Presenilin 1 Negatively Regulates β-Catenin/T Cell Factor/Lymphoid Enhancer Factor-1 Signaling Independently of β-Amyloid Precursor Protein and Notch Processing

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Abstract. In addition to its documented role in the proteolytic processing of Notch-1 and the β-amyloid precursor protein, presenilin 1 (PS1) associates with β-catenin. In this study, we show that this interaction plays a critical role in regulating β-catenin/T Cell Factor/Lymphoid Enhancer Factor-1 (LEF) signaling. PS1 deficiency results in accumulation of cytosolic β-catenin, leading to a β-catenin/LEF-dependent increase in cyclin D1 transcription and accelerated entry into the S phase of the cell cycle. Conversely, PS1 specifically represses LEF-dependent transcription in a dose-dependent manner. The hyperproliferative response can be reversed by reintroducing PS1 expression or overexpressing axin, but not a PS1 mutant that does not bind β-catenin (PS1Δcat) or by two different familial Alzheimer's disease mutants. In contrast, PS1Δcat restores Notch-1 proteolytic cleavage and Aβ generation in PS1-deficient cells, indicating that PS1 function in modulating β-catenin levels can be separated from its roles in facilitating γ-secretase cleavage of β-amyloid precursor protein and in Notch-1 signaling. Finally, we show an altered response to Wnt signaling and impaired ubiquitination of β-catenin in the absence of PS1, a phenotype that may account for the increased stability in PS1-deficient cells. Thus, PS1 adds to the molecules that are known to regulate the rapid turnover of β-catenin.

Key words: presenilin • β-catenin • Notch-1 • β-amyloid precursor protein • cyclin D1

Introduction

Presenilin 1 (*PS1*)[1] is the major gene responsible for familial Alzheimer's disease (FAD). Inherited mutations in *PS1* cause an early-onset form of Alzheimer's disease (AD) with an autosomal-dominant pattern (Levy-Lahad et al., 1995; Sherrington et al., 1995). PS1 is required for constitutive proteolytic cleavage of the β-amyloid precursor protein (APP) to generate amyloid β-peptide (Aβ), while mutations in *PS1* selectively increase the longer forms of Aβ (De Strooper et al., 1998; reviewed by Haass and De Strooper, 1999). PS1 is a multiple-pass transmembrane protein localized mainly to the endoplasmic reticulum and Golgi membranes in nonneuronal cells as well as in cell bodies and dendrites in neurons (Cook et al., 1996; Annaert et al., 1999), and is expressed in all tissues examined (Doan et al., 1996; Kovacs et al., 1996; De Strooper et al., 1997; Lehmann et al., 1997; Li et al., 1997). Recent studies have also shown that PS1 interacts with calsenilin (Buxbaum et al., 1998) and is present in cadherin/catenin complexes and is localized in the intercellular contacts (Georgakopoulos et al., 1999). Full-length PS1 undergoes constitutive endoproteolytic processing to generate an amino-terminal fragment of ∼28 kD and a carboxy-terminal fragment of ∼17 kD that remain stable as part of the same complex (Thinakaran et al., 1996a; Zhang et al., 1998b). PS1 null mice die late in embryonic development or shortly after birth, exhibiting cerebral hemorrhages and abnormal patterning of the axial skeleton and spinal ganglia (Shen et al., 1997; Wong et al., 1997). In *Caenorhabditis elegans*, the PS1 homologue Sel-12 functions to facilitate Lin-12/Notch signaling (Levitan and Greenwald, 1995). Recent reports provided convincing evidence that this activity is highly conserved. Specifically, there is an

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[1]*Abbreviations used in this paper:* Aβ, amyloid β-peptide; APC, adenomatous polyposis coli; APP, β-amyloid precursor protein; FAD, familial Alzheimer's disease; GSK, glycogen synthase kinase; LEF, T cell factor/lymphoid enhancer factor-1; NICD, Notch intracellular domain; PS1, presenilin 1; RT, reverse transcription.

absolute requirement for PS1 in the proteolysis of the Notch transmembrane domain after ligand binding with consequent release of the Notch intracellular domain (NICD), which translocates into the nucleus (De Strooper et al., 1999; Struhl and Greenwald, 1999; Ye et al., 1999).

PS1 has been shown to interact with β-catenin and this association has been implicated in modulating the Wnt-β-catenin signaling pathway (Zhou et al., 1997; Murayama et al., 1998; Yu et al., 1998; Kang et al., 1999; Stahl et al., 1999). Wnt proteins represent a family of developmentally important signaling molecules, at the heart of which is the regulation of the cytosolic pool of β-catenin (reviewed in Willert and Nusse, 1998). In the absence of Wnt, cytosolic β-catenin is rapidly turned over by the proteasome, in a pathway requiring phosphorylation by glycogen synthase kinase-3β (GSK-3β) and ubiquitination. Adenomatous polyposis coli (APC) and axin combine to positively modulate GSK-3β activity and promote β-catenin degradation (Hart et al., 1998; Ikeda et al., 1998). Binding of Wnt ligands to cell surface receptors triggers a pathway that antagonizes β-catenin degradation by inactivating GSK-3β, leading to β-catenin stabilization and subsequent translocation to the nucleus. In the nucleus, β-catenin binds to the T cell factor/lymphoid enhancer factor-1 (LEF) family of transcription factors and mediates transcriptional activation of downstream target genes (Behrens et al., 1996).

A number of genes have been shown to be activated by the β-catenin/LEF complex. The first two genes identified were c-myc and cyclin D1 (He et al., 1998; Shtutman et al., 1999; Tetsu and McCormick, 1999). The cyclin D1 gene encodes the regulatory subunit of the holoenzyme that phosphorylates and inactivates the Rb tumor suppressor protein. Transcriptional induction of the cyclin D1 gene by several oncogenes (Ras, Src, Erb-B2, Rac; reviewed in Sherr, 1996) contributes to the increased abundance of cyclin D1 protein. A requirement of cyclin D1 in G1 phase progression induced by a variety of mitogens and oncogenes has been established. Fibroblasts derived from mice with the cyclin D1 gene deleted have reduced proliferative capacity and increased apoptosis, suggesting a critical role for cyclin D1 in cellular proliferation and survival (Brown et al., 1998; Albanese et al., 1999).

Although PS1 appears to modulate β-catenin stability in vitro, controversial data have been reported. While we and Murayama et al. (1998), showed that wild-type PS1 negatively regulates β-catenin stability (Kang et al., 1999), others (Zhou et al., 1997; Weihl et al., 1999) have presented evidence that wild-type PS1 stabilized β-catenin. Thus, the recent discovery that cyclin D1 is activated by LEF signaling provided the opportunity to test our hypothesis that PS1 is a negative regulator of β-catenin stability. In the present study, we showed that PS1 indeed facilitates the rapid turnover of β-catenin. In PS1 null cells, we found increased β-catenin stability, higher cyclin D1 protein levels, and increased rate of G1-to-S phase progression in the cell cycle. The hyperproliferative response can be reversed by reintroducing PS1 expression or overexpressing axin, but not by PS1 forms lacking the β-catenin binding site or harboring FAD mutations, and is independent of Notch-1 signaling and Aβ generation. Finally, we show an altered response to Wnt signaling and impaired ubiquitination of β-catenin in the absence of PS1,

offering a potential mechanism as to where PS1 facilitates β-catenin turnover.

