

Moonlighting activity of presenilin in plants is independent of γ -secretase and evolutionarily conserved

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Presenilins (PS) provide the catalytic activity for γ -secretase, which cleaves physiologically relevant substrates including Notch, ErbB4, and APP. Recent genetic studies indicated that the contribution of PS1 to mouse development includes γ -secretase-independent functions that cannot be easily explained by any of the demonstrated or hypothesized functions of this protein. To begin a nonbiased analysis of PS1 activity unencumbered by the dominant effect stemming from loss of Notch function, we characterized PS functions in the early land plant *Physcomitrella patens*, which lacks Notch, ErbB4, and APP. Removal of *P. patens* PS resulted in phenotypic abnormalities. Further assays performed to delineate the defective pathways in PS-deficient *P. patens* implicated improper function of the cytoskeletal network. Importantly, this characterization of a nonmetazoan PS uncovered a previously undescribed, evolutionarily conserved function (human PS1 can rescue the growth and light responses) that is γ -secretase-independent (mutants with substitutions of the catalytic aspartyl residues retain the activity). Introduction of PpPS into PS-deficient mouse embryonic fibroblasts rescues normal growth rates, demonstrating that at least some metazoan functions of PS are evolutionarily conserved.

evolution | iCLIP | Alzheimer's disease | cytoskeleton | *Physcomitrella*

Presenilin (PS) proteins are polytopic transmembrane domain (TMD) proteins discovered independently as loci frequently mutated in familial forms of Alzheimer's disease (AD) (1–3) and as modifiers of Notch signaling (4, 5). PS proteins form the catalytic center of γ -secretase, an aspartate protease complex that cleaves type I substrates sequentially within their TMD to release soluble C- and N-terminal peptides (6, 7). Enzymatic activity of PS is unique among aspartyl proteases in the location (within the TMD) and the context of conserved catalytic residues (YD/GxGD) and is among the founders of a growing family of intramembrane cleaving enzymes (8, 9). Mutation of either one of the two conserved aspartate residues results in the loss of γ -secretase activity (10–12). PS proteins must associate with at least three other transmembrane proteins [nicastrin (Nct), Aph-1, and Pen-2] to assemble a functional γ -secretase (13). Whereas PS, Nct, Aph-1, and Pen-2 are essential and sufficient for reconstitution of an active γ -secretase, other cellular factors [e.g., TMP21 and CD147 (14, 15)] may play regulatory roles.

Clinically relevant substrates of γ -secretase include Notch and amyloid precursor protein [APP (16, 17)]. Hydrolysis of APP forms A β peptides, which cause AD (18). Notch is receptor in a signaling pathway regulating development and tissue renewal in the adult; mutations in Notch cause developmental syndromes (19) and contribute to adult-onset disease such as CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) (20), aortic valve disease (21), cancer (22, 23), and possibly multiple sclerosis (24). The recent realization that Notch signaling may be critical in maintaining cancer stem cells revived the interest in γ -secretase as a therapeutic target (25–29).

Interestingly, some defects resulting from loss of PS might not be due to loss of γ -secretase activity. The clearest genetic evidence of γ -secretase-independent PS functions in mice

emerged from experiments examining the role of Notch signaling in somite development where formation of discernable somites (anterior and posterior) was completely abrogated in embryos deficient in both PS paralogs, PS1 and PS2. However, mouse embryos lacking other components of γ -secretase or missing Notch pathway components developed anterior somites despite complete loss of Notch signaling (30).

To explain this phenotype, we postulated that the involvement of PS in Wnt signaling might impact the somite. If loss of PS1 affects both Notch and Wnt signaling (30), no somite may form. However, a PS–Wnt connection does not have support from genetic experiments reported in the literature (31) or from mice with compound mutations in Wnt3A and PS1; the compound mutants were not any worse than Wnt3A heterozygotes, 50% of which are born with defective tail vertebra, the product of somites (R.K., unpublished observations). More recently, PS were shown to possess ER-Ca²⁺ leak channel activity (32). However, several PS mutant proteins lacking ER-Ca²⁺ channel activity (32) can still rescue the somite defects in mice with limiting amounts of PS1 (33).

