produces somatic defects suggestive of loss-of-function mutations in Notch pathway genes (4, 5, 6, 7, 8, 9, 10). The C. elegans homologues of the presenilins, sel-12 and hop-1, genetically interact with the homologues of Notch, lin-12 and glp-1, and play a positive role in the transmission of Notch signals (11, 12, 13, 14). In addition, loss-of-function mutations in the single Drosophila PS homologue results in a Notch-like neurogenic phenotype in embryos, as well as wing “notching” and wing blade thickening in adult animals (15, 16, 17). Recently, Notch1 and PS1 were shown to interact physically in both mammalian and Drosophila cells (18, 19). These studies suggest that the requirement for PS genes in embryogenesis is largely a factor of their effect upon the Notch signal transduction pathway (13, 16, 17, 20, 21, 22). Whether the Notch pathway also plays a role in the pathogenesis of FAD, however, remains unknown.

Notch belongs to a family of conserved transmembrane receptors that influence a wide variety of cell fate decisions in organisms from worms to man (reviewed in 23, 24). Notch signaling is activated by binding of the DSL (Delta-Serrate-Lag-2) family of ligands to the extracellular domain of Notch. Ligand binding is thought to activate two sequential proteolytic steps; first on the extracellular domain (S2 cleavage) (25, 26), and
then an intramembranous endoproteolytic cleavage (S3 cleavage) that releases the cytoplasmic domain of Notch (NICD) (27, 28, 29, 30, 31). The cytoplasmic domain interacts with proteins of the CSL (CBF1-SuH-Lag1) family of transcription factors, and the complex migrates to the nucleus where it binds to transcriptional elements and activates genes such as HES1 (32). Truncated forms of Notch that consist solely of the intracellular domain have constitutive signal transducing activity and localize primarily to the nucleus, while membrane-tethered Notch constructs show constitutive activity because of spontaneous intramembraneous cleavage prior to nuclear localization (33, 34, 35, 36, 37, 38).

Recent studies have shown that presenilins are required for the intramembraneous cleavage of Notch (13, 16, 17, 20, 21, 22) as well as other type I membrane spanning proteins (39, 40, 41). In mammalian tissues, however, a deficiency of PS1 did not reduce signals through Notch1, despite a marked reduction in the intramembraneous cleavage of Notch1 (20), and Notch signaling appears intact in a PS1 conditional knockout in the brain (42). The ability of Notch signaling to proceed in the absence of PS1 could possibly be a consequence of the expression of PS2 and the functional redundancy of the two mammalian PS genes (4, 5, 20). In mammalian cells, two groups have been unable to detect evidence of γ-secretase-like cleavage of Notch1 or APP (43, 44) in cells derived from PS1/PS2 double knockout (PS1/2 null) embryos. Although these studies reveal a requirement for PS1 or PS2 in the production of the NICD fragment, only a preliminary investigation of the Notch signaling pathway in PS1/2 null cells was presented.

We sought to examine the effect of eliminating both PS homologues on Notch1 cleavage, nuclear localization and signaling in mammalian cells. We found that a
deficiency in the genes for both PS1 and PS2 markedly reduced the quantity of Notch1 cleaved at the intramembraneous site compared to control cells. In addition, membrane-tethered intracellular Notch proteins were precluded from the nucleus in PS1/2 null cells. Surprisingly, the lack of detectable cytoplasmic or nuclear NICD fragment led to only a partial reduction in HES1 activation by membrane-tethered intracellular Notch proteins or signaling elicited by Delta1 instead of the total impairment predicted by models in which either PS1 and 2 are strictly required for Notch signal transduction. PS1, PS2 or FAD-linked PS mutants restored PS1/2 null cells to wildtype levels of Notch1 cleavage and HES1 activation indicating that PS genes contribute to a fraction of Notch signaling, and that the FAD mutants tested are not markedly impaired with respect to promoting Notch signaling and cleavage. Additionally, a potent γ-secretase inhibitor (45) greatly diminished both S3 cleavage of a membrane-tethered Notch1 construct and Notch signaling in wildtype and PS1/2 null cells, suggesting the PS-null cells utilize a proteolytic mechanism for Notch signaling. We also observed that PS1/2 null cells do not elicit UAS activation through a Notch1-Gal4-VP16 (Notch1ΔE-G4V16) fusion protein suggesting that no intramembranous cleavage occurs, similar to that seen for APP in PS null cells. However, HES1 signaling was observed with Notch1ΔE-G4V16, suggesting a site for the putative PS1/2-independent protease distal to the Notch1 transmembrane domain or another uncharacterized mechanism. In conclusion, these data provide evidence that both PS-independent and PS-dependent mechanisms play a role in Notch signal transduction, and suggest that an novel mechanism supports Notch signal transduction when PS1 and 2 are absent.
EXPERIMENTAL PROCEDURES

Primary cells. On embryonic day E3.5 blastocysts were flushed from wildtype or PS1+/-; PS2+/- intercrossed mice (4) into M2 medium and plated on mitotically inhibited feeder cells. Five days later the inner cell mass was removed, trypsinized and plated onto new feeders cells. After 12 days the cells were replated onto gelatin-coated dishes, grown until confluent and then maintained in embryonic stem cell media (high glucose DMEM, 15% FBS, 2 mM L-glutamine, 1% penicillin-streptomycin, 1x non-essential amino acids, sodium pyruvate, plus recombinant LIF) on uncoated plates. For each cell line PCR genotyping was performed as described previously (3, 4). SV40 transformation was used to generate rapidly growing cell lines as described (46). PCR and RT-PCR were performed on immortalized primary cells grown for many generations without feeders. For RT-PCR, total RNA was prepared from immortalized primary cells (wildtype and PS1/2 null) using TRIZOL Reagent (Gibco BRL). mRNA (500ng and 2000ng for Fig 1B and Fig 1C respectively) was then used to perform an RT-PCR with the Titanium One-Step RT-PCR kit (Clontech). Primers specific for published murine PS1 sequence (PS1 EXON 4: 5’ TGA GAG GCA GAG ACT TGA CAA C, 5’CGG GGA CAA AGA GCA TGA TGA, PS1: 5’ACA CCC CAT TCA CAG AAG ACA A, 5’ ATG GCA ATC ATC CCG ACC AC ) and for published murine PS2 sequence (PS2 EXON 5: 5’ TAT TCG TGC CTG TCA CGC TG, 5’ GCA TCA GGG AGG ACA TGA TCA G, PS2: 5’ GCA ACG GAG ACT GGA ACA CTA C, 5’ GGC AGA GCC TTC TTG AAC ACA G) were used. Mouse β-actin primers provided by the kit were used as a positive control. The PCR was done as indicated by the manufacturer with the following modifications, 30 cycles were performed with an annealing temperature of 58°C.
DNA constructs. Notch1 contains the entire coding region of mouse Notch1 (37). NIC-myc (also known as Notch1IC) contains the entire intracellular domain of mNotch1 (amino acids 1744-2531) with a single amino-terminal myc epitope (37). NICD-myc\textsubscript{6} encodes the intracellular domain of mouse Notch1 terminating at amino acid 2185 (the XhoI site) with a carboxy-terminal hexameric myc tag (30). HA-TMIC-myc contains the transmembrane and intracellular domains of mNotch1 with an amino-terminal hemagglutinin (HA) tag and a single myc epitope tag inserted at the HindIII site (amino acid 2295) (27). Notch1\textsubscript{ΔE} (also known as \textsubscript{ΔE} or TMIC-myc 6) contains the transmembrane and intracellular domains of mNotch1 terminating at amino acid 2185 (the XhoI site) with a hexameric myc epitope at the carboxy-terminus (27). This construct has also been modified to replace a transmembrane domain methionine residue with valine (M1726V) in order to prevent intermediate translational initiations (27). Notch1\textsubscript{ΔE} -V\textsubscript{1744K} (V-K) contains the V1744K mutation (30). HES1-luc and HES1\textsubscript{ΔAB}-luc were previously described (32). Notch1\textsubscript{ΔE}-G4VP16 is derived from the Notch1\textsubscript{ΔE} construct with an insertion of Gal4VP16 after the transmembrane domain. Gal4 and Gal4VP16 were used respectively as negative and positive controls for the UAS-luc reporter (these 3 plasmids are kind gifts from Urban Lendhal, Johan Lundkvist and Helena Karlstrom). hAPP\textsubscript{swe} is a kind gift from Robert Vassar.