Materials and Methods

Antibodies

PS1 polyclonal antibodies J27 (against residues 27–42), PS1NT (1–65), αPS1Loop (against residues 319–442), and monoclonal antibody PSN2 (against residues 31–56) have been described (Thinakaran et al., 1996b, 1998; Zhang et al., 1998b; Kang et al., 1999). APP antibodies 3134, 26D6, and CT15 have been described (Soriano et al., 1999; Lu et al., 2000). Polyclonal antibody against c-myc was from BabCO. Other monoclonal antibodies used were against β-catenin (Transduction Laboratories and Sigma-Aldrich), ubiquitin, cyclin D1, cyclin A, and Cdc2 (Calbiochem), β-tubulin (Amersham Pharmacia Biotech) and 9E10, against c-myc (DSHB), used against c-myc–tagged ΔEMV and ICV Notch-1 proteins.

cDNA Constructs

A cDNA encoding the wild-type PS1 sequence lacking amino acids 330–360 (PS1Δcat) was generated by PCR-mediated deletion and confirmed by sequencing. Wild-type PS1, PS1Δcat, and axin (a gift of Frank Costantini, Columbia University, New York, NY) were subcloned into pIND (Invitrogen) or pBabe-puro (Morgenstern and Land, 1990). The latter vector was used to generate replication-incompetent retroviruses in GP+E-86 packaging cell line. Myc-tagged NH$_2$-terminal truncated Notch-1 construct (ΔEMV, in which methionine 1726 has been mutated to valine to eliminate translation initiation at that site; described in Kopan et al., 1996) and Notch-1 intracellular domain (ICV, in which valine 1744 is the first amino acid after the initiating methionine) were provided by Dr. R. Kopan (Washington University, St. Louis, MO). The former is an artificial construct specifically designed to examine the transmembrane cleavage of Notch-1 protein (Kopan et al., 1996). The human cyclin D1 promoter reporter constructs −163CD1Luc, −163ΔlefCD1Luc, −1745CD1Luc, as well as the PALUC reporter, which contains 7 kb of the human cyclin A promoter and the cyclin E promoter were previously described (Shtutman et al., 1999).

Cell Lines

PS1−/− and PS1+/− fibroblasts were prepared from whole embryos at embryonic day 15.5. Head and liver were discarded and the bodies were triturated in 5 ml of a 0.25% trypsin/PBS solution and incubated at 37°C for 15 min with continuous rotation. Tissue debris was allowed to settle at 4°C and supernatant with cells was transferred to a new tube. Fresh trypsin was added to the remaining tissue and the process was repeated a total of three times. Cells from all incubations were pooled, spun at 200 *g*, and resuspended in RPMI medium containing 10% FCS. Cells were plated onto 25-cm^2 flasks and allowed to reach confluence (usually ∼16 h). Cells from early passages (between two and five) were used in all experiments. To generate primary fibroblasts expressing wild-type PS1 or PS1 lacking amino acids 330–360 (PS1Δcat), early passage primary fibroblasts were infected with the corresponding retroviruses in the presence of 10 μg/ml polybrene for 8 h and allowed to grow to confluence without antibiotic selection. Cells were then trypsinized and used directly for the indicated experiments. Only cells passaged once after infection were used. Expression levels of both wild-type PS1 and PS1Δcat in PS1−/− cells were detected by Western blotting and were comparable with those in PS1+/− cells. Immortalized fibroblasts (used in Notch-1 transfections and transcriptional activation studies), and EcR293 cells inducibly expressing wild-type PS1 and PS1Δcat have been described (Kang et al., 1999; Soriano et al., 2001).

Immunoprecipitation and Western Blotting

Cultured cells were lysed in 1% 3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPS) buffer containing 50 mM Tris, 150 mM NaCl, 100 μg/ml amino ethyl benzenesulfonyl fluoride (AEBSF), and 10 μg/ml leupeptin on ice for 20 min. Lysates were precleared with normal rabbit serum and protein A agarose (for rabbit polyclonal antibodies) or with anti–mouse IgG agarose (for mouse monoclonal antibodies). Cleared lysates were then incubated with primary

antibody and protein A agarose or anti–mouse IgG agarose for 3 h at 4°C as described (Kang et al., 1999). Immunoprecipitates were washed twice with CHAPS buffer, and proteins were separated on SDS-PAGE and transferred to nitrocellulose membranes. Coimmunoprecipitated proteins were detected by Western blotting as above.

Turnover of Cytosolic β-Catenin

Overnight cultures of embryonic fibroblasts from six-well tissue culture plates were incubated in methionine-free medium for 20 min, followed by metabolic labeling with 200 μCi/ml [^{35}S]methionine for 20 min and chasing for the indicated periods. At each time point, cells were placed on ice and washed twice with ice-cold PBS. To release cytosolic proteins, cells were incubated twice for 10 min with 0.1% saponin buffer containing 25 mM Hepes and 75 mM potassium acetate. The two soluble fractions were pooled and β-catenin immunoprecipitated with a β-catenin monoclonal antibody. Immunoprecipitates were washed twice with buffer containing 50 mM Tris, 150 mM NaCl, and 1% NP-40 and proteins separated by standard SDS-PAGE. Dried gels were either exposed to film or quantitated by phosphorimaging (Bio-Rad Laboratories). All experiments were performed at least three times and results from either a representative experiment or averages ± SD are shown.

Transient Reporter Assays

Reporter gene assays in PS1−/− and +/− were performed with the Dual-luciferase reporter assay system (Promega) following the manufacturer's instructions after transfection with −163CD1 and −163ΔLefCD1 reporter constructs (Shtutman et al., 1999). 50 ng pRL-TK renilla luciferase were cotransfected in each sample as an internal control for transfection efficiency. Results are expressed as an average of three independent experiments performed in triplicate. In experiments with 293 cells, transient transfection of cyclin D1, cyclin A, and cyclin E promoter constructs was carried out with calcium phosphate precipitation for 6 h. 24 h later, luciferase activity was determined by comparison with that of an equal amount of empty vector (Albanese et al., 1999). Results are expressed as mean ± SEM of seven separate transfections.

Reverse Transcritption PCR

Total RNA was extracted with Trizol (Life Technologies). Reverse Transcription (RT) PCR (25 ng total RNA/reaction) was performed using Superscript II RNase H⁻ Reverse Transcriptase (Life Technologies) to synthesize first-strand cDNA and Taq DNA polymerase (Promega) for PCR reactions. Semi-quantitative PCR was carried out in which the number of cycles produced amplified products of β-catenin, cyclin, and actin cDNAs within a linear range. The optimized cycle profile for cyclin D1 was: 1 cycle, 94°C, 3 min; 24 cycles, 94°C, 1 h; 55°C, 45 min; 72°C, 1 h.

BrdU Staining

Primary fibroblasts from embryonic day 15.5 embryos were cultured on glass coverslips overnight and incubated with BrdU for 45 min at 37°C and fixed and stained for BrdU incorporation using the BrdU labeling and detection kit I (Boehringer) according to the manufacturer's instructions. Nuclei were counterstained with propidium iodide to assay for total cell numbers.