Other proposed activities of PS that are independent of γ -secretase include a role in transport of several membrane proteins (34–36), autophagy and protein degradation (37, 38), or act as a scaffold in Erk activation (39, 40). However, the dominant phenotype caused by loss of Notch signaling (41–43) severely limits the ability to identify and interrogate the physiological relevance of the entire spectrum of PS functions. These observations suggest that an essential function provided by PS in the somite and possibly elsewhere may have escaped detection because of dominant Notch phenotypes. A system, which will allow an unbiased investigation, will have to shift to organisms encoding PS but lacking *Notch*.

Notch receptors and APP proteins are metazoan proteins, i.e., they are only present in genomes of multicellular animals. In contrast, PS and related proteins have been identified in both metazoans and plants (44–48). Similar to the genomes of *Arabidopsis* (Dicot) and *Oryza sativa* (monocot), whose complete genome sequences are available, an early land plant, the moss *Physcomitrella patens*, also harbors all four components of γ -secretase. As a laboratory organism in which to interrogate PS functions, *P. patens* has many additional advantages. It has few cell types and offers excellent cytology because it is mostly single cell

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Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; MEF, mouse embryonic fibroblasts; PS, presenilin; TMD, transmembrane domain; Pp Δ psn, null phenotype of *P. patens* PS; Nct, nicastrin; LS, least square; Hyg, hygromycin.

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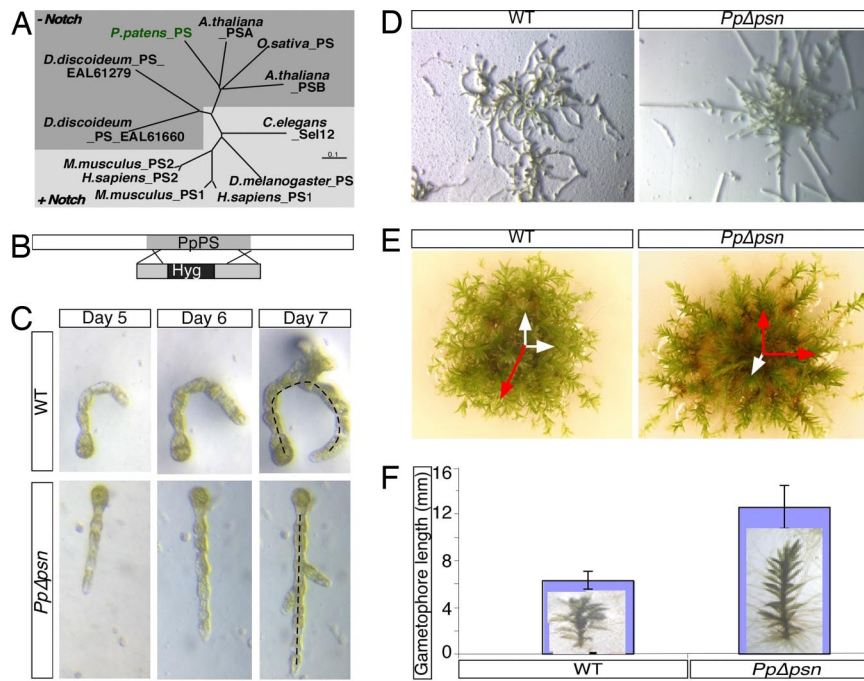


Fig. 1. Loss of PpPS displays pleiotropic phenotype. (A) ClustalW dendrogram depicting PS homologues from selected species. (B) Schematic representation of the *PpPS* KO construct used in the study. Gray, *PpPS*; black, the Hyg cassette used to disrupt the *PpPS* ORF. (C and D) Filaments regenerating from WT and *PpΔpsn* protoplasts photographed for several days. Note that WT protonemal filaments curve whereas *PpΔpsn* colonies are straight. (E) Fifteen-week-old gametophores. *PpΔpsn* gametophores spread out, whereas WT grows toward the light. Preferred axes of gametophore growth are represented in red. (F) Mean length of each gametophore is graphically represented. (Insets) Pictures of *PpΔpsn* and WT gametophore.

layer thick (see ref. 49). The complete genome of *P. patens* has been sequenced (www.mossgenome.org), and asexual plants grown in the lab are haploid. Most importantly, tissue from vegetative stages grown in the lab can be disrupted and treated to remove the cell wall generating protoplasts that can be used for transformation and other molecular procedures. *P. patens* can be used to study gene function, either by RNA interference [RNAi (50, 51)], or, because it can undergo homologous recombination at a high frequency, by specific gene deletion and/or replacement (51–54).

