Presenilin 1 Regulates Epidermal Growth Factor Receptor Turnover and Signaling in the Endosomal-Lysosomal Pathway

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Mutations in the gene encoding presenilin 1 (PS1) cause the most aggressive form of early-onset familial Alzheimer disease. In addition to its well established role in Aβ production and Notch proteolysis, PS1 has been shown to mediate other physiological activities, such as regulation of the Wnt/β-catenin signaling pathway, modulation of phosphatidylinositol 3-kinase/Akt and MEK/ERK signaling, and trafficking of select membrane proteins and/or intracellular vesicles. In this study, we present evidence that PS1 is a critical regulator of a key signaling receptor tyrosine kinase, epidermal growth factor receptor (EGFR). Specifically, EGFR levels were robustly increased in fibroblasts deficient in both tor (EGFR). While FAD mutations showed partial loss of activity, the C-terminal fragment of PS1 was sufficient to fully reduce EGFR turnover of EGFR protein. Stable transfection of wild-type PS1 but not PS2 corrected EGFR to levels comparable to FAD mutations in PS1 and PS2 alter the activity of the γ-secretase complex, leading to the change in the ratio of Aβ to that favoring Aβ42 generation and accelerated amyloid deposition in brain (6). Although the amyloid hypothesis is the leading model for AD pathogenesis, the aggressive early age of disease onset by PS1 FAD mutations have led many to suggest that other cellular perturbations of presenilin mutations might also contribute to the early disease phenotype. Recent studies have clearly demonstrated that presenilins are multifunctional proteins. First, we previously demonstrated that PS1 negatively regulates the Wnt/β-catenin signaling pathway through facilitating the paired phosphorylation and degradation of β-catenin by acting as a scaffold upon which a priming kinase, glycogen synthase kinase-3, and β-catenin are assembled (7–9). In a different modality, we and others also reported that PS1 and PS2 FAD mutations also differentially modulate phosphatidylinositol 3-kinase/Akt and MEK/ERK signaling, a pathway that leads to glycogen synthase kinase-3 inactivation and reduced Tau phosphorylation (10–13). Finally, a role for presenilins in the trafficking and maturation of select membrane proteins and/or intracellular vesicles has been shown, although the mechanisms of this activity are unclear. For example, Naruse and colleagues demonstrated that the glycosylation, trafficking, and signaling of TrkB are altered in PS1-deficient neurons (14). Moreover, the turnover of other membrane proteins, such as α-synuclein and tenecephalin, are delayed by PS1 deficiency, and the modified Eagle’s medium; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; PBS, phosphate-buffered saline; CHX, cycloheximide; APP, amyloid precursor protein; BACE, β-site APP-cleaving enzyme; DAPT, N-(3,5-difluorophenacetyl)-L-ananyl)-S-phenylglycine-β-butyl ester.

Mutations in genes encoding presenilin 1 (PS1) and presenilin 2 (PS2) account for the vast majority of early onset familial Alzheimer disease (FAD) (1, 2). Presenilins are multipass transmembrane proteins that form the core enzymatic activity of the γ-secretase complex together with nicastrin, pen-2, and aph-1. These four proteins are required for regulated intramembrane proteolysis of multiple type I transmembrane proteins, including APP and Notch, such that the loss of any one of these four proteins destabilizes the complex and abrogates γ-secretase activity (3). Accordingly, null mice for any one of these components display embryonic lethality associated with severe malformations of the axial skeleton and cerebral hemorrhage, resembling that of Notch deficiency (3). As expected, this activity is conserved in Caenorhabditis elegans and Drosophila where the presenilin complex functions to facilitate Notch signaling (4, 5).
ecules accumulate in large vacuolar structures resembling autophagosomes (15, 16). The role of PS1 in membrane protein trafficking also extends to components of amyloidogenic processing, amyloid precursor protein (APP) and β-site APP-cleaving enzyme (BACE1) (17–20).

We recently hypothesized that presenilins may be involved in the regulation of various signaling cell surface receptors that could alter the state of Tau phosphorylation and neuronal viability through the phosphatidylinositol 3-kinase/Akt pathway (11). For example, we found that in cells genetically deficient in PS1 and PS2, PDGF receptors α and β are down-regulated, a phenotype that was rescued by PS2 but not PS1. However, FAD PS1 mutations, by reducing PS2 fragments, modified PDGFR levels and activity (11). In this study, we hypothesized that a related cell surface signaling receptor, EGF receptor (EGFR/ErbB1), might also be regulated by presenilins. Surprisingly, EGFR was dramatically elevated in cells deficient in PS1 and PS2, a phenotype that was fully rescued by PS1 but not PS2 or FAD PS1 mutations. Consequently, EGFR–dependent signaling was markedly prolonged by the loss of PS1 resulting from defects in ligand induced EGF degradation. We further demonstrate that PS1 is involved in the trafficking of EGF from early endosomes to lysosomes and that EGFR levels are dramatically elevated in brains of mice conditionally ablated for PS1 and skin tumors associated with PS1 deficiency.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—The monoclonal antibody PSN2 (against residues 31–56 of human PS1), and polyclonal antibodies against PS1 hydrophilic loop region (490D) and APP (CT15) have previously been described (11, 19). The polyclonal antibodies against EGFR, PS2-CTF, phospho-EGFR PY845, phospho380-Akt, Akt, ERK1/2, and phospho-ERK1/2, and monoclonal antibody against phospho-EGFR PY1068 were purchased from Cell Signaling. Monoclonal antibodies against biotin (Jackson ImmunoResearch), ubiquitin (Santa Cruz Biotechnology), and β-tubulin (Iowa Hybridoma Bank) were purchased from commercial sources. The 1005/sc-03 polyclonal antibody against EGFR and blocking peptide for immunohistochemistry were purchased from Santa Cruz Biotechnology. Other reagents EZ-Link Sulfo-NHS-LC-Biotin (Pierce), DAPT (Calbiochem), EGFR (Sigma), cycloheximide (Calbiochem), puromycin (Calbiochem), blecocin (Calbiochem), EGFR-Alex488 (Invitrogen), LysoTracker Red (Invitrogen), Texas Red-transferrin (Invitrogen), and 125I-EGF (Amersham Biosciences) were purchased from the indicated vendors.

cDNA Constructs—cDNAs encoding wild-type PS1, PS1 D257A, PS1 A246E, PS1ΔX9, and wild-type PS2 were subcloned into the retroviral vector pBabe-puro. PS1N-PS2C, and PS2N-PS1C were also subcloned into pBabe-bleo (Fig. 1). The PS1N-PS2C construct fuses the NTF of PS1 (residues 1–280) with CTF of PS2 (residues 287–448), and the PS2N-PS1C construct fuses the NTF of PS2 (residues 1–286) with CTF of PS1 (residues 281–467), obtained from Dr. Gopal Thinakaran (21). All constructs were transfected together with a plasmid encoding the vesicular stomatitis virus membrane glycoprotein envelope into 293GP packaging cells, and the resulting supernatants were prepared for retroviral transduction as previously described (8).