APP Processing in Mouse Embryonic Fibroblasts

APP COOH-terminal fragments were identified in PS1 null cells infected with retroviruses containing PS1 wild-type or PS1Δcat sequences using antibody CT15. Aβ secretion was analyzed by immunoprecipitation of conditioned media using antibody 3134 followed by immunoblotting with 26D6.

Wnt Stimulation, β-Catenin Phosphorylation and Ubiquitination Studies

Previously described Wnt3a-transfected L cells (Shibamoto et al., 1998) were used as a source of Wnt-3a–conditioned medium. Cells were Wnt-stimulated for 2 h and washed extensively, and fresh medium was added for 2.5, 5, and 7.5 h. Levels of cytosolic β-catenin were determined as described above. In experiments where the phosphorylation state of axin was analyzed, cells were lysed in NP-40 in the presence of phosphatase inhibitors (0.4 μM microcystin-LR, 1 mM vanadate, 50 mM NaF) through-

out the experiment, and axin bands were detected with an anti–axin antiserum, previously described by Willert et al. (1999). For in vivo phosphorylation studies, cell monolayers ∼70% confluent (10-cm² plates) were treated with Wnt3a-CM or control medium. After 90 min, both media were aspirated and replaced with phosphate-free media with or without Wnt3a-CM, together with 500 μCi/ml [^{32}P] orthophosphate. After a 30-min incubation, media were aspirated and cells were washed once with ice-cold PBS and lysed with NP-40 lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris, pH 8.0, 5 μg/ml leupeptin, 100 μg/ml AEBSF, 0.4 μM microcystin-LR, 1 mM vanadate, and 50 mM sodium fluoride). Lysates were precleared for 10 min at 10,000 g and supernatants were used for β-catenin immunoprecipitation as described above. Radiolabeled β-catenin was separated by SDS-PAGE and visualized by phosphorimager (Bio-Rad Laboratories) or autoradiography.

For ubiquitination studies, cell monolayers at ∼70% confluence in six-well plates were treated with 10 μM of the proteasome inhibitor MG-132 (Calbiochem). At the indicated time points, cells were lysed with NP-40 lysis buffer and accumulation of ubiquitinated β-catenin was analyzed directly by Western blot with an antibody against β-catenin or by immunoprecipitation with the same antibody followed by Western blot with a polyclonal anti–ubiquitin antibody.

Results

Enhanced β-Catenin/LEF-dependent Cyclin D1 Transcription and Accelerated Cell Proliferation in PS1 Null Primary Mouse Embryonic Fibroblasts

We previously reported that the turnover rate of β-catenin in PS1-deficient immortalized fibroblasts was slower when compared with hemizygous PS1+/− cells, resulting in higher levels of cytosolic β-catenin (Kang et al., 1999). Accordingly, based on the recent finding that cyclin D1 transcription is activated by β-catenin/LEF, we predicted comparable alterations in cyclin D1 transcription in primary fibroblasts from early passages. Western blotting showed that PS1−/− cells contained higher amounts of β-catenin and cyclin D1 protein (Fig. 1 a). In contrast, levels of cdc2 and cyclin A, two other related genes not subject to β-catenin–mediated transcription (Tetsu and McCormick, 1999), were not elevated in PS1−/− cells. This was confirmed by quantitative RT-PCR, which showed that PS1−/− cells expressed ∼ 2.5-fold higher levels of cyclin D1 mRNA than that of PS1+/− controls (2.53 ± 0.1, mean ± SD, $n = 3$) while β-catenin mRNA levels were unchanged (Fig. 1 b). Consistent with our previous report using immortalized PS1-deficient cell lines (Kang et al., 1999), pulse-chase experiments in primary fibroblasts showed that β-catenin turnover was reduced in PS1−/− cells (data not shown, see Fig. 3). No difference in cyclin D1 turnover was detected (Fig. 1 c), demonstrating that differences in cyclin D1 levels occurred at the transcriptional level.

To determine whether higher levels of cyclin D1 resulted in increased rate of entry into S phase in PS1−/− fibroblasts, we assayed BrdU incorporation in primary fibroblasts from PS1+/− and PS1−/− embryos. Indeed, BrdU incorporation was approximately twofold higher in PS1−/− than PS1+/− cells (Fig. 1 d). Therefore, PS1 deficiency in mouse embryonic fibroblasts is accompanied by increased levels of β-catenin and cyclin D1, and accelerated cell proliferation.

The sequence −81 to −73 in the cyclin D1 promoter contains a consensus LEF binding site and has been shown to represent the main contributor to β-catenin transactivation (Shtutman et al., 1999; Tetsu and McCormick, 1999). To confirm that elevation of cyclin D1 mRNA seen in
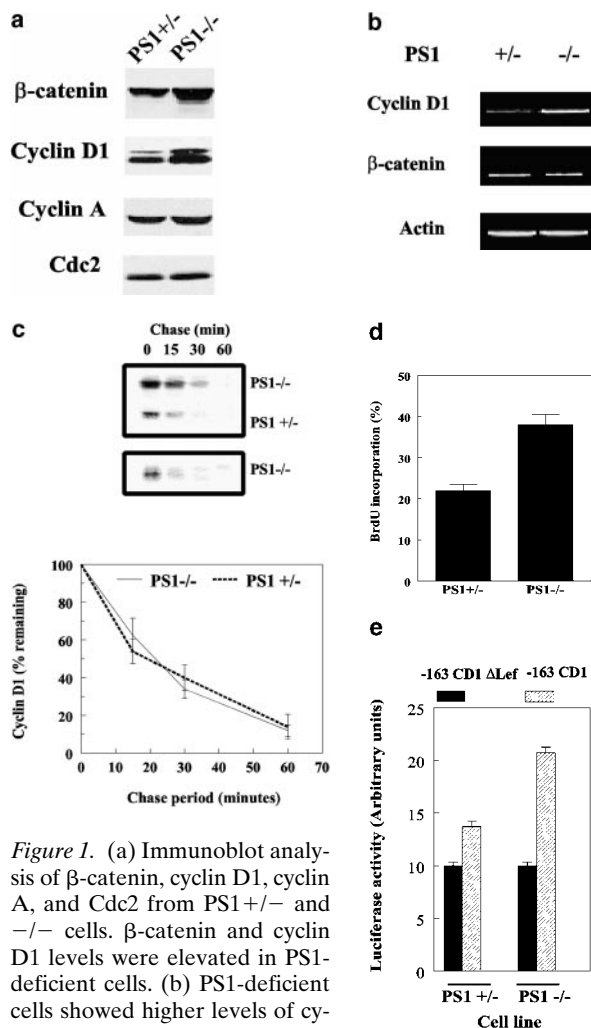
Figure 1. (a) Immunoblot analysis of β-catenin, cyclin D1, cyclin A, and Cdc2 from PS1+/− and −/− cells. β-catenin and cyclin D1 levels were elevated in PS1-deficient cells. (b) PS1-deficient cells showed higher levels of cyclin D1 mRNA. Semiquantitative RT-PCR analysis of β-catenin, cyclin D1, and actin from PS1−/− and +/− primary fibroblasts. 25 ng of total RNA-derived cDNA were used for PCR reactions. Controls without reverse transcriptase yielded no bands (not shown). Results from a representative experiment are shown. (c) Loss of PS1 did not affect the turnover rate of cyclin D1. The cells were labeled for 20 min and chased for 15, 30, and 60 min. (Middle) Because PS1−/− cells have higher constitutive levels of cyclin D1, a shorter film exposure of the same autoradiogram that is comparable with that seen in PS+/− cells is shown. Quantitation from three independent experiments is shown (Bottom). (d) BrdU incorporation in PS1+/− and −/− cells. Primary fibroblasts from embryonic day 15.5 embryos were incubated with BrdU for 45 min at 37°C and BrdU incorporation was determined as described in Materials and Methods. Results are shown as the proportion of total cells (calculated from propidium iodide-stained nuclei) that were positive for BrdU staining, and represent the average of two independent experiments, each performed in triplicate (1,000 cells from at least 10 independent fields were scored for each sample). (e) Enhanced β-catenin/LEF-induced activation of the cyclin D1 promoter in PS1-deficient cells. PS1+/− and PS1−/− fibroblasts were transfected with constructs containing luciferase reporter driven by the cyclin D1 promoters. 1 μg of −163CD1 (wild type) or −163ΔlefCD1 (lacking the main LEF binding sequence) were used together with 50 ng of pRL-TK *renilla* luciferase construct in each sample as an internal control for transcription efficiency. Results are shown as the ratio of reporter luciferase to control *renilla* luciferase and represent the mean ± SD of three independent experiments performed in triplicate.