In this article, we report the initial characterization of the null phenotype of *P. patens* PS (*PpΔpsn*) and demonstrate, using multiple lines of evidence and approaches, that evolutionarily conserved functions underlying the phenotype associated with loss of PS in this basal organism are independent of γ -secretase and may be placed in a pathway regulating the function of the cytoskeleton. This genetically tractable system adds a powerful new tool to the investigation of PS proteins and their cellular partners.

Results

Characteristics of PS Homologues in *P. patens*. A full-length PS cDNA from *P. patens* (*PpPS*) was cloned based on sequence information available from the EST database, PHYSCObase (moss.nibb.ac.jp). *PpPS* cDNA contains a single, intron-less ORF that encodes a polypeptide of 477 aa, with 49% and 46% similarity to human PS1 and PS2, respectively. Based on the length of hydrophilic loop, *PpPS* is a γ -type PS similar to the rice (*O. sativa*) and both *Arabidopsis* homologues (55) (Fig. 1A). Characteristics of PS family, i.e., the catalytic aspartates embedded in motifs YD and GxGD and the PAL motif, are conserved in *PpPS* [supporting information (SI) Fig. 6]. The complete genome sequence suggests that *PpPS* exists as a single copy in its haploid genome, which we confirmed by Southern blot analysis (data not shown).

Mutant Phenotypes Because of the Loss of PS. To evaluate the role of PS in *P. patens*, we created a null mutant of *PpPS* (*PpΔpsn*) by homologous recombination (Fig. 1C). RT-PCR analysis confirmed that this plant lacked the *PpPS* transcript (data not shown); genomic PCR using sequences flanking the ORF identified integration into the *PpPS* locus (data not shown).

P. patens has alternating sexual and asexual stages in its life cycle.

Diploid stage plants (sporophyte) generate haploid spores, which germinate into vegetative tissue that further develops into leafy shoots (or gametophores) bearing male and female gametes. Fertilization of these gametes results in diploid sporophytic generation. *PpΔpsn* plants displayed a unique phenotype early in development: filaments regenerating from *PpΔpsn* protoplasts grew in straight lines and did not curl like WT filaments (referred to as “coiled” morphology hereafter; Fig. 1C). These differences remained clearly evident throughout filament growth (Fig. 1D and E). Whereas the number of leaves per unit length was unchanged in mature gametophores, *PpΔpsn* gametophores exhibited three distinct phenotypes: First, shoots were longer and the total number of leaves was much greater than in WT gametophore (Fig. 1E and F). Second, the *PpΔpsn* gametophore did not grow in a predominant upward direction (Fig. 1E, red arrows), suggesting a defect in sensing of light or gravity. Third, WT *P. patens* became fertile within three months, but *PpΔpsn* plants were still unable to complete their life cycle (i.e., formation of spores) after a four-month period (data not shown). This inability may indicate a form of sterility, which will not be investigated further here.

Mutant Phenotypes Implicate the Cytoskeleton. The phenotypes described above could result from a number of defective processes, including the reception and integration of polarizing signals (light, gravity), cytoskeleton organization, membrane and vesicle cycling, and/or cell wall composition and organization. To investigate which processes depend on PpPS, we grew *P. patens* under different light conditions. When WT filaments were subjected to unidirectional red light, the chloroplasts rearrange and localize primarily to the cell-cell contact zones (56) (Fig. 2A). The redistribution of chloroplasts to contact zones was not observed in *PpΔpsn*; instead, chloroplasts remained randomly oriented (Fig. 2A). Chloroplast relocation in *P. patens* requires an intact photoreceptor complex, signal transduction to downstream effectors, and a dynamic actin and tubulin filament networks (57). To determine which of these pathways have become impaired in *PpΔpsn*, we subjected WT and *PpΔpsn* filaments to polarized red light and monitored their growth response. WT filaments responded by aligning themselves parallel to the plane of polarized light, whereas *PpΔpsn* filaments aligned perpendicular to the plane of polarized light (Fig. 2B). Because