Immunoprecipitation, SDS-PAGE and Western Blots. For RBP co-IPs cells were plated at a density of 3x10\textsuperscript{6} in 10 cm plates and transfected the following day with 15ug DNA (1:1:1 ratio between Notch, RPB3-Flag and PS) using Lipofectamine Plus (Gibco BRL). The amount of DNA in each well was equalized by the addition of a control plasmid, pCDNA3. Cells were harvested 48 h after transfection and immunoprecipitation
studies were performed as described previously (20). Myc-tagged Notch1 constructs were detected with an anti-myc monoclonal antibody (9E10). Polyclonal antibodies against the amino-terminal domain of hPS1 (47) and the carboxy-terminal domain of hPS2 (48) were used to detect PS expression using 30µg of protein. Bound antibodies were detected with a chemiluminescent substrate (Pierce). For Jagged 1, IPs cells were harvested in cold RIPA buffer containing protease inhibitors and lysates were subjected to centrifugation at 10,000g for 10 min to remove cellular debris. Lysates were precleared by the addition of purified IgG1 (Sigma) followed by incubation with protein G beads (UltraLink, Pierce). Precleared lysates were then incubated with 2 µg of Jagged1 antibody (C20, Santa Cruz Biotechnologies) per 250 µg protein at 4 °C for 16hr, and immune complexes were pulled down with protein G beads, after an additional 30 min incubation at 4 °C. Jagged1 expression was then determined using the same Jagged antibody. For Notch1 blots, a polyclonal antibody against a region encompassing the RAM domain and part of the ankyrin domain (NRC, amino acids 1820-1835) was used to detect Notch1 expression at a dilution of 1:2000 using 50ug of protein. For β-galactosidase blots, a polyclonal antibody (Cappel) was used. For APP blots, 22C11 antibody (Chemicon) was used.

Immunofluorecence and nuclear counts. Cells were transfected using Lipofectamine Plus with 12ug Notch DNA per 10cm plate. Twenty-four hours post-transfection cells were harvested with trypsin (Gibco BRL) and replated onto 4 well Permanox slides (Nalge Nunc International) at a density of 1x10^5 cells/well. The following day the cells were fixed with 4% paraformaldehyde and stained with 9E10 antibody followed by FITC-conjugated anti-mouse antibodies to detect expression of the
myc tagged Notch constructs, and Hoechst dye to detect nuclei. In a blinded manner, labeled cells were scored for nuclear or cytoplasmic localization of the myc epitope. No myc-immunoreactivity was observed in the absence of transfected myc-tagged Notch constructs.

**Luciferase assays.** Cells were plated in 6-well dishes at a density of 4x10^5 and transfected the following day with Lipofectamine Plus (Gibco BRL). The amount of DNA (1.3 µg) in each well was equalized by the addition of a control plasmid, pCDNA3. Cells were harvested 48 h post transfection and luciferase activity was measured using a luminometer (Zylux). To ensure equivalent transfection between lines a control reporter gene (thymidine kinase-renilla luciferase; TK-luc, or Rous sarcoma virus-firefly luciferase; RSV-luc) was included with each experiment. Corrected luciferase activity was defined as the ratio of HES1-luc values divided by TK-luc values. For co-culture assays (48), QT6-RCAS-GFP or QT6-RCAS-Delta1 cells (a kind gift of Olivier Pourquie) were plated at a density of 1.5x10^6 24 h after transfection and harvested 24 h later for luciferase measurements.

**Northern Blot Hybridization.** Total RNA was prepared from primary cells using TRIZOL Reagent (Gibco BRL). PolyA mRNA was isolated from total RNA using the PolyATtract mRNA Isolation System III (Promega). One µg of polyA mRNA was run on a 1.2% agarose gel containing 5mM methylmercury hydroxide and was transferred to nylon by electroblotting. ^32^P labeled probes were prepared using the PrimeIt II kit (Stratagene). Hybridization was performed in Church buffer (1% BSA, 7% SDS, 0.5M NaH_2PO_4 pH 7.0, 1mM EDTA, 100ug/ml salmon sperm DNA) at 65 °C for 16 h. cDNA
for rat HES1 and HES5 were gifts from Ryoichiro Kageyama. A probe to human γ-actin was used as a loading control (50).

**γ-secretase inhibitor treatment of cells.** Compounds A and B (structures and method of synthesis appear in ref. 45) were resuspended in DMSO. Cells were transfected as described above. The following day cells were treated with the indicated amount of inhibitor for 17 hours in serum-free medium (with 0.2% BSA) such that the final concentration of DMSO in the media was 0.1%. Control wells were treated with 0.1% DMSO alone. Aβ1-40 was measured by ELISA as described (45) using a sandwich technique with an antibody to the amino acid 40 carboxy terminus and a biotinylated mAb that recognizes Aβ residues 10-20.
RESULTS