PS1−/− cells represents enhanced β-catenin–mediated transcription, we used reporter constructs containing the −81 to −73 LEF binding site (−163CD1), or with the LEF-binding domain deleted (−163CD1ΔLEF) (Shtutman et al., 1999) and determined the LEF-dependent transcriptional activation of the cyclin D1 promoter by measuring the reporter luciferase activity in PS1+/− and PS1−/− cells. As seen in Fig. 1 e, luciferase activity derived exclusively from the −81 to −73 LEF binding site (−163 CD1 subtracts −163 CD1ΔLEF) was threefold higher in PS1−/− cells when compared with PS1+/− cells (10.7 ± 0.2 vs. 3.7 ± 0.25, average ± SD, $n = 3$). These results demonstrated that higher levels of cytosolic β-catenin in PS1−/− cells correlated with enhanced Lef-dependent transcription of cyclin D1.

The preceding observations can be argued to suggest that, in hyperproliferating PS1-deficient cells, cyclin D1 transcription would be augmented in parallel, as an epiphenomenon of faster cell growth. Therefore, it is essential to corroborate that PS1 expression can specifically modulate cyclin D1 transcription. To determine whether the cyclin D1 promoter was a target of PS1 repression, we assessed transcriptional activity from luciferase reporter constructs containing the sequences for cyclin D1, as well as those of cyclin A (which, despite containing a putative LEF-1 binding site, is not activated by β-catenin/Lef; Tetsu and McCormick, 1999) and cyclin E (which lacks a LEF-1 consensus site) promoters in 293 cells. As shown in Fig. 2 a, wild-type LEF (Topflash) reporter was repressed in a dose-dependent manner by PS1. −1745 CD1Luc reporter (wild-type cyclin D1 promoter), which was induced ∼10-fold by β-catenin (Shtutman et al., 1999), was significantly repressed by PS1. Repression of reporter activity by PS1 was maintained in the −163 CD1Luc, which contains the consensus LEF binding site. However, mutation or deletion of the LEF site abolished repression (Fig. 2 b). Importantly, neither cyclin A nor E reporter activity was repressed by PS1 (Fig. 2 c). These results provide strong evidence in favor of the concept that the enhanced levels of cyclin D1 seen in PS1-deficient fibroblasts originate, at least in part, from dysregulation of β-catenin turnover and subsequent elevation of β-catenin/Lef-mediated activity, rather than constituting an epiphenomenon of faster cell growth.

Modulation of β-Catenin Signaling by PS1 Requires the Interaction of both Proteins

Earlier studies indicated that PS1 interacts with β-catenin through its hydrophilic cytoplasmic loop domain, although the precise binding site has not been carefully mapped (Zhou et al., 1997; Yu et al., 1998; Stahl et al., 1999). We performed pull-down assays using glutathione-S-transferase–PS1 loop fusion proteins in cell homogenates and confirmed that amino acids 330–360 within the loop of PS1 are required for its association with β-catenin (Saura et al., 2000). Based on these data, we generated a PS1 deletion mutant lacking residues 330–360 (PS1Δcat) to determine whether the effects of PS1 on β-catenin signaling and cell proliferation require the interaction between PS1 and β-catenin. Using the previously described muristerone-inducible EcR293 cells (Kang et al., 1999), we confirmed
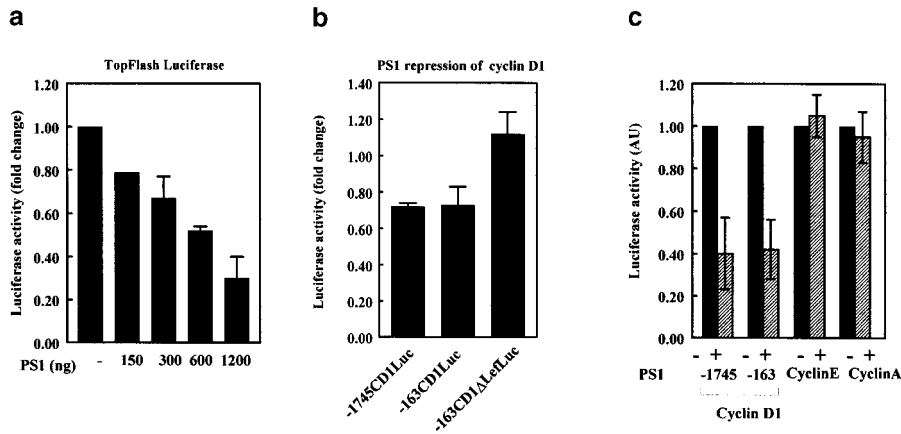
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Figure 2. (a) PS1 represses transcription from the LEF-binding sequence linked to a minimal promoter (Top-Flash) in a dose-dependent manner. TopFlash luciferase reporter (1.2 μg) was cotransfected in 293 cells with increasing amounts of wild-type PS1 (150–1,200 ng). Transcriptional repression is shown by its comparison with the effect on equal amounts of empty expression vector (arbitrarily assigned a luciferase activity of 1). (b) The TCF site in the cyclin D1 promoter is required for repression by PS1. Repression of the cyclin D1 promoter after PS1 transfection (300 ng) was maintained when the sequences between −1745 to −163 of the promoter were deleted. In contrast, deletion of the LEF binding site (−163CD1ΔLefLuc) completely abrogated PS1 repression. (c) PS1 specifically regulates transcription of cyclin D1. Wild-type cyclin D1 promoter (−1745CD1) and −163CD1, both containing the main LEF-binding site, are inhibited by PS1 expression. In contrast, neither cyclin A nor E transcription is affected by PS1.

that induction of PS1Δcat resulted in the production of a shortened full-length PS1Δcat that was processed into a ~28-kD amino-terminal fragment (NTF) indistinguishable from wild-type NTF and an ~14-kD carboxy-terminal fragment lacking amino acids 330–360. Immunoprecipitation experiments showed the lack of binding of PS1Δcat to β-catenin (Soriano et al., 2001).