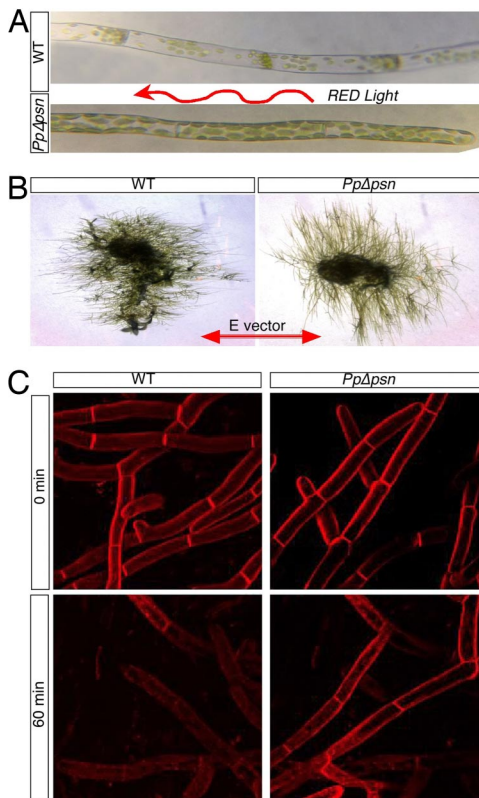


Fig. 2. Differential responses of WT and *PpΔpsn* filaments. (A) Chloroplasts relocation differs between WT and *PpΔpsn* cells exposed to unidirectional red light (arrow represents the light vector). (B) *PpΔpsn* filaments align perpendicular to the E-vector under polarized red light whereas WT filaments align parallel to the E-vector. (C) Clearance of FM4-64 fluorescence from the membrane is delayed in *PpΔpsn* filaments.

filaments did align in response to light (albeit in the incorrect plane), the defect in *PpΔpsn* is likely downstream of light detection in either a signaling system (which, as in metazoans, may require γ -secretase activity) or in the cytoskeletal network(s) or both.

To further define the step altered in *PpΔpsn*, we performed a light- and gravity-independent assay involving the function of the cytoskeleton. WT and *PpΔpsn* protoplasts were stained for two minutes with FM4-64 (a lipophilic dye used to stain membranes), washed and chased for one hour in the absence of the dye. Immediately after staining, both WT and *PpΔpsn* filaments stained bright red, but after the 1-h chase, fluorescence was lost in WT filaments because of surface membranes recycling via endocytosis. In contrast, *PpΔpsn* filaments retained significant fluorescence (Fig. 2C), most likely reflective of a slower rate of endocytosis and/or membrane recycling. Together, these experiments point to a defect in cytoskeletal related responses in *PpΔpsn*.

Transient Silencing of *PpPS* by RNAi Mimics Deletion of *PpPS*. An unfortunate consequence of altered membrane dynamics in *PpΔpsn* protoplasts is that they are refractory to transformation; this phenotype prevented us from performing rescue experiments directly in *PpΔpsn*. Also, as the knockout was created by disrupting the ORF and not by deletion, an amino-terminal truncated product may be produced, contributing to the observed phenotypes. We therefore asked whether RNAi could mimic faithfully the effects of *PS* loss. RNAi lines were established in the NLS4 background, which constitutively expresses a nuclear green fluorescent protein (GFP) (50, 51). When this line is used in conjunction with a vector that expresses complementary RNA strands for targeted gene-fragments contiguous with GFP sequences (pUFGI-*PS-KD*), effec-