In mammalian cells, Notch1 signaling has been shown to induce expression of the HES1 gene (32, 49) through an association of the intracellular domain of Notch with a CSL protein (24). In order to evaluate the effect of PS deficiency on Notch1 signaling, we generated primary embryonic cell cultures from PS1/PS2 (PS1/2) double heterozygous intercrosses (Fig. 1A). PCR of genomic DNA from PS1/2 null cells revealed the predicted size products for the deleted exons, and lacked visible amplification products from wildtype PS1 or PS2 genomic sequences. In order to ensure that we had a pure population of PS1/2 null cells, we also performed RT-PCR on RNA from wildtype and PS1/2 null cells (Fig. 1B, C). In Figure 1B, when primers specific for mouse PS1 exon 4 or mouse PS2 exon 5, the exons targeted in PS1 and PS2 knockout mice (3, 4), respectively, were used, no RT-PCR product was detected in PS1/2 null cells (lanes 8 and 12). In contrast, strong bands of the predicted size for wildtype products (PS1-151bp, PS2-263bp) were amplified from the RNA from wildtype cells (lanes 7, 11). Sequences outside of the deletion were transcribed and apparently stable in PS1/2 null cells, as primers directed at untargeted exons showed specific bands in wildtype and null cells (PS1-294bp, lanes 5 and 6; PS2-99bp, lanes 9 and 10). To measure the sensitivity to detect contamination by wildtype RNA, we mixed decreasing amounts of wildtype RNA with RNA from PS1/2 null cell and performed RT-PCR (Fig. 1C). We were able to detect a PCR signal from amplification of wildtype PS1 exon 4 when as little as 5 ng wildtype RNA was mixed with 1995 ng PS1/2 null cell RNA. This indicates that contamination with wildtype PS1 RNA in PS1/2 null cells is less than 0.25%. As an additional control mouse β-actin was amplified to an equivalent degree in both wildtype
and PS1/2 null cells (Fig 1B, lanes 3 and 4; Fig. 1C, lower panel) and no bands were detected in the absence of added primers (Fig.1B, lanes 1 and 2). Taken together, the experiments showing a lack of detectable wildtype DNA or mRNA indicate that PS1/2 null cells lack wildtype PS1 and PS2 genes.

We next examined the endogenous expression of Notch1 and one of its ligands Jagged 1 in both cell types. Both PS1/2 null and wildtype cells expressed endogenous Notch1 and Jagged1 at equivalent levels (Fig. 1D). This provides an opportunity for endogenous Notch-ligand interactions for cells grown in contact. Signaling through Notch1 has been shown to induce the expression of target genes including HES1 and HES5 genes in mammalian cells and embryos (4, 8, 32, 49, 51, 52). We therefore examined the steady state levels of endogenous transcription of the HES1 and HES5 genes in wildtype and PS1/2 null cells and found that both cell types showed roughly equivalent expression of these mRNAs (Fig. 1E). As a more sensitive measure of the rate of transcriptional activation due to endogenous signals, these cells were transfected with a HES1-luciferase (HES1-luc) reporter construct. A 2.5 ± 0.65 (x ± S.E.M.; n=6) fold higher level of corrected luciferase activity (HES1-luc/TK-luc) was observed in wildtype cells compared to PS null cells, indicating that PS1 and 2 influence basal HES1 transcription possibly via their influence on endogenous Notch signals.

In order to examine the effect of PS1/2 deficiency on ligand-independent Notch1 signaling, we utilized membrane-tethered and non-membrane-tethered intracellular Notch constructs (see Fig. 1F) and compared the magnitude of HES1 activation resulting from their activity. Transfection of control cells with non-membrane-tethered, intracellular forms of Notch1, NICD-myc6 (also known as NICD) and NIC-myc (also known as
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Notch1IC), resulted in HES1 activation of approximately five fold and 25-30 fold, respectively (Fig. 2A). In PS1/2 null cells HES1 activation by these constructs was approximately equivalent to that seen in control cells (Fig. 2A). Transfection of control cells with membrane-tethered Notch constructs, HA-TMIC-myc or Notch1ΔE (also known as ΔE), elicited an approximate 4.5 fold induction of HES1 transcription (Fig. 2B). In contrast, transfection of PS1/2 null cells with these membrane tethered Notch1 constructs resulted in an approximate 2.8-fold induction, corresponding to 40% less activation of HES1 transcription (Fig. 2B). Since the baseline HES1 transcription is 2.5 fold lower in PS null cells, the absolute level of signaling is approximately 16% of wildtype cells. These results demonstrate that the activity of membrane-tethered Notch1 proteins, but not intracellular forms, is largely, but not exclusively dependent on the presence of the mammalian PS genes.

We next asked whether overexpression of human PS1 (hPS1) or hPS2 could restore maximal activation by membrane-tethered Notch constructs in PS1/2 null cells. We found that transient overexpression of hPS1 or hPS2 in PS1/2 null cells resulted in a 40-60% increase in HES1 activation above the activation elicited by HA-TMIC-myc (Fig. 2C) or Notch1ΔE (Fig. 2D) alone. Thus hPS1 or hPS2 enhanced the signaling in PS1/2 null cells to nearly the levels of activation observed in control cells (Fig. 2C), indicating that the impairment was a consequence of PS deficiency and that the PS genes are redundant in promoting Notch signal transduction. Finally, we assessed the ability of an FAD-linked mutant of hPS1 (A246E) to rescue the deficit in HES1 activation elicited by membrane-tethered Notch1 proteins in PS1/2 null cells. We found that transient overexpression of the A246E variant of hPS1 functioned similarly to hPS1 and hPS2 in
enhancing HES1 activation to control levels (Fig. 2C). These experiments demonstrate that an FAD-mutant form of PS1 can functionally substitute for wildtype PS1 or PS2 in promoting Notch signaling.

Ligand-induced activation of the HES1 gene has been demonstrated by use of a co-culture assay where cells expressing Notch ligands are grown with cells expressing Notch1 (48). We made use of this assay system to address whether ligand-dependent Notch1 signaling was affected by the simultaneous loss of PS1 and PS2. We observed an enhancement of HES1 promoter activity when wildtype or PS1/2 null cells were co-cultured with Delta1-expressing QT6 quail cells (QT6-Delta) in the absence of exogenously added Notch1 in both serum-containing medium (Fig. 3A,B) or serum-free medium (Fig. 3C,D). However, the PS1/2 null cell line showed a maximal HES1 transcription after co-culture with Delta1 cells in medium containing serum, and in serum-free medium (Fig 3C,D). HES1 activation was also seen when exogenous full-length Notch1 was co-transfected (not shown). To ensure that the HES1 activity in this assay was specific for the CSL-dependent pathway of Notch signaling, a mutant form of the HES1 promoter was used in which the two CSL-binding sites have been deleted (HES1ΔAB)(32). This promoter failed to respond to co-culture with QT6-Delta cells (Fig. 3A,B). These experiments illustrate that ligand-dependent Notch1 signaling through endogenous Notch receptors or transfected Notch1 can proceed in the absence of the two known PS genes, but that the degree of ligand-activated Notch signaling was markedly reduced.

To explore if transcription of the HES1 gene was indeed a consequence of endogenous Notch signals, we made use of a known physiological inhibitor of Notch,
Numblike (Nbl)(52). Nbl belongs to the Numb family of proteins, which have been shown to inhibit Notch signaling during cell fate determination possibly through a direct interaction with the intracellular domain of Notch (54, 55, 56, 57, 58). We observed Nbl could effectively inhibit HES1-luc activation of an exogenously added intracellular Notch construct (NIC-myc) in wildtype cells (Fig. 3E). We then asked whether Nbl could inhibit HES1 transcription in wildtype and PS1/2 null cells and found that Nbl inhibited endogenous HES1-luc activity in a dose-dependent manner in both wildtype and PS1/2 null cells, resulting in a 50% (null) to 75% (wildtype) maximal inhibition at the highest DNA levels used (Fig. 3F). The absolute value of HES1 transcription after Nbl was approximately equal in wildtype and PS1/2 null cells when maximally inhibited by Nbl. Since Nbl was able to reduce HES1 activity in PS1/2 null cells, we conclude that endogenous Notch signals persist in cells lacking PS1 and PS2.