Cell Lines and Transfections—All cell lines were grown in DMEM containing 10% fetal bovine serum unless explicitly stated otherwise. PS1+/− cells, PS1−/− cells (genetically deficient in PS1 and PS2), and PS1−/− cells have previously been described (7, 8). To generate stable cell lines expressing presenilin variants, corresponding retroviral supernatants from 293GP cells were added in the presence of 10 μg/ml Polybrene and selected with 3 μg/ml puromycin or 100 μg/ml bleocin. Resistant cells were pooled and maintained in the presence of appropriate antibiotic without clonal selection as previously described (11).

Cell and Brain Tissue Lysis, Biotinylation, and Signaling Assays—Cells were lysed in buffer containing 1% CHAPS, 0.1% SDS, 50 mM Tris (pH 8.0), 150 mM NaCl, 0.002% sodium azide, 400 nM Microcystin-LR, 0.5 mM sodium vanadate, and 1 μM protease complete inhibitor mixture (Sigma). For signaling assays, overnight confluent cultures were serum-starved in DMEM for 4 h, and EGF (30 ng/ml) was added for the indicated times. Protein quantitations from cell lysates were performed by using the micro-BCA method (Pierce), and equal amounts of protein were then subjected to either direct immunoblotting with the indicated antibodies or immunoprecipitated with EGFR antibody and immunoblotted with ubiquitin antibody. For cell surface biotinylation experiments, adherent cells were washed three times with ice-cold 1× phosphate-buffered saline, incubated with 1 ml of biotin label (Pierce EZ-Link Sulfo-NHS-Biotin) at 2 mg/ml for 1 h at 4 °C, washed three times with ice-cold 1× phosphate-buffered saline, and subjected to cell lysis as described above. Lysates were then immunoprecipitated with anti-biotin antibody and/or subjected to immunoblotting for EGFR. To extract protein from mouse brains, hippocampi, and frontal cortices were micro-dissected form 1-year-old mouse brains and homogenized with 30 strokes at 4 °C in lysis buffer containing 62.5 mM Tris, pH 6.8, 2% Triton X-100, 10% glycerol, 10 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, and 1 mM sodium vanadate. The homogenates were then centrifuged (13,000 × g, 4 °C, 15 min) to remove nuclei and debris, and protein concentration was measured as above. All experiments were performed at least...
three times, and representative experiments and/or quantitations are shown.

Reverse Transcriptase PCR Analysis—PS−/−, PS+/− (hPS1), and PS−/− (hPS2) cells were grown to confluence, and total RNA was isolated using the RNeasy mini kit from Qiagen. Two micrograms of total RNA was subjected to reverse transcriptase first strand synthesis using the Superscript kit (Invitrogen) according to the manufacturer’s instructions. Equal amounts of the reverse transcriptase product were then used for PCR of EGFR within a linear range of amplification, empirically determined to be between 16 and 22 cycles. Quantitations were performed using a digital camera-based imaging system. All experiments were performed at least three times, and results were normalized to PS−/− cells.

125I-EGF Internalization and Degradation Assays—To assess ligand internalization, cells cultured in 12-well dishes were incubated for 1 h in HEPES binding media (Hanks’ balanced salt solution containing 50 mM HEPES, pH 7.2, 0.1% bovine serum albumin). Cells were then incubated with a low concentration of 125I-EGF (1.5 ng/ml) in HEPES binding media for 1–5 min at 37 °C. At the indicated times, one set of cells was washed three times with ice-cold Hanks’ balanced salt solution on ice, lysed in 1 N NaOH, and used to determine total cellular 125I-EGF. In a replicate set of cells, cell surface-bound 125I-EGF was removed by two rapid acid washes (50 mM glycine, 100 mM NaCl, pH 3.0) on ice, lysed with 1 N NaOH, and used as a measure of internalized 125I-EGF. Percent 125I-EGF internalization was calculated by γ-radiation measured from intracellular 125I-EGF (after removing cell surface 125I-EGF) divided by total cellular 125I-EGF. For 125I-EGF degradation assays, cells were incubated with 3 ng/ml 125I-EGF for 10 min at 37 °C in binding medium, washed 3 × with PBS on ice, and further incubated at 37 °C in serum-free DMEM for 1.5 or 3 h. The medium was then collected, and cells were lysed with 1 N NaOH. Radioactivity from the lysate and trichloroacetic acid-soluble medium was measured by γ-radiation counter. Degradation of 125I-EGF was calculated by subtracting nonspecific surface 125I-EGF binding from controls for each condition co-incubated with 200-fold excess unlabeled EGF. Non-competable 125I-EGF binding represented <10% of total binding in all experiments. Experiments were performed in triplicate.

Fluorescent EGF Trafficking Assays—Mouse embryo fibroblasts were grown on coverslips and incubated with 500 ng/ml EGF-Alexa488 (Invitrogen) for 10 min at 37 °C (pulse period). After two washes with ice-cold PBS, cells were incubated for 0, 1, or 2 h at 37 °C to monitor intracellular trafficking of the internalized EGF-Alexa488 to lysosomes (chase period). To mark lysosomes, cells were incubated with 75 nm LysoTracker Red DND-99 (Invitrogen) for 30 min at 37 °C immediately prior to the end of the chase period. After each indicated time point, mouse embryo fibroblasts were washed with ice-cold PBS on ice and fixed in 4% formaldehyde for 20 min on ice. Cells were then visualized with the Olympus IX81 microscope.

Immunohistochemistry—Paraffin sections were immunostained using the Vectastain ABC Elite Kit (Vector Laboratories) with rabbit polyclonal anti-EGFR (1005) antibody at a dilution of 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA). Tissue sections were pretreated with microwave for antigen recovery. Sections were blocked for endogenous peroxidase for 10 min using 3% H2O2 in deionized water and incubated with a blocking solution containing 5% goat serum and 0.3% Triton X-100. Tissues were then incubated with primary antibody at 4 °C overnight, washed, and incubated with secondary antibody (1:250) for 30 min at room temperature. After washing, slides were incubated in the ABC complex for 30 min at room temperature, and then stained with diaminobenzidine (Sigma). Sections were counterstained with hematoxylin I (Richard-Allan Scientific) and mounted in VectaMount (Vector Laboratories). The specificity of the immunoreaction was verified by the lack of immunoreactivity when stained with primary antibody preabsorbed with the blocking peptide (sc-03, Santa Cruz Biotechnology) or secondary antibody alone.