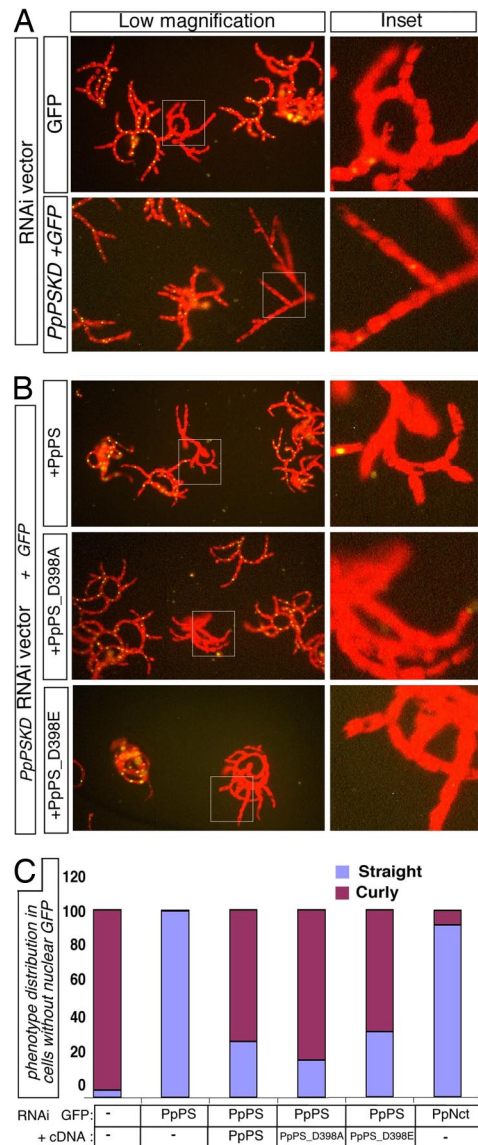


Fig. 3. Transient silencing of *PpPS* by RNAi mimics genetic loss of *PpPS*. (A) Fluorescent images of filaments regenerating from protoplasts transformed with *PpPSKD* construct or the corresponding vector alone. Nuclear GFP fluorescence overlaps with the red autofluorescence of chlorophyll in untransformed cells; straight filaments in GFP-negative cells are observed with *PpPSKD*, but not vector controls. (B) *PpPSKD* filaments cotransformed with WT or aspartate mutants of *PpPS* display normal phenotype despite loss of GFP expression (reflective of silencing). (C) Quantitative analysis of three independent experiments.

tive reduction of both GFP and target gene expression is achieved. Protoplasts transformed with the vector alone (harboring RNAi for GFP) lost GFP expression but displayed the WT, coiled filament morphology (Fig. 3A) and chloroplast realignment (data not shown). When transformed with a RNAi vector targeting both GFP and *PpPS*, filaments lacking GFP also exhibited the mutant, straight filaments morphology observed in *PpΔpsn* (Fig. 3A). Silencing of an unrelated protein involved in assembly of actin-based structures (58) in *P. patens* (51) did not result in the straight filament phenotype (data not shown) demonstrating that the phenotype was not a general response to RNAi.

To address whether the other γ -secretase components contributed to straight filament phenotype, we asked whether PpNct is involved in the pathway leading to the *PpΔpsn* phenotype. *PpNct* knockdown by RNAi (*NctKD*) resulted in protoplasts with straight

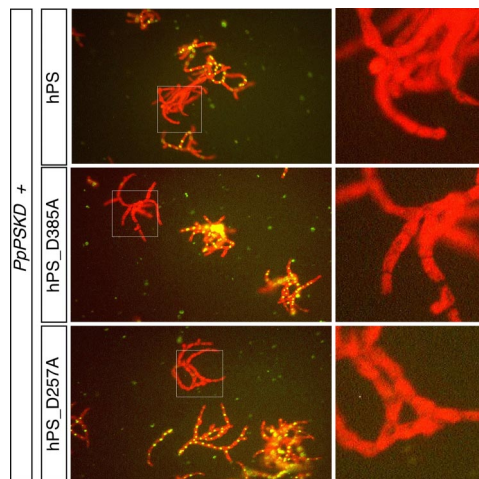


Fig. 4. Mammalian PS retained the activity responsible for *PpPSKD* phenotype in moss. *PpPSKD* protoplasts cotransformed with human PS constructs display WT growth morphology.