We next examined the consequences of PS1Δcat expression in PS1−/− primary fibroblasts after retroviral-mediated infection. In PS1Δcat infected PS1−/− fibroblasts, the NH₂- and COOH-terminal fragments, but not full-length PS1, were detected, and lack of binding of PS1Δcat to β-catenin in these cells was confirmed (Fig. 3 a). Expression of wild-type PS1 resulted in a reduction of cytosolic β-catenin levels, whereas introducing PS1Δcat had no significant effect (Fig. 3 b, top; tubulin levels are shown at bottom as loading control). By pulse-chase paradigm, β-catenin turnover was increased in wild-type PS1-infected PS1−/− cells as expected, but expressing PS1Δcat in PS1−/− cells did not have a significant effect (Fig. 3 c). As predicted from our earlier results, the recovery of β-catenin turnover in primary PS1−/− cells after reintroduction of wild-type PS1 resulted in a concomitant decrease in BrdU incorporation (and therefore in cell proliferation; Fig. 3 d). In marked contrast, entry into S phase in PS1−/− cells was not modified by reintroducing PS1Δcat or vector control.

Axin, in association with APC and GSK-3β, regulates β-catenin degradation. Thus, mutations in axin were recently detected in hepatocellular carcinoma, and in these tumor cells overexpression of wild-type axin was shown to decrease β-catenin levels and suppression of cell growth (Satoh et al., 2000). Taking advantage of this observation, we expressed axin in PS1-deficient fibroblasts to determine whether the hyperproliferative effect can be blocked by specific inhibition of the β-catenin signaling pathway. As shown in Fig. 3, axin overexpression resulted in a decrease of both β-catenin levels and cell proliferation in PS1−/− cells to a degree that was comparable with the expression of wild-type PS1 (Fig. 3, b and d). These results argue that upregulated β-catenin signaling plays a functional role for the accelerated cell proliferation in the absence of PS1.

Effects of PS1 FAD Mutations on β-Catenin Signaling

The effects of FAD mutations on β-catenin turnover have also been controversial. We previously reported that, in cultured cells and transgenic mice expressing FAD mutations, turnover of endogenous β-catenin was delayed, suggesting a partial loss of function (Kang et al., 1999). In view of the preceding observations, we examined the levels of cytosolic β-catenin levels and BrdU incorporation in PS1 null cells expressing these FAD PS1 mutations. As shown in Fig. 4 a, expression of PS1 M146L or PS1 ΔX9, unlike wild-type PS1, failed to downregulate cytosolic β-catenin levels. Consistent with this finding, BrdU incorporation in these cells remained unchanged as compared with expression of wild-type PS1 (Fig. 4 b). Thus, FAD mutations in PS1 resulted in a loss of function with respect to its ability to modulate β-catenin turnover.

Both Wild-Type PS1 and PS1Δcat Restore Normal Proteolytic Cleavage of Notch-1 and γ-Secretase Activity

Recent reports have shown that PS1 plays a central role in the proteolytic release of the NICD (De Strooper et al., 1999; Struhl and Greenwald, 1999; Ye et al., 1999), the signaling domain of the Notch-1 receptor that translocates into the nucleus to activate transcription of downstream genes. In addition, the role of PS1 in Notch processing can be separated from its role in the modulation of γ-secretase activity (Kulic et al., 2000). Therefore, while studies described above have provided compelling evidence that one of the functions of PS1 is to regulate cytosolic β-catenin turnover, a role that requires interaction of both proteins, we cannot exclude the possibility that the cellular consequences we have defined are due to the concomitant impairment of Notch signaling and/or γ-secretase activity. To address this issue, we analyzed Notch-1 and APP processing in PS1 null cells infected with retroviruses expressing either wild-type PS1 or PS1Δcat to determine whether PS1Δcat is capable of restoring Notch-1 processing and Aβ generation. In PS1−/− fibroblasts, NICD was not cleaved from the ΔEMV Notch construct (Myc-tagged NH₂-terminal trun-
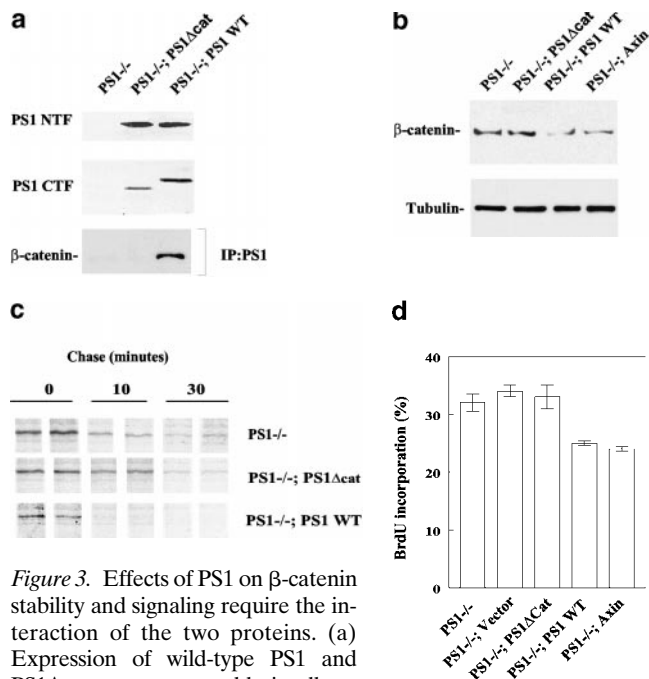
Figure 3. Effects of PS1 on β-catenin stability and signaling require the interaction of the two proteins. (a) Expression of wild-type PS1 and PS1Δcat were comparable in all experiments, and similar to those in PS1+/− cells (not shown). As ΔCTF lacks amino acids 330–360, a domain recognizable by the αPS1Loop antibody, its signal appears to be weaker than wild-type carboxy-terminal fragment. Immunoprecipitation with J27, an antibody against the NH₂ terminus of PS1, shows that PS1Δcat does not interact with β-catenin. (b, Top) Retroviral infection of wild-type PS1 or axin, but not PS1Δcat, in PS1 null cells results in reduction of cytosolic β-catenin levels. (Bottom) β-Tubulin blot shown as loading control. (c) Expression of wild-type PS1 in PS1 null cells restores efficient degradation of β-catenin. In contrast, expression of PS1Δcat has no significant effect. Cells were metabolically labeled with [³⁵S]methionine for 20 min, chased for 0, 10, and 30 min, and immunoprecipitated for cytosolic β-catenin as described in Materials and Methods. Results from a representative experiment performed in duplicate are shown. (d) Decrease in BrdU incorporation in PS1−/− cells after expression of wild-type PS1 and axin, but not PS1Δcat. Cells were infected with the indicated retroviral vectors, and BrdU incorporation was performed as described 48 h after plating when the cells reached 70–80% of confluence.