RESULTS

Presenilin 1 Deficiency Dramatically Elevates EGF Receptor Levels by Reduced Turnover at Steady State—We previously showed that PDGFR levels were reduced in PS−/− fibroblasts that are genetically deficient in both PS1 and PS2, a phenotype that was rescued by reintroduction of PS2 but not PS1 (11). This led us to hypothesize that other cell surface signaling receptors might also be deregulated by the loss of presenilins. Although we previously showed that EGF-induced Akt and ERK1/2 phosphorylation was largely normal in PS−/− cells up to 1-h of exposure, we had not performed an exhaustive study of EGF levels and downstream signaling events (11). As an initial attempt to characterize signaling via other cell surface receptors, we were surprised to find that EGFR levels were dramatically elevated in PS−/− cells (genetically deficient in both PS1 and PS2) compared with PS+/+ cells (Fig. 2A), despite a comparable level of Akt and ERK1/2 activation within a 1-h period of EGF exposure seen in our previous study (11). To ascertain that this difference in EGFR level is not an artifact of comparing two different cell lines, we stably transfected PS−/− cells with human PS1 (PS−/− (hPS1)) or PS2 (PS−/− (hPS2)). Indeed, stable transfection of PS1 but not PS2 into PS−/− cells markedly reduced EGFR to levels similar to that seen in PS+/+ cells (Fig. 2A), demonstrating that the loss of PS1 at least in part underlies this phenotype. Quantitations from multiple experiments (n = 7) showed that EGFR levels in PS−/− (hPS1) cells were dramatically reduced by ~66% from that of parental PS−/− cells (see Fig. 4, A and B). Similarly, in fibroblasts genetically deficient in PS1 alone (PS−/−), EGFR was also elevated compared with control PS1+/+ fibroblasts, and this was again corrected by stable transfection of PS1 (Fig. 2B).

The marked change in EGFR levels by the loss of PS1 may be attributable to increased EGFR mRNA levels, as we had found for PDGFR α and β (11), or due to turnover of the EGFR protein. To test for the former possibility, we conducted quantitative reverse transcriptase-PCR analysis within an empirically determined linear range of amplification from equal amounts total RNA. PS−/− cells contained ~50% higher EGFR mRNA levels compared with PS+/+ cells (Fig. 2C). However, this difference is likely attributable to arbitrary non-PS1-related differ-
ences between the two cell lines, because we could not detect significant changes in EGFR mRNA levels between parental PS−/− cells and PS−/− cells stably transfected with human wild-type PS1 or PS2 in three separate experiments (Fig. 2 C). Thus, the marked accumulation and reduction of EGFR protein in PS−/− and PS−/− (hPS1) cells, respectively, cannot be attributed to the abundance of EGFR mRNA. To determine whether the turnover of EGFR protein is altered by PS1, we used cycloheximide (CHX, 50 μg/ml) to inhibit protein synthesis for increasing time intervals and monitored EGFR protein levels. In previous studies, EGFR without ligand activation, was shown to be a long-lived protein with a half-life of ~10 h (22). In PS−/− cells rescued with PS1, turnover of EGFR was detected starting from 3 h and progressed rapidly over a 9- to 24-h period (Fig. 3, A and B), with a half-life within the range of ~10 h as previously seen in other cell lines (Fig. 3B). In contrast, the turnover of EGFR was virtually absent over a 9- to 24-h period in parental PS−/− cells (Fig. 3, A and B), with a half-life of EGFR not measurable within the 24-h range of CHX chase experiment. Quantitations of EGFR levels after a 24-h CHX treatment from three experiments showed that levels of EGFR relative to no CHX

**FIGURE 2.** Elevated EGFR protein but not mRNA by the loss of PS1 and correction by PS1 but not PS2. A, confluent overnight cultures were lysed, and equal protein amounts were subjected to immunoblotting for EGFR, PS1, and PS2. A representative experiment is shown. B, cells were lysed, and equal protein amounts were subjected to immunoblotting for EGFR and PS1. A representative experiment is shown. C, total RNA was isolated from overnight confluent cultures, and equal amounts of RNA were subjected to quantitative reverse transcriptase-PCR within a linear range of amplification. Experiments were conducted three times, and graphs show means ± S.E. values normalized to PS−/− cells.

**FIGURE 3.** Turnover of EGFR is dramatically slowed in PS−/− cells and is restored by reconstitution of PS1. A, confluent overnight cultures were treated with 50 μg/ml CHX for the indicated times and lysed, and equal protein amounts were subjected to immunoblotting for EGFR. Exposure of EGFR was adjusted to reflect similar amounts at time 0 in both PS−/− and PS−/− (hPS1) cells. A representative experiment is shown. B, the graph shows another representative experiment treated with cycloheximide (50 μg/ml) for the indicated times. Percent EGFR remaining represents the amount of EGFR remaining at each time point of CHX treatment as a percentage of EGFR without CHX treatment (time 0). C, graph shows multiple experiments (n = 3) with cells treated with or without cycloheximide (50 μg/ml) for 24 h. Percent EGFR remaining represents the amount of EGFR remaining after 24 h of CHX treatment as a percentage of EGFR without CHX treatment (time 0). Error bars represent ± S.E.
treatment in PS/−/− and PS/−/− (hPS1) cells were 73 and 15%, respectively (Fig. 3C), demonstrating a defect in the turnover of EGFR by PS1 deficiency at steady-state.

PS2 and FAD PS1 Mutations Fail to Fully Correct Steady-state EGFR Levels: Role of PS1 CTF—PS1 and PS2 exert both unique and additive functions that may contribute to embryonic development and adult physiology. Taking advantage of the observation that PS2 did not contribute to the reduction in EGFR (Fig. 2, A and C), we next utilized PS1/PS2 chimeras to determine which region of PS1 is sufficient to rescue this phenotype. The PS1N-PS2C chimera in which PS1 NTF (residues 1–280) was fused to PS2 CTF (residues 287–448) completely failed to reduce EGFR levels (Fig. 4A). In contrast, the PS2N-PS1C chimera in which PS2 NTF (residues 1–286) was fused to PS1 CTF (residues 281–467) reduced steady-state EGFR levels as effectively as wild-type PS1 (Fig. 4A). Thus, the CTF of PS1 appears to be sufficient to reduce EGFR levels. PS1 FAD mutations cause the most aggressive form of AD, at least in part, by increasing the relative amount of biotinylated cell-surface EGFR and amyloid deposition. It has been proposed that other aspects of PS1 dysfunction associated with PS1 FAD mutations also contribute to AD pathogenesis. Indeed, two FAD mutations tested (ΔX9 and A246E) largely failed to correct EGFR levels compared with wild-type PS1 (Fig. 4B), suggesting implications of these mutations in EGFR turnover and trafficking in AD. Specifically the PS1 A246E only partially (by ~30%) reduced EGFR levels, whereas the PS1 ΔX9 mutations completely failed to reduce EGFR levels in PS/−/− cells (Fig. 4B). Interestingly, the steady-state level of total EGFR was in direct proportion with cell surface EGFR in all of the PS1 variant cell lines (including FAD PS1 mutations) tested, indicating that surface EGFR is elevated by nearly 5-fold in PS/−/− cells (hPS1 A246E) cells). Quantitations from PS/−/− and PS/−/− (hPS1) cells (n = 3 each) showed that surface EGFR was elevated by nearly 5-fold in PS/−/− cells (Fig. 4C, upper panel), which was directly proportional to the elevation in total EGFR in PS/−/− cells seen from these biotinylation experiments (Fig. 4C, lower panel).