To investigate the mechanism of the PS-dependent and PS-independent pathways of Notch signaling, we studied the production of intracellular Notch fragments (NICD) by intramembraneous cleavage (S3 site) in PS1/2 null cells. Since it is known that RBP3 associates with and stabilizes the NICD form of Notch (29), we performed co-immunoprecipitation studies with Notch1ΔE and a FLAG-tagged CSL protein RBP3 to enrich for NICD. In comparison to control cells (Fig. 4A, lane 3), we observed a virtual absence of detectable NICD fragment in PS1/2 null cells (Fig. 4B, lane 3). No immunoprecipitation of NICD was seen when RBP3 was not co-expressed (Fig. 4A-B, lane 2). When PS1 alone was deficient, NICD was greatly reduced but not abolished (20, and BEB and JN unpublished observations). In contrast, PS2 deficient cells showed no measurable reduction in the cleavage of Notch1 compared to wildtype cells (BEB, DD,
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JSN unpublished observations). These results support prior observations that mammalian PS homologues are required to promote the intramembraneous cleavage of Notch1. Additionally, the data indicate that PS1 and 2 are functionally redundant, and that PS1 has a quantitatively greater role in supporting Notch cleavage.

To assess whether the two mammalian PS homologues could functionally rescue the cleavage defect in PS1/2 null cells, we transiently overexpressed hPS1 and/or hPS2 in control and PS1/2 null cells. Overexpression of hPS1 in control cells revealed a small increase in the ratio of NICD to uncleaved TMIC (Fig. 4A, lane 4), however, we cannot exclude that this increase could have been the result of a more efficient transfection. This increase is similar to that observed when the FAD mutant forms of hPS1 A246E (Fig. 4A, lane 5) or M146L (not shown) were used. More dramatically, overexpression of hPS1 or hPS2 in PS1/2 null cells restored the absolute level of NICD and the ratio of NICD to TMIC to that of control cells (Fig. 4B, lanes 4 and 8). To examine whether FAD-linked mutant forms of PS genes could also restore NICD formation in PS1/2 null cells, we transiently transfected various FAD-linked forms of hPS1 (M146L, A246E, H163R) and hPS2 (M293V). We observed that the FAD mutant forms of hPS1 and hPS2 were able to restore cleavage of the NICD (Fig. 4B, lanes 4-9). We therefore conclude that PS1 and PS2 have comparable activity in promoting Notch1 cleavage, and that FAD-causing PS mutations have similar levels of activity.

In light of our observation that Notch signaling was partially preserved in PS1/2 null cells (figures 2 and 3), we asked if Amyloid-β was produced in these cells. Human APPswe (hAPPswe), a mutant that shows increased γ-site cleavage (for review see 59), was transfected and a highly sensitive sandwich ELISA was used to detect Aβ1-40. In
wildtype cells, we observed a 100-hundred fold increase in the production of Aβ1-40 after transfection with hAPPswe (Fig 4C, graph). However, PS1/2 null cells showed no production of Aβ1-40 despite expression of transfected hAPPswe and co-transfected β-galactosidase at equal or higher levels than wildtype cells (compared Fig 4C lanes 2, 3 and 4 with lanes 6, 7 and 8). As further evidence that the lack of presenilins alone was responsible for the lack of Aβ1-40 production, PS1/2 null cells transfected with hAPPswe produced significant levels of Aβ1-40 when co-transfected with hPS1. Thus, using a highly sensitive means to detect γ-secretase activity, these data indicate that the production of Aβ strictly requires either PS1 or PS2 as previously noted (43, 44) and is completely abolished by compound A (45).

We next examined the effect of PS1/2 deficiency on nuclear localization of Notch1 protein. Wildtype and PS1/2 null cells were transfected with plasmids encoding myc-tagged forms of membrane-tethered (Notch1ΔE) or non-membrane-tethered intracellular (NICD-mycΔ6) Notch1, and the expressed protein was visualized with antibody to the myc epitope in fixed cells. The majority of cells expressing the non-membrane-tethered, NICD-mycΔ6 showed a predominately nuclear localization (~87%) of the Notch protein, which was not influenced by PS1/2 deficiency (Fig. 5A-F, M). Wildtype cells expressing the membrane-tethered form of Notch1, Notch1ΔE, displayed a slightly reduced nuclear localization (~70%) of Notch protein with visible staining of intracellular and plasma membranes (Fig. 5G-I, N). In contrast to wildtype cells, PS1/2 null cells expressing Notch1ΔE showed a dramatic shift in subcellular localization, characterized by an exclusion of myc immunoreactivity from the nucleus in 100% (252/252) of cells examined (Fig. 5J-L, N). Instead, Notch1ΔE immunoreactivity was
observed in intracellular membraneous structures and the plasmalemma. We also used an antibody against a region of Notch encompassing the RAM domain and part of the ankyrin repeats (NRC, amino acids 1820-1835) and were unable to detect any nuclear Notch in the PS1/2 null cells (data not shown). This demonstrates the absence of PS genes results in a dramatically reduced quantity of Notch1 in the nucleus.

Since Notch1 proteins still retain their ability to activate promoters in PS1/2 null cells despite the apparent dependence of PS genes on Notch cleavage and nuclear accumulation, we wondered whether a protease with similarities to the γ-secretases might underlie Notch signaling in PS1/2 null cells. We therefore examined the effect of γ-secretase inhibitors (45) on Notch cleavage and signaling. Several classes of inhibitors have been shown to block APP cleavage at the intramembraneous site, reduce Aβ production, and target PS proteins (45, 60, 61, 62). Although data suggests that γ-secretase inhibitors block Notch cleavage (21, 63), they have not been tested on PS-independent Notch signaling.

We therefore examined the effect on Notch signaling of compound A and compound B, potent and weak γ-secretase inhibitors, respectively, that were described in ref. 45. Serum free medium was used to enhance the bioavailability of the inhibitors. We found that compound A (4000nM) but not compound B was able to inhibit Notch signaling elicited by QT6-Delta cells in wildtype and PS1/2 null cells (Fig 3C, D). These data show that γ-secretase inhibitors can block ligand-activated Notch signaling in wildtype and PS1/2 null cells. Interestingly, in serum free medium, HES1 activation was elicited by QT6 cells and partially inhibited by compound A, consistent with the notion
that compound A also inhibited signaling elicited by endogenous Notch ligands on QT6 cells interacting with the endogenous Notch receptors on PS1/2 null and wildtype cells.