filaments (Fig. 3C), similar to the *PpPSKD* (Fig. 3D). The similarity between the phenotype observed in *PpPS* and *PpNct* knockdown could result from loss of γ -secretase activity or by means other mechanisms, including loss of PS protein in Nct-deficient cells (59–61). To test whether indeed γ -secretase activity was involved in the phenotype, we asked whether the *PpPSKD* phenotype could be rescued with WT *PpPS* or *PpPS* cDNAs containing substitutions of D398, replacing the conserved aspartyl in the essential GxGD motif to glutamate (D398E) or alanine (D398A). Interestingly, the WT as well as either D398 substitution in *PpPS* could rescue filament morphology in *PpPSKD* plants (Fig. 3B), presumably by overriding the RNAi effect. The ability of *PpPSKD* to phenocopy *Pp Δ psn*, and the ability of WT *PpPS* to rescue this phenotype, confirmed that the straight filament phenotype is caused by loss of PS.

Human PS Can Rescue *PpPSKD* Independent of γ -Secretase Function.

The results described above suggest that the coiled morphology of regenerating filaments is independent of proteolytic activity associated with PS proteins because aspartyl mutations in PS proteins eliminate enzymatic activity; however, we were unable to demonstrate any enzymatic activity for PpPS. To ask whether the loss of proteolytic activity contributed to the *PpPS* phenotype, we next tested whether the human PS (hPS) could provide an evolutionarily conserved activity and rescue the *PpPSKD* phenotype. hPS rescued coiled growth (Fig. 4A) and chloroplast alignment (data not shown). This conservation enabled us to test the rescuing ability of PS mutants lacking γ -secretase activity. Both the hPSD257A and hPSD385A substitutions abolish enzymatic activity [they are unable to reconstitute γ -secretase activity in PS-deficient mouse embryonic fibroblasts (MEFs) (7)]. Nonetheless, both aspartyl-substituted hPS proteins were capable of rescuing the growth phenotype of *PpPSKD*. This observation confirmed that retention of γ -secretase activity was not required for the growth phenotype in *P. patens*. We conclude that a conserved function of PS proteins is γ -secretase-independent.

Plant PS Fail to Rescue γ -Secretase Activity in PSDKO Cells. The ability of a mammalian protein (hPS) to rescue a plant PS deficiency prompted us to ask whether PpPS could rescue any of the documented defects in PS1, PS2 deficient mouse fibroblasts (PSDKO MEF; ref. 62). To this possibility, we established several clones of mammalian PSDKO cells stably expressing PpPS, PpPSD398A and PpPSD398E (collectively, PSDKO_PpPS lines). PSDKO_PpPS cell

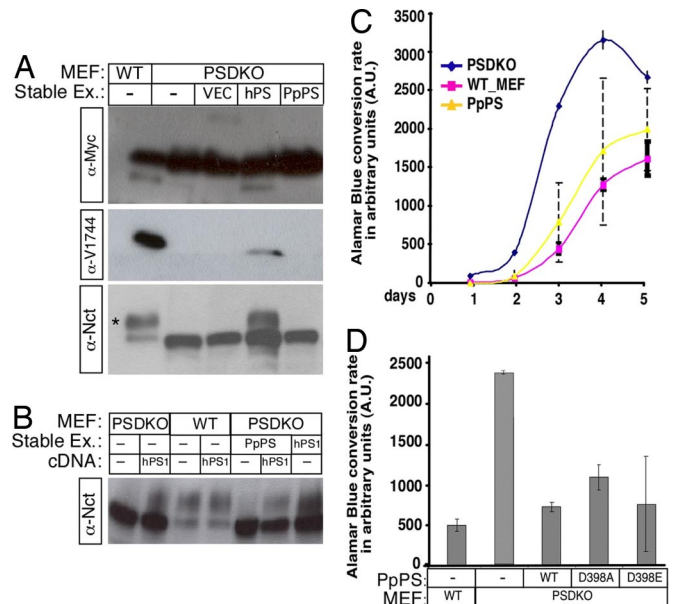


Fig. 5. *PpPS* can suppress accelerated proliferation in PS-deficient embryonic fibroblasts. (A) Analysis of the Notch cleavage in stable MEF lines expressing WT or mutant PpPS. Stable PpPS expression cannot rescue NCT maturation (*) or Notch substrate cleavage in PSDKO. (B) Nct maturation can be restored by transiently transfecting hPS into the PSDKO_PpPS MEFs. (C) Growth curves of WT ($n = 4$), PSDKO ($n = 4$), and PSDKO_PpPS clones ($n = 4$) were determined by alamarBlue conversion in 1 h as a reflection of increase in cell number. (D) alamarBlue conversion on day 3 is shown for each PSDKO_PpPS clone (average of 8 replicates).