To test whether γ-secretase activity of PS1 might be involved in the regulation of EGFR, we next tested the PS1 D257A mutation, which lacks γ-secretase activity presumably solely via a catalytic enzymatic defect (23). Interestingly, expression of PS1 D257A mutation in PS/−/− cells completely failed to reduce the steady-state level of EGFR (Fig. 4B), suggesting that catalytic γ-secretase activity may be involved in regulating EGFR levels. To further test this hypothesis, we treated PS/−/−, PS/−/−, and PS/−/− (hPS1) cells with two potent γ-secretase inhibitors,
brane proteolysis, we treated it itself might be a substrate for presenilin-dependent intramembrane proteolysis. As the catalytic function of the PS1-mediated regulation of EGFR is likely not solely dependent on the catalytic γ-secretase activity of PS1 as measured by APP CTF accumulation, but the CTF of PS1 is necessary and sufficient to regulate EGFR turnover. Moreover, FAD PS1 mutations (AX9 and A246E) likely represent partial loss of function mutations with regards to EGFR regulation.

Prolonged EGF-induced Akt and ERK1/2 Activation and Delayed Ligand-induced EGF Degradation by Presenilin 1 Deficiency—In our previous study, we showed that loss of presenilins reduces the activation of Akt and ERK1/2 by PDGF-BB or PDGF-AA. The reduction in responsiveness to PDGF was due to a decrease in PDGF receptors at the level of mRNA, which was restored by PS2 but not PS1 (11). We also showed that EGF-induced Akt and ERK1/2 phosphorylation was relatively unaltered in PS−/− cells up to 1 h of ligand exposure, which we interpreted to be normal (11). Because we found a marked elevation in EGFR in PS−/− cells in this study, which was reversed by PS1, we assessed whether Akt and ERK1/2 signaling might instead be prolonged beyond the 1-h period of EGF exposure. In PS−/− (hPS1) cells, exposure to 30 ng/ml EGF induced a peak in Akt and ERK phosphorylation within 15–30 min and gradually declined over a 4-h EGF induction period (Fig. 6A). In contrast, EGF exposure to PS−/− parental cells markedly prolonged Akt and ERK1/2 phosphorylation such that substantial activation was seen up to 4 h of EGF exposure (Fig. 6A). Thus, PS1 deficiency appears to preferentially prolong the period of active signaling.

Whereas the unoccupied EGFR is slowly degraded with a half-life of ~10 h, the binding of EGF to EGFR normally induces its rapid dimerization and degradation in lysosomes with a half-life of ~1 h (~10-fold acceleration of turnover) (22). Because we observed prolonged EGF-induced Akt and ERK1/2 phosphorylation, we next examined whether ligand-induced degradation of EGFR was affected by PS1 deficiency. Upon exposure to EGF (30 ng/ml), turnover of EGFR was rapid in PS−/− (hPS1) cells, with substantial decay apparent within 1 h (Fig. 6B). In contrast, EGF-induced degradation of EGFR was largely lost in the parental PS−/− cells up to 3 h (Fig. 6B), consistent with our finding that EGF-induced Akt and ERK1/2 activation was also prolonged by PS1 deficiency.

As PS1/PS2 chimeras and PS1 variants showed differential effects on steady-state levels of EGFR, we also examined whether EGF-induced turnover of EGFR is also altered by these presenilin variants. As expected, those mutants that showed elevated steady-state levels of EGFR also demonstrated slowed EGF-induced EGFR degradation. Specifically, the PS1N-PS2C chimera, which failed to reduce EGF levels at steady state, also failed to correct EGF-induced turnover of EGFR compared with wild-type PS1 (Fig. 6B). Likewise, the PS2N-PS1C chimera, which reduced the steady-state level of EGFR, also demonstrated rapid EGF-induced degradation of EGFR, similar to that seen in wild-type PS1 (Fig. 6B). Thus, it appears that the steady-state level of EGFR reflects the ligand-induced degradation of EGFR, the latter however occurring at least an order of magnitude faster than the unoccupied EGFR.

Presenilin 1 Does Not Alter the Ligand-induced Autophosphorylation, Ubiquitination, and Internalization of EGFR—Following ligand binding and dimerization of EGFR on the cell surface, EGFR undergoes rapid autophosphorylation of its cytoplasmic tail via intrinsic tyrosine kinase activity. This tyrosine phosphorylation is not only required for the recruitment of signaling adaptor proteins such as Grb2 and Shc, but also for the accelerated turnover of EGFR upon ligand exposure (24). Thus, we examined whether PS1 affects the autophosphorylation of EGFR after treatment with EGF for varying time points. Similar to that seen with Akt and ERK1/2 activation, we observed no defect in tyrosine phosphorylation EGFR but rather increased initial EGFR phosphorylation followed by slower dephosphorylation over a 1-h period in PS−/− cells compared with PS−/− (hPS1) cells (Fig. 6C). This also correlated with EGF-induced degradation of total EGFR, suggesting that reduced ligand-induced EGFR turnover accounts for the
intracellular 125I-EGF divided by total cellular 125I-EGF. Specific 125I-EGF binding was calculated by subtracting nonspecific surface 125I-EGF binding from calculated by dividing trichloroacetic acid-soluble counts in the medium divided by total counts in the lysate. Specific 125I-EGF binding was calculated by 1 N NaOH, and used as a measure of internalized 125I-EGF.

performed in triplicate.

prolonged P-Akt and P-ERK1/2 activation in times. Equal protein amounts were subjected to immunoblotting for phospho-Akt (P-Akt), phospho-ERK1/2 (P-ERK1/2), total Akt, and total ERK1/2. Note the prolonged P-Akt and P-ERK1/2 activation in PS/−/− cells. A representative experiment is shown. B, confluent overnight cultures were serum-starved for 4 h in DMEM, and EGF (30 ng/ml) was added for the indicated times. Equal protein amounts were subjected to immunoblotting for EGFR. Note that cells lacking PS1 CTF do not promote EGF-induced degradation of EGFR. EGF exposure was adjusted to show similar amounts at time 0. A representative experiment is shown. C, confluent overnight cultures were serum-starved for 4 h in DMEM, and EGF (30 ng/ml) was added for the indicated times. Equal protein amounts were subjected to immunoblotting for phospho-EGFR. Note the initial increase in EGFR phosphorylation followed by a delay in EGFR dephosphorylation in PS/−/− cells compared with PS/−/− (hPS1) cells. A representative experiment is shown. D, confluent overnight cultures were serum-starved for 4 h in DMEM, and EGF (100 ng/ml) was added for the indicated times. Equal protein amounts were subjected to immunoprecipitation for EGFR and immunoblotting for ubiquitin or EGFR. Note that ligand-induced ubiquitination of EGFR is intact and comparable between PS/−/− and PS/−/− (hPS1) cells. E, confluent cells were incubated with 125I-EGF (1.5 ng/ml) for 1–5 min at 37 °C. At the indicated times, one set of cells was washed three times with ice-cold Hanks' balanced salt solution on ice, lysed in 1 n NaOH, and used to determine total cellular 125I-EGF. In a replicate set of cells, cell surface-bound 125I-EGF was removed by two rapid acids on ice, lysed with 1 n NaOH, and used as a measure of internalized 125I-EGF. Graph shows percent 125I-EGF internalization, which was calculated by γ-radiation measured from intracellular 125I-EGF divided by total cellular 125I-EGF. Specific 125I-EGF binding was calculated by subtracting nonspecific surface 125I-EGF binding from controls for each condition co-incubated with 200-fold excess unlabeled EGF. Experiments were performed in triplicate. Error bars represent standard deviation.

reduced decay of phospho-EGFR (Fig. 6B). Thus, perturbations in tyrosine autophosphorylation of EGFR do not account for the slowed rate of ligand-induced EGFR turnover by the loss of PS1.