To assess the effect of γ-secretase inhibitors on Notch signal transduction and cleavage with ligand-independent Notch constructs, cells were transfected with HES1-luc along with Notch1ΔE and then treated with either compound A or compound B in serum free medium (Fig. 6). We found that compound A, but not compound B caused a significant reduction in Notch signaling in both wildtype and PS1/2 null cells at 400-40,000 nM (Fig. 6A). Both cell types showed equivalent inhibitory potencies and an enhancement of Notch signaling over baseline in the range of 0.4-40 nM when compound A was used. These data indicate that a γ-secretase inhibitor is capable of blocking both PS-dependent and PS-independent Notch signal transduction elicited by membrane-tethered Notch constructs. We note, however, that that even maximal concentrations of compound A did not reduce HES1 transcription elicited by Notch1ΔE to basal values in either cell type, suggesting that a mechanism not involving a γ-secretase supports a significant fraction of Notch signaling.

We next examined the intramembranous cleavage of Notch and the production of the NICD fragment in the presence of γ-secretase inhibitors. In order to enrich for the NICD fragment, SV40 immortalized cells were used and were transfected with Notch1ΔE along with a Flag-tagged RBP3. Treatment of the cells with compound A caused a dose-dependent reduction in the amount of NICD produced as visualized by the extracts alone (extract) or by co-immunoprecipitation with RBP3 (Flag-IP) (Fig. 6B). In the presence of serum, only small inhibition was observed when the cells were treated with 1000nM that was further inhibited at 2000 and 4000nM treatment. In the absence of serum,
compound A was more bioavailable and was effective at reducing cleavage at 40 and 400 nM (Fig 6C). As a control, we observed that treatment of the cells with the less potent compound B did not affect NICD production even at the highest concentration used (Fig 6B and not shown). These data demonstrate that a γ-secretase inhibitor reduces S3 cleavage of Notch. However, the concentrations required to block Notch signaling are significantly higher than those needed for blocking NICD production or for blocking Aβ production (45).

To explore the dependence on intramembrane cleavage of PS-independent signaling, we studied the effect of a mutation in a transmembrane valine (V1744K, V-K), a version of Notch1 that had been previously shown to partially decrease Notch cleavage and signaling (30, 64). In wildtype cells, a maximal activation of HES1 (approximately fifteen-fold) was reached when 100ng of DNA was transfected (Fig.7A). In contrast, signaling by Notch1ΔE-V1744K in wildtype cells was reduced and reached a maximal activation of six-fold at 100ng of DNA (Fig 7A). In PS1/2 null cells, signaling by Notch1ΔE, reached a peak of approximately 3 fold at 1000ng of DNA (Fig. 7C). Importantly in PS1/2 null cells, the concentration dependence of the V-K mutant on HES1 activity was very similar to the profile elicited by Notch1ΔE, consistent with a model in which intramembrane cleavage does not play a major role in Notch signaling in PS1/2 null cells.

In light of the apparent lack of requirement for either an intramembranous endoproteolysis for Notch signaling in PS null cells, we explored the effect of compound A on signaling by Notch1ΔE-V1744K in wildtype and PS1/2 null cells (Fig 7B and 7D). Using 1000ng of Notch1ΔE-V1744K, the maximally activating concentration in PS1/2
null cells, we observed a significant inhibition by compound A (4000 nM) in both wildtype and PS1/2 null cells (Fig 7B,D). These data further indirectly support the conclusion that PS-independent signaling is independent of intramembraneous proteolysis and that compound A is acting at a site other than the γ-secretase in PS null cells.

The prior experiments show that Notch signaling is partially preserved in PS1/2 null cells despite an absence of cleavage at the S3 site as measured by a western blot or nuclear translocation as visualized by immunocytochemistry. The S3 site cleavage of Notch proteins can be assayed with greater sensitivity by measuring the nuclear access and activation of UAS promoters by a fusion protein in which Gal4-VP16 has been inserted into the Notch intracellular domain (31). Using a derivative of Notch1ΔE, (Notch1ΔE-G4VP16) that contains Gal4-VP16 immediately following the membrane spanning domain (Fig 1E), we observed an activation of UAS promoters in wildtype cells by Notch1ΔE-G4VP16 but not in PS1/2 null cells (Figure 8A). Transfected Notch1ΔE-G4VP16 DNA (from 0.1 µg to 1 µg) activated the UAS promoter in wildtype cells but was unable to activate UAS-luc activity in PS1/2 null cells even at the highest concentration used (not shown) despite a high activity of Gal4-VP16 alone (Fig 8B). This provides strong evidence that the Gal4-VP16 domain of Notch1ΔE-G4VP16 was not released in PS1/2 null cells, consistent with the absence of intramembraneous endoproteolysis. In line with this conclusion, compound A reduced the UAS activity of Notch1ΔE-G4VP16 in wildtype cells (P < 0.05) but not in PS1/2 null cells. As a further indicator of the specificity of compound A, UAS activation by untethered Gal4-VP16 was unchanged by the addition of the inhibitor.
The previous experiment indicating that Notch constructs are not cleaved at the intramembraneous site in PS null cells raise the question as to whether Notch1ΔE-G4VP16 is also able to activate HES1 in PS1/2 cells. In contrast to the lack of UAS signaling seen above, we observed a marked activation of HES1 promoters by Notch1ΔE-G4VP16 in both wildtype and PS1/2 null cells (Fig 8C). The ability of Notch1ΔE-G4VP16 to activate HES1 but not UAS suggests a novel mechanism, such as proteolysis of Notch distal to the Gal4-VP16 domain that releases most of the Notch1IC domain. However, we have been unable to detect a product consistent with this predicted site of proteolysis (unpublished). We note that the activation of HES1 by Notch1ΔE-G4VP16 was not inhibited by compound A in wildtype or PS1/2 null cells (Fig 8C) unlike activation elicited by Notch1ΔE or Notch1ΔE-V1744K (Figs 6, 7). Thus, any conclusions regarding the mechanism of Notch1ΔE-G4VP16 activation of HES1 must be interpreted with caution.
DISCUSSION

The present study demonstrates that a novel mechanism exists to facilitate Notch signal transduction in the absence of PS1 and PS2 in mammalian cells. Since the deficiency of both PS1 and PS2 produced a dramatic reduction of the proteolytic release of the Notch IC domain and a lack of detectable nuclear NICD, the present data support the contention that presenilins play an important role in the metabolism of Notch proteins during signal transduction, as indicated by previous investigations. However, we observed that Notch signaling was partially preserved in PS null cells, consistent with either a novel mechanism of Notch signal transduction or the production of a small amount of an active Notch fragment by a non-PS1, non-PS2 proteolytic activity. The observation that γ-secretase inhibitors reduce Notch signaling in PS1/2 null cells is consistent with a model in which an uncharacterized protease with pharmacological similarities to the PS-linked γ-secretases does indeed support Notch signaling. However, this protease is unlikely to act at the transmembrane domain of Notch. The present study clarifies the role of presenilins in Notch metabolism and further illuminates the emerging differences between the mechanism of Notch signal transduction and APP cleavage (65, 66).