lines were first tested for their ability to cleave Δ ice (63). Neither α -MYC antibodies, which can recognize cleaved and uncleaved Δ ice molecules independent of scissile bond selection, nor α -V1744 antibodies, which can recognize the amino terminus of the common γ -secretase-cleaved Notch1-based substrates, detected any significant cleavage in PSDKO_PpPS. Both antibodies readily recognized cleavage of Δ ice in cell line expressing human PS1 (PSDKO_hPS; Fig. 5A).

Lack of Δ ice cleavage in PSDKO_PpPS can be due to either the inability of PpPS to perform proteolysis, or to the possibility that PpPS cannot associate with mammalian partners because of sequence divergence and hence cannot incorporate itself into γ -secretase complex (64). To differentiate between these possibilities, we examined the glycosylation of Nct [a measure of Nct maturation and functional assembly of γ -secretase complex, (65–68)]. Mature Nct was not observed in PSDKO_PpPS lines (Fig. 5A and B), indicating that PpPS failed to assemble into functional γ -secretase complexes in the mammalian cells. To confirm that cells expressing plant proteins have not lost the ability to assemble γ -secretase, PSDKO_PpPS lines were transiently transfected with hPS; significant amounts of mature Nct were detected (Fig. 5B) indicating recovery of the complex. These results demonstrate that no γ -secretase complex is present in PSDKO_PpPS lines.

PpPS Reverts Accelerated Proliferation in PS-Deficient Mouse Embryonic Fibroblasts.

We next asked whether PpPS could complement a γ -secretase-independent function of PS. The most obvious difference between PSDKO and WT MEF lines derived from heterozygote littermates is their growth rates (69). To evaluate growth, we assayed all our MEF sublines for their ability to convert alamarBlue, a redox dye whose metabolism in live cells leads to increase in fluorescence which correlates well with cell numbers (70). Fluorescence from WT MEFs, PSDKO cell lines and stable lines expressing human or plant PS were recorded over several days and

generate stable lines, transformants were alternately grown for a week each in the presence or absence of Hyg.

Mammalian Cell Culture and Transfection. All cell lines were maintained in Dulbecco's modified Eagle's medium with 10% FBS, 4 mM glutamine, and antibiotics. HEK 293 cells were transfected by calcium phosphate in *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES)-buffered saline. PS1^{-/-} PS2^{-/-} dKO mouse embryonic fibroblasts (PSDKO) were transfected by using Lipofectamine (Invitrogen). All mutant PS constructs were generated by using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and were subsequently sequenced to confirm that only the desired mutations were introduced. To generate PSDKO cell lines expressing mPS1, WT PpPS, D398E PpPS, or D398A PpPS, appropriate constructs were cotransfected with the plasmid, pBabePuro, conferring antibiotic resistance, at a ratio of 10:1. The next day, cells were trypsinized and plated on P150 plates, and, after 24 h, 4 μ g/ml puromycin was added to the medium. Stable lines were subjected to several rounds of antibiotic selection and tested for expression of appropriate PS proteins by Western analysis.

Western Blotting. Western Blotting was performed by fractionating cellular lysates on SDS/PAGE and transferring them onto nitrocellulose membranes. Membranes were then blocked in 10% milk

(PBS and 0.1% Tween 20) and incubated in primary antibody (1:1,000 in 5% milk, PBS, and 0.1% Tween 20) followed by HRP-conjugated secondary antibody (1:5,000 in 5% milk, PBS, and 0.1% Tween 20; Pierce).

Microscopic Observations. Microscopic images of moss were taken with a Spot RT Slider camera (Diagnostic Instruments, Sterling Heights, MI) on either a Zeiss inverted microscope or an Olympus dissecting microscope. Images were then processed by using Canvas X and Adobe Photoshop CS software.

Statistical Analysis of Growth. For statistical analysis of growth, see *SI Text*.

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