cated Notch-1, in which methionine 1726 has been mutated to valine to eliminate translation initiation at that site; Kopan et al., 1996). However, in both PS1 wild-type and PS1Δcat-infected PS1−/− cell lines, the NICD release from ΔEMV Notch was clearly restored (Fig. 5 a). Consistent with this restoration of Notch cleavage, expression of both PS1 wild-type and PS1Δcat also restored Aβ secretion and normal turnover of APP COOH-terminal fragments (Fig. 5 b). It is also noteworthy that a PS1 form lacking the entire cytosolic loop that does not bind β-catenin also restores Notch-1 cleavage in PS1 null cells (Saura et al., 2000), in agreement with the above results. Thus, increased cytosolic β-catenin stability and downstream signaling in PS1−/− cells are not the consequences of impaired Notch-1 signaling or γ-secretase activity. By extension, the upregulation of cyclin D1 transcription and enhanced cell proliferation in the PS1−/− cells is most likely due to perturbed β-catenin signaling, rather than impaired Notch-1 or APP signaling.
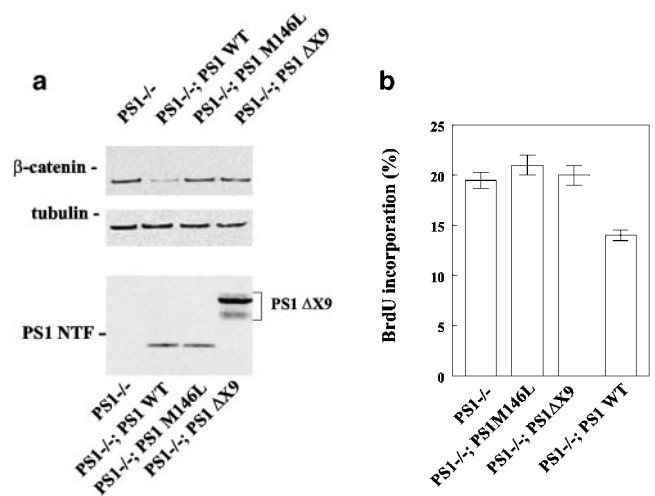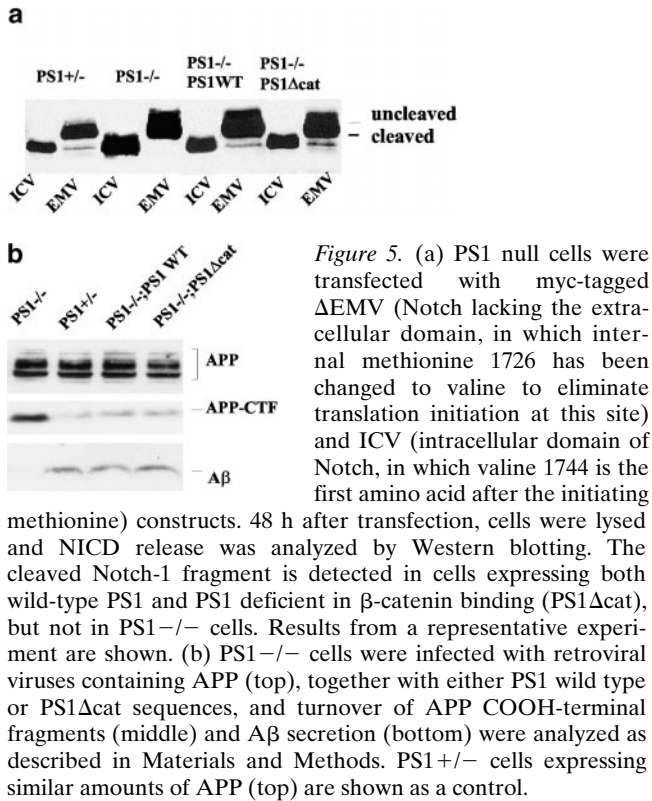

Figure 4. (a, Top) Retroviral infection of wild-type PS1, but not FAD mutants M146L and ΔX9, results in reduction of cytosolic β-catenin levels. (Middle) β-Tubulin blot shown as loading control. A representative experiment is shown. (b) Decrease in BrdU incorporation in primary PS1 null cells after expression of wild-type PS1, but not FAD mutants M146L and ΔX9. Cells were infected with the indicated retroviral vectors and BrdU incorporation was performed as described 48 h after plating, when the cells reached 70–80% of confluence. Results from two independent experiments performed in triplicate are shown.

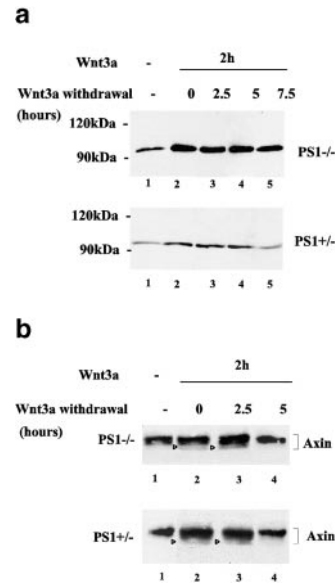## Abnormal Modulation of β-Catenin Levels by Wnt3a Signaling in the Absence of PS1

Because aberrant Wnt stimulation is believed to result in tumorigenesis in several tissues (reviewed in Barker et al., 2000), we asked whether loss of PS1 alters the response to Wnt signaling. In primary mouse embryonic fibroblasts with or without PS1, the levels of cytosolic β-catenin and axin phosphorylation, parameters that have been shown to be modulated by Wnt signaling (Willert et al., 1999, and references therein) were determined after Wnt-3a stimulation. Specifically, we examined axin phosphorylation and β-catenin levels under the following conditions: (a) basal, unstimulated state, (b) Wnt stimulation, and (c) after removal of the Wnt signal. We chose these conditions because an adequate response to Wnt signaling in vivo involves not only the adequate upregulation of β-catenin levels and nuclear signaling after stimulation, but presumably the timely restoration of basal levels once Wnt activation is switched off. As shown before, under basal conditions, β-catenin levels were higher in the absence of PS1 (Fig. 6 a, lane 1, compare top and bottom). After treatment with conditioned medium from Wnt-3a–transfected cells (Wnt3a-CM) for 2 h, β-catenin increased substantially in both PS1+/− and −/− cells, likely due to inactivation of GSK-3β (Fig. 6 a, lane 2). Concomitantly, there was the appearance of a faint, faster migrating, dephosphorylated axin species (Fig. 6 b, lanes 2 and 3, arrowheads), consistent with dephosphorylated forms of axin, as previously described (Willert et al., 1999). This species was indistinguishable between PS1+/− and −/− cells, and was no longer detectable 5 h after Wnt3a withdrawal (Fig. 6 b, lane 4). In contrast, while cytosolic levels of β-catenin were consistently restored in PS1+/− cells within 7.5 h af-

a

PS1+/− | PS1−/− | PS1−/− PS1WT | PS1−/− PS1Δcat

ICV EMV ICV EMV ICV EMV ICV EMV

— uncleaved
— cleaved

b

PS1−/− | PS1+/− | PS1−/−;PS1 WT | PS1−/−;PS1Δcat

] APP

APP-CTF

Aβ

Figure 5. (a) PS1 null cells were transfected with myc-tagged ΔEMV (Notch lacking the extracellular domain, in which internal methionine 1726 has been changed to valine to eliminate translation initiation at this site) and ICV (intracellular domain of Notch, in which valine 1744 is the first amino acid after the initiating methionine) constructs. 48 h after transfection, cells were lysed and NICD release was analyzed by Western blotting. The cleaved Notch-1 fragment is detected in cells expressing both wild-type PS1 and PS1 deficient in β-catenin binding (PS1Δcat), but not in PS1−/− cells. Results from a representative experiment are shown. (b) PS1−/− cells were infected with retroviral viruses containing APP (top), together with either PS1 wild type or PS1Δcat sequences, and turnover of APP COOH-terminal fragments (middle) and Aβ secretion (bottom) were analyzed as described in Materials and Methods. PS1+/− cells expressing similar amounts of APP (top) are shown as a control.