Notch cleavage and nuclear access of the intracellular domain have been shown to be PS dependent (16, 17, 20, 21, 39, 44, 58, 67, 68). While we confirm the need for PS proteins to promote the cleavage and release of the NICD fragment, this study shows that the Notch molecules are capable of eliciting HES1 activation in PS1/2 null cells. Membrane-tethered intracellular Notch1 constructs showed signaling at about 16% of the absolute levels found in control cells. Signaling in PS1/2 cells was enhanced following
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transient expression of either PS1 or PS2. These data reveal that transduction initiated by membrane-tethered Notch1 constructs is partly PS-dependent and partly PS-independent. Similarly, ligands for Notch were able to activate HES1 transcription in PS1/2 null target cells albeit at lower levels than in wildtype cells. Delta1-mediated activation of the HES1 promoter required intact CSL binding sites, demonstrating that the PS-independent transduction proceeded through a CSL-dependent pathway of Notch signaling.

In the present study, some of the evidence for PS-independent signaling relied upon overexpressed Notch1 or its TMIC form, which could possibly be subject to overexpression artifacts. However, we also found that signaling through endogenous Notch receptors was PS-independent. The experiments showing Nbl inhibition of HES1, and experiments in which Delta1 triggered HES1 transcription in the target cells also suggest that at least part of the HES1 transcription, and presumably the part of the HES5 mRNA expression, were elicited by signaling by endogenous Notch in PS null cells. These studies reduce the concern that the PS-independent Notch signaling that we observed was due to transfection artifacts that produced aberrant forms or localization of Notch protein.

The present study fits with the conclusion that Notch signaling utilizes cleavage in vitro and in vivo (30, 38, 64), but reveals that the two mammalian presenilins are not strictly required for all of Notch signaling. An additional cleavage of Notch is implied by the observation that both ligand-activated and ligand-independent Notch signaling in the absence of PS1 and 2 were sensitive to a γ-secretase inhibitor, although we have not provided direct evidence that cleavage occurs. Additionally, the observation that a strong γ-secretase inhibitor blocked PS-independent Notch signaling while a weak one was
ineffective implies some structural similarities to the \( \gamma \)-secretase. A thorough structure-activity profile of \( \gamma \)-secretase inhibitors would be required to establish a true pharmacological similarity. We also note that the blockade of signaling in both wildtype and PS-null cells required 5-10-fold higher concentrations than those reported for blocking APP processing or Notch cleavage (in wildtype cells). This could reflect the action of compound A on an uncharacterized protease that is not part of the \( \gamma \)-secretase, or an intrinsic property of Notch cleavage by the \( \gamma \)-secretase. Interestingly, Notch signaling in PS1/2 null and wildtype cells was enhanced at concentrations that showed inhibitory effects upon Notch cleavage in wildtype cells. The enhancement of Notch signaling by \( \gamma \)-secretase inhibitors is reminiscent of the enhancement of A\( \beta \)(1-42) production at low concentrations of inhibitor where A\( \beta \)(1-40) production was inhibited (69 and data not shown). However, whether or not the putative PS-independent Notch protease has structural similarities, shared components, or a similar pattern of cleavage with the \( \gamma \)-secretase is an open question.

Interestingly, the present study provides indirect and preliminary evidence that the PS-independent mechanism of Notch cleavage occurs distal to the transmembrane domain. First, experiments showing that the V-K mutation made little difference on PS-independent signaling suggest that the intramembraneous sequence was not the main substrate for the putative PS-independent protease. Secondly, studies with the Notch1\( \Delta E \)-G4VP16 construct in PS null cells showing a strict dependence upon presenilins for the release of Gal4-VP16 and initiation of UAS transcriptional activation imply that intramembraneous endoproteolysis was absent in PS1/2 null cells. Despite the lack of an intramembraneous endoproteolysis, the fusion protein is capable of triggering
HES1 activity in PS null cells. The retention of HES signaling in the absence of UAS activation could be explained by a distinct protease that recognizes and cleaves a site distal site to the GAL4-VP16 domain and releases the NICD fragment. At present, however, we lack direct evidence for such a protease and other mechanisms may be operative as indicated above (see results). Nevertheless, these studies provide evidence that Notch signals can trigger transcriptional activation without perturbing the transmembrane domain of Notch.

Previous genetic studies have illuminated only a PS-dependent pathway of Notch signal transduction (11, 13, 14, 16, 17). In C. elegans, the two presenilins, sel12 and hop1 show redundant but unequal function, whereas in Drosophila a single presenilin gene is present. The similarity of the animals with reduced sel12 and hop1 function and the lin12 and glp1 double mutants in C. elegans (12, 14), and the similarities between Notch mutants and presenilin mutants in Drosophila, support the contention that all Notch functions are PS dependent (15, 70). Moreover, chimeras of Notch and Gal4-VP16 were utilized to demonstrate that PS genes are required for nuclear access of the Notch intracellular domain, consistent with a role for PS proteins in the intramembraneous cleavage of Notch (16, 39).

In mammals, only two PS genes have been identified. The two PS genes are similarly redundant but unequal, as PS1 null mice die around birth (2) and PS2 null mice have minimal phenotypes (5). PS1 mice show a phenotype that is reminiscent of the Notch1 null embryos with axial truncations and cortical abnormalities but is considerably weaker (71, 72). Studies of PS1/2 knockout mice are also consistent with a dependence of Notch signaling on the redundant mammalian PS genes because phenotypes such as
severe somitic abnormalities, ectopic Delta1 expression, reduced HES5 expression, and somitic defects were present in PS1/2 null embryos. These phenotypes were generally stronger than those found in Notch1 or RBP-Jk deficient embryos, consistent with the argument that PS genes are downstream of multiple Notch proteins (4, 5).

However, studies of PS and Notch pathway mutant mice have not formally excluded the possibility of a component of Notch signaling that is PS-independent in mammalian tissues. First, it is not yet clear what the phenotype of a total loss of Notch function would be in a vertebrate. Importantly, Notch1 is expressed in the gastrula, but Notch1 null embryos show no apparent defects in gastrulation, consistent with some redundancy of Notch genes in a putative function during gastrulation (73, 74). Since four Notch homologues are known in mammals only a quadruple knockout would answer this question. Second, no studies have examined double mutants of Notch and PS genes to evaluate if they are epistatic. For instance, if a PS1/2 null; Notch1 null embryo were more severely affected than the PS1/2 null, it would be consistent with a PS independent pathway of Notch signaling. This result is plausible since the recently discovered deficits of vasculogenesis in Notch1 and Notch1;Notch 4 null embryos (75) were not observed in the initial studies of the PS1/2 null embryos (4, 5).