Secondary to autophosphorylation, EGFR also undergoes ubiquitination via the recruitment of Cbl family of ubiquitin ligases, a process important for targeting EGFR to lysosomes for degradation (24, 25). Thus, we tested whether defects in ubiquitination of EGFR might account for the slowed turnover of EGFR after ligand exposure in PS/−/− cells. Cells were treated with or without EGF for the indicated times, and lysates were subjected to immunoprecipitation of EGFR and detection of ubiquitin conjugates. In the absence of EGF stimulation, no ubiquitinated EGFR could be detected in either PS/−/− or PS/−/− (hPS1) cells (Fig. 6D). After a 5-min EGF exposure, robust ubiquitination of EGFR was seen in both PS/−/− and PS/−/− (hPS1) cells, which quickly diminished (i.e. deubiquitinated) within 15 min in both cell lines (Fig. 6D). Thus, these results demonstrate that the slowed rate of ligand-induced EGFR degradation in PS/−/− cells is not due to a defect in the ubiquitination of EGFR.

Endocytosis of surface EGFR after ligand exposure occurs within minutes and represents a key step in the eventual degradation of EGFR in lysosomes (26). To determine whether defects in the endocytosis of EGFR might underlie its delayed
turnover by the loss of PS1, we next conducted EGFR internalization experiments using 125I-EGF. Because ligand-induced internalization of EGFR is extremely rapid, we examined the ratio of internalized versus total 125I-EGF after 1 and 5 min of radiolabeled ligand exposure at 37 °C. Percent-internalized EGFR was calculated by subtracting nonspecific 125I-EGF counts (after stripping surface label with acid washes) by total 125I-EGF counts after 1 and 5 min, respectively (Fig. 6E). Thus, the delayed degradation of EGFR by PS1 deficiency cannot be attributed to defects in EGFR internalization.

Loss of Presenilin 1 Impairs the Degradation of 125I-EGF and Trafficking of EGF Receptor from Early Endosomes to Lysosomes—The amount of secreted trichloroacetic acid-soluble 125I-EGF is a reliable and quantitative surrogate measure of EGFR degradation via the endosomal-lysosomal pathway (27). Thus, we next treated cells with 125I-EGF at 37 °C for 10 min to allow for surface binding and internalization (25). The excess unbound material was extensively washed and replaced with serum-free DMEM for 1.5 and 3 h to monitor the appearance of trichloroacetic acid-soluble 125I-EGF in the medium and remaining total counts in the lysate. After 1.5 h in PS/−/− (hPS1) cells, trichloroacetic acid-soluble counts in the media were 1.84 times those in lysates (Fig. 6F). During the same period in PS/−/− cells, trichloroacetic acid-soluble counts were only 48% of those remaining in lysates (Fig. 5F), clearly showing an impairment in the degradation of 125I-EGF by the loss of PS1. After 3 h, the difference in the ratio of trichloroacetic acid-soluble versus total lysate counts was even further widened with 7.99 and 1.31 in PS/−/− (hPS1) and PS/−/− cells, respectively (Fig. 6F). These data conclusively demonstrate that PS1 promotes

the ligand-induced degradation of EGFR following its internalization, consistent with our immunoblot analysis (Fig. 6B).

Upon internalization from the cell surface, EGFR is initially delivered to early endosomes. At this point, the default pathway is to recycle EGFR back to the cell surface. However, upon ligand exposure, EGFR is instead sorted to late endosomes/multivesicular bodies that fuse with lysosomes, resulting in the rapid degradation of EGFR (26, 28). Having quantitatively shown that the loss of PS1 impairs 125I-EGF degradation, we next examined whether the sorting of EGFR from early endosomes to lysosomes is delayed in PS/−/− cells. If so, this site could account for the accumulation of EGFR by PS1 deficiency. Endocytosis of ligand-occupied EGFR is such that the majority of EGFR is localized to endosomes after 10 min of ligand exposure (25). Thus, we treated cells with Alexa488-labeled EGF for 10 min to permit internalization to endosomes, washed out the unbound ligand, and monitored the trafficking of Alexa488-EGF to lysosomes for 0, 1, or 2 h with LysoTracker Red, a lysosome marker. After the initial exposure to ligand for 10 min, Alexa488-EGF was seen in a punctate vesicular pattern in both perinuclear locations and peripheral cell edges but did not colocalize with LysoTracker Red in either PS/−/− and PS/−/− (hPS1) cells as expected (Fig. 7). The lysosome marker, LysoTracker Red, showed primarily perinuclear staining in both cell lines (Fig. 7). After 1 h of chase, PS/−/− cells continued to show punctate EGF localization throughout the cell but showed little or no colocalization with LysoTracker Red (Fig. 7). At the same time (1 h), cell periphery localization of EGF signal was absent in PS/−/− (hPS1) cells and began to show occasional colocalization with lysosomes (Fig. 7). After 2 h of chase, there was frequent colocalization of Alexa488-EGF with LysoTracker in PS/−/− (hPS1) cells (Fig. 7). In contrast, although the Alexa-EGF signal showed a largely perinuclear pattern in PS/−/− cells at this time, there was still little to no localization of EGFR in lysosomes even after 2 h of chase (Fig. 7). Together with the preceding data demonstrating that EGFR autophosphorylation, ubiquitination, and internalization are not affected by PS1 deficiency, we conclude that the degradation of EGFR by the loss of PS1 is delayed at a step between the trafficking of EGFR from endosomes to lysosomes.

Accumulation of EGF Receptor in Brains of PS1-conditional Knockout Mice and Skin Hyperplasias/Tumors Associated with PS1 Deficiency—Our findings thus far showed that the loss of PS1 results in the accumulation of EGFR by a mechanism involving the delayed trafficking of EGFR to lysosomes for degradation without altering the autophosphorylation, ubiquitination, and internalization of the receptor. These observations were all made in immortalized cultured cells genetically deficient in PS1. To determine whether these findings could be confirmed in vivo, we examined brains of 1-year-old mice that were conditionally ablated for PS1 by Cre recombinase driven by the α-CKII promoter in a conventional PS2 knock-out background (cPS1-flox; PS2−/−) compared with PS2 knock-out alone (cPS1; PS2−/−) (29, 30). As expected, Cre-mediated ablation of PS1 led to a strong accumulation of APP CTFs (Fig. 8A). As seen in cultured fibroblasts, EGFR levels were markedly elevated in both hippocampi and frontal cortices of mice conditionally lacking PS1 compared with control littermates (Fig. 8, PS1 in EGFR Turnover and Signaling
^levels were increased by an average EGFR levels compared with controls (not shown). Quantita-
cPS1; PS2 in a conventional PS2 knock-out background (PS1^null). Thus, the loss of those in skin (31). The preceding results showed that key event in many human neoplastic malignancies, including not only in cultured cells but also in adult brain, the latter that (Fig. 8)