ter Wnt3a withdrawal (Fig. 6 a, bottom, lane 5), β-catenin levels remained consistently elevated at this same time point in PS1−/− (Fig. 6 a, top, lane 5). Thus, our results showed that, although Wnt stimulation properly elevates β-catenin levels in PS1 null cells, the signal is abnormally prolonged.

To determine which pool of cytosolic β-catenin was abnormally elevated in the absence of PS1, the levels of phosphorylated β-catenin were assessed in the presence or absence of Wnt3a-CM. By labeling with ^{32}P, the levels of phosphorylated β-catenin were markedly elevated under basal conditions in PS1−/− cells as compared with PS1+/− cells (Fig. 7 a, compare lane 1 with 3, top). After Wnt3a stimulation, levels of phosphorylated β-catenin were markedly reduced in both cell types (Fig. 7 a, top). The rapid reduction in phosphorylated β-catenin levels, attributable to GSK-3β inactivation, indicated that, in response to Wnt3a stimulation, this step of β-catenin dephosphorylation was unaffected by the absence of PS1.

In the current model of β-catenin degradation, phosphorylated β-catenin should have been rapidly ubiquitinated and degraded by the proteosome (Orford et al., 1997). The accumulation of phosphorylated β-catenin suggested to us that, in PS1−/− cells, β-catenin ubiquitination (i.e., downstream of phosphorylation) may be abnormal. Inhibition of the proteasome results in the accumulation of the ubiquitinated forms of proteins normally degraded by the ubiquitin–proteasome pathway. Accordingly, high molecular weight (HMW) β-catenin species accumulate after proteosome inhibition (Aberle et al., 1997; Orford et al., 1997; Salomon et al., 1997). In PS1+/− control cells, HMW β-catenin species were detectable after 30 min of treatment with the proteasome inhibitor MG-132,

a

Wnt3a − 2h

Wnt3a withdrawal (hours) − 0 2.5 5 7.5

120kDa −
90kDa − PS1−/−
1 2 3 4 5

120kDa −
90kDa − PS1+/−
1 2 3 4 5

b

Wnt3a − 2h

Wnt3a withdrawal (hours) − 0 2.5 5

PS1−/−] Axin
1 2 3 4

PS1+/−] Axin
1 2 3 4

Figure 6. (a) Abnormal β-catenin turnover after Wnt stimulation in the absence of PS1. Primary PS1−/− and PS1+/− fibroblasts were incubated with Wnt-3a–conditioned medium (Wnt-CM) as described in Materials and Methods. After 2 h of treatment, Wnt-CM was removed, and cells were washed extensively and incubated for the indicated time points with fresh normal medium. Shown are the levels of cytosolic β-catenin. Results are representative of three independent experiments. (b) Cells were treated with Wnt-CM exactly as described for Fig. 5 a, and axin levels were analyzed by Western blot. Note that the axin phosphorylation pattern in response to Wnt is not different between PS1+/− and −/− cells. In marked contrast, restoration of β-catenin levels is delayed in PS1−/− cells with respect to control cells (a).

and further increased after 120 min (Fig. 7 b, bottom). These HMW β-catenin bands were identical to the species demonstrated to represent ubiquitinated β-catenin (Aberle et al., 1997; Orford et al., 1997). In contrast, HMW forms of β-catenin were apparent in PS1-deficient cells only after 60 min of treatment, and the overall response was blunted even after 120 min (Fig. 7 b, top), suggesting that the ubiquitinated β-catenin pool was diminished. Thus, higher levels of phosphorylated β-catenin in PS1-deficient cells apparently corresponded with delayed ubiquitination, coincidental with reduced degradation of β-catenin.

Discussion

In addition to its documented role in the proteolytic processing of Notch and APP, PS1 has been shown to interact with β-catenin. However, the biological consequences of this latter interaction remain controversial. In the present study, we provided evidence that loss of PS1 function results in increased stability of cytosolic β-catenin, leading to enhanced β-catenin/Lef-dependent signaling, among which is elevation of cyclin D1 levels. This, in turn, is associated with increased entry into the cell cycle and cell proliferation, a phenotype that can be reversed by expressing wild-type PS1, but not a PS1 form that does not associate with β-catenin. Conversely, overexpression of PS1 in vitro results in LEF-mediated repression of cyclin D1 transcription in a dose-dependent manner. All these effects on β-catenin turnover by PS1 are independent of APP and notch proteolysis and appear to be mediated at the level of β-catenin phosphorylation/ubiquitination. Finally, FAD mutations in PS1 cannot efficiently restore β-catenin degradation in PS1 null primary fibroblasts.

Using primary mouse embryonic fibroblasts deficient in PS1, we demonstrated that PS1 negatively modulates the cytosolic (and hence signaling) pool of β-catenin. We
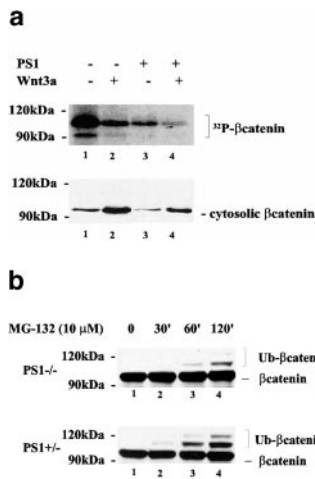
a

PS1   − − + +
Wnt3a − + − +

120kDa −
90kDa −   ] ³²P-βcatenin
                1 2 3 4

120kDa −
90kDa −   − cytosolic βcatenin
                1 2 3 4

b

MG-132 (10 μM)   0   30' 60' 120'

PS1−/−   120kDa −   Ub-βcatenin
         90kDa −   − βcatenin
                    1   2   3   4

PS1+/−   120kDa −   Ub-βcatenin
         90kDa −   − βcatenin
                    1   2   3   4

Figure 7. (a, Top) Enhanced phosphorylation of β-catenin in PS1 null cells. Cells were metabolically labeled with ³²P in the absence or presence of Wnt3a-CM as described in Materials and Methods. Note the accumulation of phosphorylated β-catenin in PS1 null cells both in basal conditions and after Wnt stimulation. (Bottom) Experiment performed in parallel and identical to the one described (top), except that ³²P labeling was omitted. (b) Abnormal accumulation of high molecular weight species of β-catenin in PS1 null cells after proteasome inhibition. Cells were treated with MG-132 as described in Materials and Methods and β-catenin accumulation was monitored at the indicated time points.