In contrast to the present study, one prior study has examined Notch signaling in PS1/2 null cells and concluded that it was abolished (43, 44, 76). We note that this study did not examine ligand activated Notch signal transduction and only evaluated a single concentration of Notch1ΔE. In agreement with the published work, we observed virtually no activation of HES1 promoters at low concentrations of Notch1ΔE, and that higher levels were required to see PS-independent signaling (Fig 7C).
What biological function the PS-independent activity serves in embryogenesis or in adult tissues is an open question. The answer to this question could require the isolation of the putative PS-independent Notch protease and then loss-of-function experiments. Nonetheless, these studies do indeed show that γ-secretase inhibitors act upon both PS dependent and PS independent Notch cleavage and signaling, and therefore these results have significant implications for development of drugs that block APP processing but not Notch signal transduction. An inhibitor that blocked only the PS-dependent γ-secretase and spared Notch could possibly avoid toxicities related to the blockade of Notch signaling (63). Although it is conceivable that small amounts of APP are cleaved at the γ-site in PS1/2 null tissues, it seems likely that the novel PS-independent activity prefers Notch to APP as a substrate. This interpretation fits with the emerging concept that differences between Notch and APP cleavage could be exploited to develop γ-secretase inhibitors that are selective for APP (66, 77).

Finally, the identification and characterization of PS-independent Notch signaling offers insights into the pathogenesis and therapy of Alzheimer’s disease. Since we observed that FAD-mutant PS proteins were functionally equivalent to wildtype PS proteins, and that PS1 and PS2 have redundant functions with respect to Notch signaling and cleavage, the present study differs from published data suggesting that FAD mutations show a partial loss of function for Notch cleavage and signaling (22, 78, 79). We could not exlude a small impairment of function, however. Understanding the possible effects of PS genes and their mutations on Notch function is especially important given recent evidence that Notch signals can affect the architecture of the neuritic tree in
mammals (80, 81, 82), which may possibly be important to neuronal plasticity in adults and the pathogenesis of Alzheimer’s disease.
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FIGURE LEGENDS

Figure 1. PS1/2 null cells express Notch signaling components. (A) PCR genotyping of genomic DNA extracted from individual immortalized primary cell cultures derived from PS1/2 heterozygous intercrosses. Amplification of endogenous and targeted PS1 alleles produce fragments of ~500 bp and ~370 bp, respectively. The corresponding PS2 alleles produce fragments of 640 bp (endogenous) and 380 bp (targeted). (B) RT-PCR on total RNA extracted from individual immortalized primary cell cultures. Amplification of endogenous β-actin, intact and targeted exons for PS1 and PS2, respectively. (C) RT-PCR of co-mixtures of RNA from wildtype cells and PS1/2 null cells. Decreasing quantities of wildtype RNA were added to PS1/2 null cell RNA to a total of 2 µg and RT-PCR was performed. Wildtype PS1 exon 4 (top panel) and β-actin (lower panel) were detected with specific primers. (D) Notch1 and Jagged1 protein expression in wildtype and PS1/2 null cells. The p120 polypeptide of Notch1 and the intact Jagged1 protein (200 kDa) were detected using antibodies to the carboxy terminus of each protein after immunoprecipitation (see methods). (E) Expression of HES1 and HES5 mRNA was analyzed by Northern blotting using 1ug of polyA mRNA derived from wildtype or PS1/2 null cells. Hybridization with a probe to HES1 and HES5 detected a transcript of approximately 1.7kb. Hybridization to γ-actin was used as a loading control. (F) Full length and intracellular Notch1 constructs used in this study. All constructs used have been described previously (30, 37, 38). Notch1ΔE (also known as ΔE) has the M1726V mutation. Notch1ΔE (V1744K) V-K substitutes valine 1744 with a lysine. Notch1ΔE-G4VP16 is derived from Notch1ΔE, with an insertion of Gal4-VP16 just after the transmembrane domain.
Figure 2. **HES1 activation by membrane-tethered Notch1 constructs in PS1/2 null cells and rescue with PS1, PS2 and an FAD-linked variant.** (A) Signaling elicited by intracellular Notch1 (non-membrane-tethered). (B) Signaling by membrane-tethered intracellular Notch1. The data shown in (A-B) represents the mean and SEM from 2-6 independent experiments where each point was assayed in triplicate. (C-D) Rescue of membrane-tethered Notch signaling by PS1, PS2 and an FAD-linked variant of PS1 (A246E). Transfection of PS1, PS2 or A246E (1ug) significantly increased relative HES1-luc activation by (C) HA-TMIC-myc and (D) Notch1ΔE in PS1/2 null cells. In A-D, primary cells derived from control or PS1/2 null embryos were transfected with 200 ng of the HES1-luc reporter alone or with activated intracellular Notch constructs (1ug), and luminescence was determined as described. A representative of three similar experiments is shown where each point was assayed in triplicate, and error bars represent standard error of the mean. Significance was determined using a two-tailed Student’s t test (*P < 0.05, **P < 0.01). Normalized HES1 transcription (HES1/TK) is plotted.

Figure 3. **Endogenous Notch signaling in wildtype and PS1/2 null cells.** (A,B) Primary cells derived from wildtype or PS1/2 null embryos were transfected with 500 ng of HES1-luc or a mutant form of the HES1 promoter, HES1ΔAB-luc, in which the two CSL binding sites have been deleted (29). (C,D) Primary cells derived from wildtype (C) or PS1/2 null (D) embryos were transfected with 500 ng of HES-luc. One day later QT6 or QT6-Delta cells were added and co-cultivated for 24 hours with the transfected primary cells in medium containing serum for A and B, but changed to serum-free...
medium at 10 h for C and D. For inhibitor experiments, cells were treated with DMSO containing 0, 400nM or 4000nM of compound A or compound B for 17 hours in serum free medium (45). Luciferase activity (HES/TK) is shown in A and B from a representative experiment performed in triplicate. Mean luciferase activity relative to control levels is shown in C and D from 4 experiments assayed in triplicate. (E) HES1-luc activation by intracellular Notch1 is inhibited by Nbl. Wildtype cells were transfected with 300ng HES1-luc with empty vector, NIC-myc (1µg) alone, or NIC-myc (1µg) and Nbl (1µg). A representative experiment is shown. (F) Nbl inhibits endogenous HES1-luc activity in wildtype and PS1/2 null cells. Cells were transfected with 300ng HES1-luc along with increasing amounts of Nbl cDNA (0.05-1.0µg). Data is plotted as the percent of maximal activity observed with HES1-luc alone for each cell line. Data shown is the mean and s.e.m. from two independent experiments where each point was assayed in triplicate. * indicates P< 0.05 by a two-tailed Student’s t test for the indicated pair.

**Figure 4. Absence of detectable NICD, RBP-associated NICD and Aβ (1-40) production in PS1/2 null cells.** Primary cells derived from (A) PS1/2 hetero or (B) PS1/2 null embryos were transiently transfected with Notch1ΔE, either in the presence (lanes 3-9) or absence (lane 2) of co-transfected RBP3-Flag. hPS1, hPS2 or FAD-mutant forms of these genes were transfected as indicated (lanes 4-9) and detected with either anti-PS1NT or anti-PS2loop. Immunoprecipitates produced using the anti-Flag antibody (Flag IP) and cell extracts (extracts) were analyzed by Western blotting using an anti-myc antibody (9E10). Total extract cells transfected with NICD-myc6 (30) were analyzed.
without IP (lane 1). The sizes of uncleaved Notch1ΔE and NICD-myc6 are shown (arrows). In the absence of RBP3, no proteins were co-immunoprecipitated non-specifically (lane 2) in either PS1/2 hetero or PS1/2 null cells. (C) SV40 immortalized cells derived from PS1/2 hetero or PS1/2 null embryos were transfected with β-galactosidase (0.5 µg), an empty vector, hAPPswe (Swedish form) (5 µg) and/or human PS1 (5 µg) as indicated. The following day, the transfected cells were treated with 0 or 4000nM of compound A for 17 hours in serum-free medium (45). (Top, graph) Supernatant from three separate cultures for each point were analyzed using a sandwich ELISA to detect Amyloid-β (1-40). The mean and s.d. are plotted. (Bottom, gels) Lysates were analyzed by western-blot using the anti-APP antibody (22C11) and the anti-β-galactosidase antibody.