FIGURE 8. Accumulation of EGFR in brains of conditional PS1 knock-out mice and in skin hyperplasias and tumors associated with PS1 deficiency. A, equal amounts of brain lysates from frontal cortices and hippocampi of 1-year-old mice conditionally ablated for PS1 by Cre recombinase driven by the ß-CRE II promoter in a conventional PS2 knock-out background (PS1^+/--; cPS1-floxed; PS2^+/--) and PS2 knockout alone (Cont = cPS1; PS2^−/−; Cre negative) (29, 30) were immunoblotted for EGFR, ß-tubulin, and APP-CTFs. Note the marked elevation in EGFR in brains of PS1^+/-- mice. B, graph represents quantitations of EGFR in the hippocampus and frontal cortex in PS1-floxed mice (n = 5) compared with control Cre negative littermates (n = 3). Error bars represent ±S.E. C, paraffin sections were immunostained using the Vectastain ABC Elite Kit with rabbit polyclonal anti-EGFR (1005; sc-03) antibody, incubated with secondary antibody, washed, incubated in the ABC complex, stained with diaminobenzidine (brown), counterstained with hematoxylin (blue), and mounted in VectaMount (original magnification, 60×). The specificity of the immunoreaction was verified by the lack of immunoreactivity when stained with primary antibody preabsorbed with the blocking peptide or secondary antibody alone (supplemental Fig. S3). Note the salient increase in EGFR staining in hyperplasia and tumor associated with the loss of PS1. Brackets indicate the epidermal layer of the skin.

A and B). Whole brain extracts also showed a similar increase in EGFR levels compared with controls (not shown). Quantitations in the hippocampus and frontal cortex showed that EGFR levels were increased by an average ~3-fold in PS1^floxed mice (n = 5) compared with control Cre negative littermates (n = 3) (Fig. 8B). PS2 deletion alone had no effect on brain EGFR levels (not shown). Thus, the loss of PS1 leads to elevated EGFR levels not only in cultured cells but also in adult brain, the latter that may have important consequences for neuronal physiology.

It is well established that abnormal activation of EGFR is a key event in many human neoplastic malignancies, including those in skin (31). The preceding results showed that PS1 deficiency results in the accumulation of EGFR both in cultured cells and in brain. In this context, our previous findings that PS1-deficient mice rescued with human PS1 driven by the neuron-specific Thy-1 promoter (hPS1 rescued mice) develop epidermal neoplasms take on greater importance (32). Although PS1 null mice are embryonically lethal, hPS1-rescued mice are viable and survive into adulthood. However, as the Thy-1 promoter is neuron-specific, hPS1-rescued mice lack PS1 in skin and develop epidermal hyperplasias and carcinomas with over 90% penetrance in animals older than 9 months of age (32). Thus, we next looked for evidence of EGFR accumulation in normal and abnormal epidermis from hPS1-rescued mice by immunohistochemistry. Within neoplastic and hyperplastic epidermis from hPS1-rescued mice, the intensity of EGFR staining was profoundly increased compared with that of wild-type adult skin (Fig. 8C). Furthermore, EGFR immunoreactivity was still markedly increased in hPS1-rescued epidermis lacking evidence of hyperplasia compared with wild-type adult mouse epidermis, although not as intense as hyperplastic or neoplastic tissues (not shown). Pre-absorption of the primary antibody with the peptide immunogen nearly depleted all of the EGFR staining (supplemental Fig. S3). Because overexpression of EGFR is a key feature of multiple carcinomas (33, 34), these findings suggest that elevated EGFR levels in hPS1-rescued epidermis significantly contributes to skin hyperplasia and neoplastic transformation.

DISCUSSION

Because mutations in PS1 cause the most aggressive form of AD, we and others have proposed that partial loss of activities in PS1 contribute to the unusually aggressive disease phenotype in addition to the increase in Aβ42 levels. Indeed, recent studies have implicated important roles of PS1 in ß-catenin turnover and signaling, regulation of Akt and ERK signaling, and trafficking of various membrane and membrane-associated proteins (7–12, 14–16). This way, PS1 may function as a hub of intracellular signaling and trafficking pathways that impinge on AD pathogenesis, and perturbations in these pathways may further potentiate the deleterious effects of Aβ42 accumulation. In this study, we made a number of novel observations using cultured cells and animal models regarding a physiological activity of PS1 in the regulation of EGFR that may not only impinge on AD but also in cell cycle control and tumorigenesis. Specifically, we found that EGFR levels were highly elevated in PS^−/− cells, a phenotype that was corrected by stable transfection of wild-type PS1 but not PS2. The increase in EGFR level by the loss of PS1 could be attributed to the reduced turnover of the EGFR protein but not the abundance of its mRNA. The C-terminal fragment of PS1 was sufficient to correct EGFR levels, but ΔX9 and A246E FAD PS1 mutations showed at least partial loss of activity in this phenotype. The stability of EGFR by the loss of PS1 was also seen after exposure to EGF and led to prolonged signal activation. The defect in EGFR turnover could not be attributed
to tyrosine autophosphorylation, ubiquitination, or endocytosis of EGFR, but its delayed trafficking from endosomes to lysosomes, as measured by $^{125}$I-EGF degradation and fluorescent EGF tracking experiments. Finally, we demonstrated strong in vitro evidence that conditional ablation of PS1 in brain and constitutive PS1 deficiency in skin also leads to robust elevation of EGFR, lending to the notion that the regulation of EGFR turnover by PS1 results in physiologically important consequences in vivo.

While our manuscript was under review, Zhang and colleagues (35) reported a similar finding that EGFR was markedly elevated by the loss of PS1 in cultured cells and in vivo. However, they attributed the elevated EGFR protein in PS$^{-/-}$ cells to an increase in EGFR mRNA when compared with PS WT mouse embryo fibroblasts. At the same time, the EGFR mRNA comparison was not made to PS$^{-/-}$ cells reconstituted with PS1 (35). Indeed, we also found an increase in EGFR mRNA in PS$^{-/-}$ cells compared with PS$^{+/+}$ cells, but this difference could not be restored in PS$^{-/-}$ cells when reconstituted with either PS1 or PS2. Thus, we interpreted the increase in EGFR mRNA in PS$^{-/-}$ cells compared with PS$^{+/+}$ cells to be presenilin-unrelated differences between two cell lines. Furthermore, Zhang and colleagues reported that in $[^{35}$S]methionine pulse-chase experiments, EGFR turnover was largely not affected by reconstitution of PS1 in PS$^{-/-}$ cells (35). However, closer examination of their data shows that EGFR decayed from a relative value of 1 to ~0.3 (30% remaining) in a 20-h period of chase in PS$^{-/-}$ cells, whereas EGFR decayed from a relative value of ~0.45 to ~0.05 (11% remaining) in PS$^{-/-}$ (PS1) cells during the same period (35). This represents markedly faster EGFR turnover (~2.7-fold) over a 20-h period by reconstitution with PS1. Given that EGFR has a reported half-life of >10 h in a ligand-unoccupied state (22), the comparison of EGFR turnover after a 20-h chase period seems reasonable. Thus, we interpret our EGFR turnover results in the ligand-unoccupied state to be consistent with those presented by Zhang and colleagues (35). Interestingly, Zhang and colleagues also proposed a mechanism of action in which the loss of $\gamma$-secretase activity via inhibition of AICD generation elevates EGFR. This in turn blocks the inhibitory role of AICD on EGFR expression via binding to the EGFR promoter (35). As such, they reported that in APP/APLP2 double knock-out cells, EGFR was elevated and could be suppressed by exogenous expression of APP (35).