showed that both cyclin D1 mRNA and protein were increased in PS1−/− cells and this was correlated with higher β-catenin/LEF–dependent transcriptional activity from the cyclin D1 promoter in these cells. Moreover, we observed that, as previously described for colon cancer cells, increased levels of cyclin D1 in PS1−/− cells correlated with accelerated progression through the G1 phase of the cell cycle. It is important to note that increased cyclin D1 levels are unlikely to represent an epiphenomenon of hyperproliferation in PS1 null cells. This is because PS1 expression can specifically modulate LEF-mediated transcription in a dose-dependent manner, consistent with the concept that increase in cyclin D1 levels occurs before hyperproliferation itself and as a consequence of PS1 deficiency. In other words, we have provided compelling evidence that PS1, through modulation of β-catenin signaling, directly affects not only cyclin D1 levels, but also cell growth associated with cyclin D1 activity. Finally, further support of a direct causal effect rather than merely a correlative link was demonstrated by using a mutant PS1 construct where the interaction between PS1 and β-catenin was lost (Fig. 3). In short, accumulation of β-catenin and increased cell proliferation in PS1-deficient cells can be reversed by reintroducing wild-type PS1, but not PS1Δcat, a PS1 mutant that does not bind β-catenin.

Our results also suggested a model in which PS1 functions to regulate β-catenin turnover at a step after β-catenin phosphorylation. In the absence of PS1, the rapid dephosphorylation of β-catenin after Wnt-3a stimulation was retained. Moreover, modulation of axin phosphorylation was also not altered during Wnt stimulation and withdrawal in PS1-deficient cells. However, the elevation of cytosolic β-catenin levels was seen both in basal (i.e., unstimulated) and after Wnt stimulation, and was accompanied by an increase in the levels of phosphorylated β-catenin. Thus, in the absence of PS1, turnover of β-catenin was prolonged in both Wnt-3a–stimulated and at basal conditions. Lastly, the increase in phosphorylated β-catenin, which should normally be at very low levels due to rapid degradation, was correlated with a decrease in ubiquiti-

nation. We therefore hypothesize that PS1 modulates β-catenin degradation by facilitating ubiquitination. This mechanism would be consistent with two recent reports examining the interaction of *Drosophila* presenilin homologue (DPS) and Armadillo/β-catenin (Arm/βcat). In *Drosophila*, loss of endogenous DPS resulted in the accumulation of Arm/β-cat in the cytoplasm (Noll et al., 2000), leading the authors to suggest a possible role of DPS in proteosomal degradation. In addition, in a genetic screen of candidates that modify an Arm/βcat phenotype, DPS was identified as a negative regulator of Wg/Wnt signaling (Cox et al., 2000). Therefore, both of these findings in *Drosophila* are entirely consistent with our present observations.

PS1 is required for the cleavage of Notch-1 to release the signaling intracellular domain (NICD), a step necessary for initiating downstream gene transcriptional activation (De Strooper et al., 1999; Struhl and Greenwald, 1999; Ye et al., 1999). There is evidence to suggest that the Notch and Wnt pathways are mutually inhibitory (Axelrod et al., 1996). Therefore, it can be argued that enhanced β-catenin/LEF signaling and increased cell proliferation in the absence of PS1 could be secondary to defective Notch signaling. However, Notch-1 from PS1−/− cells expressing PS1Δcat is efficiently cleaved. The fact that this mutant does not restore the β-catenin phenotype strongly suggests that Notch-1 deficiency is not the cause of the abnormal β-catenin phenotype. Thus, modulating β-catenin signaling and facilitating Notch-1 proteolytic cleavage are likely independent and parallel functions of PS1. It remains possible that the β-catenin activity facilitated by PS1 in vivo is required at later stages of development and consequently masked by its early requirement for Notch-1 signaling.

Consistent with the results on Notch-1 proteolysis, the requirement of PS1 in γ-secretase cleavage is independent of β-catenin turnover. PS1Δcat also restores efficiently γ-secretase activity in PS1−/− cells, both in terms of APP COOH-terminal fragments and Aβ generation. These findings, entirely consistent with the recent report of Saura et al. (2000) again strongly suggest that this latter activity and the role in β-catenin homeostasis are likely to be independent functions of PS1. Thus, the association of PS1 with β-catenin is not required in the intracellular compartment where APP and Notch proteolysis takes place.

Taken together, our findings provide a parsimonious interpretation that is consistent with the current model of the Wnt–β-catenin pathway in which stabilization of β-catenin, in this case through loss of PS1, leads to proliferative signal via activation of cyclin D1. These results also argue that the discrepant results reported by different laboratories may reflect measurements of dissimilar pools of β-catenin. While we examined the Wnt-3a–responsive cytosolic pool of endogenous β-catenin defined as the fraction that was saponin extractable, others assessed both cytosolic and membrane-bound β-catenin expressed by transient transfections (Zhang et al., 1998a). However, the membrane-bound β-catenin pool is not responsive to Wnt stimulation (data not shown). Furthermore, the presence of lower molecular weight β-catenin species was used to imply a stimulation of degradation without formal proof. Although the identity and functional significance of these species remain unknown, we suspect that they accumulate

when degradation of full-length β-catenin is defective (see, for example, Schlosshauer et al., 2000). In short, these discrepancies underscore the importance of defining accurately the β-catenin pool under study, since its biological role is tightly linked to its subcellular localization.

In our proposed scenario, the loss of PS1 would be similar to that postulated in a number of human cancers, including colon, melanoma, hepatocarcinomas, and pilomatricomas of the skin. In these tumors, mutations in APC, axin, or β-catenin result in abnormally high β-catenin levels, and increased β-catenin/LEF signaling is thought to contribute to neoplasic transformation (for reviews, see Polakis, 1999; Roose and Clevers, 1999). In this respect, it is of interest that epithelial hyperplasia and skin tumors have been described in the majority of PS1 null mice rescued with the human PS1 transgene (hPS1) driven by the neuronal-specific human Thy-1 (hThy) promoter (Zheng et al., 2000). PS1 null mice die either embryonically or perinatally. However, animals rescued by Thy-1–driven hPS1 survive into adulthood. Since the skin of these animals has been shown to be deficient in PS1 and to show elevated β-catenin levels, the tumor phenotype in these rescue animals is consistent with constitutively upregulated transcription of cyclin D1 in PS1-deficient skin, due to impaired attenuation of Wnt signals. However, a definitive causal link between β-catenin levels, PS1 deficiency, and tumor formation remains to be established.

In summary, our results demonstrated that PS1 is part of the β-catenin degradation machinery, thereby adding PS1 to a list of known proteins that regulate β-catenin stability (Orsulic and Peifer, 1996; Hart et al., 1998; Ikeda et al., 1998; Seeling et al., 1999; Zorn et al., 1999). We propose a model in which PS1 contributes to the rapid turnover of β-catenin after its phosphorylation, possibly by some enhancement of the ubiquitination process. We have shown that FAD mutations in PS1 represent a loss of its function as a β-catenin modulator. Whether this phenotype contributes to Alzheimer's disease is an issue not addressed in this study. Nevertheless, our results further emphasize the importance of maintaining a rapid turnover of cytosolic β-catenin in normal cell growth and differentiation.

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