**Figure 5. Subcellular localization of membrane-tethered and non-membrane-tethered intracellular Notch1 in control and PS1/2 null cells.** Primary cells derived from wildtype (A-C, G-I) or PS1/2 null (D-F, J-L) embryos were transfected with NICD-myc6 (A-F) or Notch1ΔE (G-L) and stained with an anti-myc (9E10) antibody followed by a FITC-conjugated secondary antibody (A, D, G, J). Nuclei were visualized by staining with Hoechst dye (B, E, H, K). Merged images are seen in (C, F, I, L). Stained cells were counted and the location of NICD-myc6 (M) or Notch1ΔE (N) was scored as cytoplasmic only or as nuclear (nuclear or nuclear and cytoplasmic expression). For each condition the number of labeled cells counted exceeded 200. The effect of genotype on the localization of Notch1ΔE was significant by a two-tailed Student’s t test (***P < 0.001).
Figure 6. Effect of γ-secretase inhibitors on Notch1 signaling and metabolism in wildtype and PS1/2 null cells. (A) Primary cells derived from wildtype or PS1/2 null embryos were transfected with 300 ng of HES1-luc along with Notch1ΔE (1µg). The following day the cells were treated with increasing concentrations (0-40000nM) of compound A or compound B for 17 hours in serum free medium. Control wells were treated with the appropriate concentration of DMSO. Relative HES1 transcription levels are plotted. The mean and s.e.m. from 3-5 independent experiments is shown where each point was assayed in triplicate. (B,C) SV40 immortalized cells derived from PS1/2 hetero or PS1/2 null embryos were transfected with 7µg of Notch1ΔE alone (C) or with 7 µg of Notch1ΔE and 7µg of RBP3-Flag (B) and treated with the indicated amounts of inhibitors for 17 hours in serum-containing medium (+ SERUM) (B) and serum free medium (- SERUM) (C). Immunoprecipitates produced using the anti-Flag antibody (Flag IP) and cell extracts (extracts) were analyzed by Western blotting using an anti-myc antibody (9E10). Lysates from cells transfected with Notch1ΔE were also analyzed (lane 1). The sizes of uncleaved Notch1ΔE and NICD are shown (arrows).

Figure 7. HES1 activation by membrane-tethered Notch1 in PS1/2 null resembles a partial deficiency of S3 cleavage but is still sensitive to a gamma-secretase inhibitor. (A,C) Comparison of concentration curves of Notch1ΔE and Notch1ΔE-V1744K in wildtype (A) and PS1/2 null (C). In A and C, cells were transfected with 300ng of the HES1-luc reporter vector alone or with increasing amounts of Notch1ΔE or Notch1ΔE-V1744K (10-1000ng). Mean luciferase activity relative to control levels is shown.
Immortalized primary cells derived from wildtype or PS1/2 null embryos were transfected with 300 ng of HES1-luc along with Notch1ΔE (1µg) or Notch1ΔE (V1744K) (1µg). The following day the cells were treated with 0 or 4000nM of compound A for 17 hours in serum free medium. Control wells were treated with the appropriate concentration of DMSO. Luciferase activity (HES/TK) is shown (A toD) Data shown is the mean and s.e.m. from two independent experiments where each point was assayed in triplicate. * indicates p< 0.05 by a two-tailed student’s test for the indicated pair.

Figure 8. Loss of Gal4 but not Notch Signaling with a Notch1ΔE-G4VP16 Fusion Protein. Primary WT and PS1/2 null immortalized cells were transfected with 300 ng of (A and B) UAS-luc or with 300ng of (C) HES1-luc. 100ng of an empty vector, Notch1ΔE-G4VP16, Gal4 or Gal4VP16 were co-transfected where indicated. The following day the cells were treated with 0 or 4000nM of compound A for 17 hours in serum free medium (45). Control cells were treated with the appropriate concentration of DMSO. Luciferase activity (HES/TK) is shown and represents the mean and s.e.m. from two independent experiments where each point was assayed in triplicate.
FIGURE 2
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A

B

C

D

HES1 ACTIVATION

HES1 ACTIVATION

HES1 ACTIVATION

HES1 ACTIVATION

- NICD-myc ΔE

- HA-TMIC-myc Notch1ΔE

HA-TMIC PS

- + + + +

- - PS1 PS2 A246E

- - - PS1 PS2
FIGURE 4
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A

![Image of gel electrophoresis with bands labeled Notch1ΔE, NICD, anti-myc, anti-PS1NT, and PS1FL.]

- NICD-myc
- Notch1ΔE
- RBP-FLAG
- PS

- + - - - - - - -
- - + + + + + + +
- - - + + + + + +
- - - + + + + + +

B

![Image of gel electrophoresis with bands labeled Notch1ΔE, NICD, anti-myc, anti-PS1NT, and PS2CT.]

- NICD-myc
- Notch1ΔE
- RBP-FLAG
- PS

- + - - - - - - -
- - + + + + + + +
- - - + + + + + +
- - - + + + + + +

C

![Bar graph and Western blots showing secretion of Aβ1-40.]

- WT
- PS1/2 NULL

- Anti-APP
- Anti-B gal

- hAPPsw
- B gal
- Inhibitor
- hPS1
FIGURE 5
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Figure 6

A

HES1 Activation (% Control)

Inhibitor A

Inhibitor B

Log [Inhibitor, M]

WT cells

PS1/2 Null

B

WT

PS1/2Null

Inhibitor (nM):

0 4.0 40 400 4000 4000 0 40 400

extract

FLAG IP

+ SERUM

- SERUM

C

WT

PS1/2Null

Inhibitor (nM):

0 40 400 0 40 400

Notch1αE

NICD

Notch1αE

NICD
FIGURE 7
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A

WT

Log [DNA, ng]

HES-1 FOLD ACTIVATION

B

WT

Log [DNA, ng]

HES1 ACTIVATION

C

PS1/2 null

Log [DNA, ng]

HES-1 FOLD ACTIVATION

D

PS1/2 null

Log [DNA, ng]

HES1 ACTIVATION

Inhibitor A (nM)

DNA (1000ng)

0 0 4000 0 4000

- 

Notch1ΔE Notch1ΔE V1744K

- 

Notch1ΔE Notch1ΔE V1744K

*