In our hands, however, we found no evidence that reconstitution of APP695 in APP/APLP2 double knock-out fibroblasts reduced EGFR levels (supplemental Fig. S2). Although APP intracellular domain and Fe65 may suppress the transcriptional expression of EGFR under certain conditions, it has also been reported by Almeida and colleagues (36) that EGFR levels are increased in primary neurons and brains of TG2576 APP transgenic mice. Although Zhang and colleagues reported that the $\gamma$-secretase inhibitor L-685,458 increased EGFR levels (35), we found no evidence that two other $\gamma$-secretase inhibitors, DAPT and LY-411575, had any effect on EGFR levels under condition in which APP CTFs were strongly elevated. Thus, it appears that the regulation of EGFR by PS1 is not simply a function of inhibiting $\gamma$-secretase activity. Taken together, we do not favor the interpretation that AICD plays a major role in the steady-state or ligand-induced regulation of EGFR level. Although Zhang and colleagues did not address the issue of ligand-induced EGFR turnover in their study, our data clearly demonstrated that PS1 promotes both the steady-state and ligand-induced turnover of EGFR. It remains to be seen the extent to which $\gamma$-secretase activity per se and AICD may affect the transcriptional regulation of EGFR, despite the lack of support for this model in this study.

Role of PS1 in EGFR Trafficking and Degradation—EGFR/ErbB1 is a member of the ErbB family of receptor tyrosine kinases that also include ErbB2, ErbB3, and ErbB4 (22). EGFR is one of the most studied receptor tyrosine kinases to date, and its signaling, trafficking, and degradation both in an unoccupied state and ligand-activated state are now generally well understood. Without ligand exposure, the majority of EGFR is present on the cell surface and is endocytosed at a leisurely rate with $t_{1/2}$ of ~20–30 min but is rapidly recycled to the cell surface as a default pathway (22, 26). During this period, a small fraction of EGFR is sorted to late endosomes/multivesicular bodies for fusion with lysosomes for degradation, resulting in a relatively long half-life of EGFR (~10–14 h) at steady state (22, 37). Thus, even small changes in the number of EGFR receptors sorted to lysosomes could lead to a relatively large overall change in EGFR protein. In the ligand-occupied state, EGFR undergoes rapid dimerization and tyrosine autophosphorylation in its cytoplasmic tail. This leads to the rapid recruitment of signaling adaptor proteins, such as Shc and Grb2, as well as Cbl family of ubiquitin ligases that mediate the ubiquitination of EGFR (24, 25). This process accelerates the rate of EGFR endocytosis 5- to 10-fold, suppresses the recycling pathway, and accelerates the sorting to lysosomes, resulting in a similar hastening of EGFR degradation designed to prevent overactivation (22, 28).

In this study, the loss of PS1 resulted in an average ~3-fold accumulation of EGFR at steady state due to its delayed turnover. However, the proportion of EGFR present on the cell surface versus total EGFR was not altered. This suggests that the constitutive EGFR internalization and recycling pathways are primarily not affected by PS1, but rather the fraction of EGFR that is sorted to lysosomes. Given that the majority of EGFRs are present on the cell surface, and for every ~10 EGFRs recycled to the cell surface after internalization, only 1 is degraded in lysosomes (22), only minor changes in EGFR sorting to lysosomes could lead to a 3-fold elevation in total EGFR.

In the ligand-occupied state, we found no evidence that the tyrosine autophosphorylation, ubiquitination, or internalization of EGFR was affected by the loss of PS1. This indicates that the kinase domain of EGFR, phosphatases that regulate EGFR signaling, Cbl family of ubiquitin ligases, and the entire array of machinery for EGFR internalization (i.e. clathrin-coated pits) are not significantly perturbed by the loss of PS1. However, ligand-induced degradation of EGFR was nevertheless delayed by PS1 deficiency, suggesting defects in EGFR degradation by retarding the trafficking of EGFR from endosomes to lysosomes. The impairment in the ligand-induced turnover of EGFR by the loss of PS1 was also robustly and quantitatively confirmed by $^{125}$I-EGF degradation experiments. By monitoring Alexa488-labeled EGF, we indeed confirmed that the trafficking of EGFR is markedly delayed at a point between endo-
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somes and lysosomes. This cellular phenotype is similar to that observed with mutations in a dileucine motif at position 679–680 of EGFR that favors recycling at the expense of lysosomal sorting, resulting in a defect in ligand-induced EGFR degrada-
tion without altering EGFR internalization (27, 38). Recently, it was noted that loss of PS1 results in the intracellular retention of exogenously applied transferrin (19), a phenotype that was attributed to defects in transferrin recycling to the cell surface. However, failure of EGFR to enter the recycling pathway would be predicted to alternatively favor the sorting to lysosomes by the loss of PS1, a phenotype that is exactly the opposite of what we observed. However, it remains possible that generalized sorting either to recycling or degradative pathways could be disrupted in PS−/− cells. In this model, EGFR trafficking may be compromised at the level of sorting endosomes. If so, it would be predicted that cell surface EGFR level would be reduced relative to total EGFR by the loss of PS1. However, this prediction was not met, because EGFR level on the cell surface relative to total EGFR was not affected by PS1.

We found that the γ-secretase-defective PS1 D257A mutation failed to correct EGFR levels compared with wild-type PS1. However, the PS1N-PS2C chimera that contains γ-secretase activity (21) also failed to reduce EGFR levels. Moreover, FAD PS1 mutations (A246E and ΔX9), which elevate Aβ42 but do not abrogate γ-secretase activity, also showed at least partial loss of activity in reducing EGFR levels. We found that two different γ-secretase inhibitors (DAPT and LY-411575) in PS+/+ and PS−/− (hPS1) cells failed to increase EGFR to mimic the PS null state. Finally, we did not find evidence that EGFR itself is a γ-secretase substrate. Taken together, these data indicate that γ-secretase activity of PS1 alone as measured by intramembrane proteolysis of APP cannot account for the reg-
ulation of EGFR turnover. However, we cannot rule out the possibility that γ-secretase activity on certain other substrates, which are more resistant to pharmacological γ-secretase inhibi-
tion, may contribute to EGFR regulation. Alternatively, the PS1 D257A mutation, like the FAD PS1 ΔX9 and A246E muta-
tions, may exhibit perturbations in the sorting of EGFR from endosomes to lysosomes independent of γ-secretase activity. Active PS1 fragments and nicastrin are normally localized to endosomes and lysosomes (40). Moreover, it has been reported that, in PS1-deficient neurons, telencephalin and synucleins accumulate in large degradative vesicles resembling autophagic vacuoles (15, 16), vesicles that normally fuse with multivesicu-
lar bodies and lysosomes for degradation. Thus, it is tempting to speculate that PS1 may be involved in regulating the fusion of certain subtypes of post-endosomal vesicles to lysosomal mem-
branes. Interestingly, Wang and colleagues recently docu-
mented that PS1 is involved in the maturation of melanosomes, endosomal-lysosomal-related organelles in melanocytes involved in pigmentation, and that the FAD PS1M146V muta-
tion exhibits partial loss-of-function in this process (41). Whether the defects in endosomal-lysosomal trafficking of EGFR seen in this study are mechanistically related to the defects in melanosome maturation by the loss of PS1 remains to be answered. It is also worth noting that lysosomal pathology is enhanced in human PS1 FAD cases and in transgenic mice car-
rying PS1 FAD mutations (42). Because the CTF of PS1 was required for reducing both ligand-unoccupied and occupied turnover of EGFR, further assessment of PS1 CTF will likely lead to critical clues to the precise molecular underpinnings of PS1-mediated EGFR turnover and potentially other membrane proteins normally delivered to lysosomes for degradation.

In this study, although we carefully examined total EGFR, surface EGFR, and subsequent post-translational modification, internalization, trafficking, and turnover of EGFR from the cell surface, we did not address several other aspects of EGFR reg-
ulation. As such, it should be noted that a potential role of PS1 in the biosynthesis, maturation, and/or trafficking of EGFR down the secretory pathway cannot be ruled out.

There is a growing list of membrane and membrane-associ-
ated proteins whose intracellular trafficking and turnover are affected by PS1. These include proteins that are substrates for γ-secretase activity (APP, cadherins, tyrosinase, ErbB4, and others) (17, 41, 43, 44) and those that are not γ-secretase sub-
strates (TrkB, telencephalin, α-synuclein, β-catenin, EGFR, and others) (8, 14–16). Although the precise manner in which PS1 regulates the trafficking and turnover of each of these pro-
teins may not be uniform, PS1 appears to function as a hub that modulates multiple and disparate signaling and trafficking events via both intramembrane proteolysis and other mecha-
nisms. Whether the role of PS1 in mediating the trafficking, turnover, and signaling of each of these proteins results in sig-
nificant physiological or pathological consequences is an important issue to be addressed.

In Vivo Implications of PS1 in EGFR Turnover and Signaling—It has been shown that mutations that enhance the stability, signaling, and/or expression of EGFR can all serve as oncogenic signals that promote hyperplasia and neoplastic transformation in human tissues, including skin (31, 33, 34). Thus, EGFR is a prime target for therapeutic intervention in multiple cancers by either blocking EGFR stimulation or its tyrosine kinase activity (33, 34). Thus, the observation that the loss of PS1 results in the stabilization of EGFR not only in cultured cell but also in tumors associated with chronic PS1 deficiency is intriguing. Importantly, we observed profound elevation in EGFR not only in tumors but also in hyperplastic skin compared with wild-type adult epidermis. Moreover, EGFR staining was still noticeably elevated in PS1-negative epidermis even without evidence of hyperplasia compared with wild-type adult skin (not shown). Thus, these findings strongly suggest that stabilization of EGFR by PS1 deficiency likely serves as a strong contributing factor in hyperplasia and neoplastic transformation in the epidermis of hPS1-rescued mice.

Previously, we had proposed that accumulation and activation of β-catenin signaling, in particular the S45 phosphoryla-
ted species, contribute to tumorigenesis in hPS1-rescued mice (8, 32). It is now evident that both PS1-associated pathways likely play contributing roles in this process. In the vast majority of tumorigenic phenotypes, multiple hits are required to pro-
duce malignant transformations. Moreover, it has been demonstrated that activation of β-catenin and EGFR pathways can reinforce each other. For example, activation of EGFR leads to tyrosine phosphorylation of β-catenin, which promotes its detachment from cell surface cadherin complexes and enhances nuclear localization (45, 46). Second, it was recently
reported that phosphorylation of β-catenin by Akt, an intermediate in EGFR signaling, promotes β-catenin transcriptional activity (47). Third, it has been shown that β-catenin nuclear signaling can up-regulate EGFR expression (48). Although, in our cultured cells, we observed no evidence for the regulation of EGFR mRNA by PS1 in cultured cells in this study, Zhang and colleagues presented evidence for the regulation of EGFR mRNA by AICD (35). Indeed, EGFR expression at the transcriptional level remains a distinct possibility in the oncogenic setting. Taken together, we hypothesize that activation of EGFR and β-catenin pathways by the loss of PS1 mutually reinforce each other and contribute to the tumorigenesis seen in the skin of hPS1-rescued mice.

EGFR knock-out mice have highlighted the role of EGFR in embryonic development, and in particular, development of the nervous system. EGFR null mice have smaller brains, abnormal astrocyte development, and excessive neuronal death, resulting in death between mid gestation and postnatal day 20 depending on the genetic background (49). Moreover, the role of EGFR in maintaining growth and subsequent differentiation of neural stem cells is well known. Indeed, we previously showed enhanced proliferation of neural progenitor cells in the hippocampus of mice overexpressing the PS1 A246E mutation compared with wild-type PS1 (50), which, in part, might be explained by elevated EGFR levels. However, there is little known about the role of EGFR signaling in mature neurons of adult brain. Recently, it was shown that EGFR activation inhibits its axon regeneration mediated by myelin and chondroitin sulfate proteoglycans in the adult mouse central nervous system (51). Some evidence suggests that EGF may play a neuroprotective role against excitotoxic and nitric oxide-mediated neuronal injuries (52, 53). However, Cha and colleagues (54) also demonstrated that prolonged exposure to EGF induces oxidative neuronal death that could be blocked by inhibitors of EGFR tyrosine kinase activity and ERK1/2.

Importantly, it was reported that the accumulation of Aβ impairs multivesicular body sorting of EGFR, leading to its robust elevation in both primary neurons and in brains of Tg2576 APP transgenic mice (36). Indeed, EGFR immunoreactivity was previously shown to be strongly increased in neurites surrounding neuritic plaques in AD (55). In this context, the observation that postmitotic neurons in AD brains and transgenic mice with amyloid accumulation aberrantly demonstrate markers of cell cycle activation is intriguing (56–58). This neuronal cell cycle re-entry phenotype is associated with ectopic expression of various cyclins, cyclin-dependent kinases, and at times chromosomal replication (56–58). However, postmitotic neurons fail to undergo mitosis, and experimental evidence shows that such neurons become dysfunctional and eventually degenerate (59, 60). We hypothesize that the stabilization of EGFR by both PS1 FAD mutations and Aβ accumulation promotes aberrant cell cycle re-entry of postmitotic neurons and render them more vulnerable for neurodegeneration. In addition, we previously showed that FAD PS1 mutations may contribute to neurodegeneration and aberrant cell cycle re-entry by stabilizing both EGFR and β-catenin while simultaneously driving Aβ42 deposition. Similar mechanisms involving EGFR, β-catenin, and Aβ may also contribute to neurodegeneration in sporadic AD.

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