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| Creators | Watanabe, Hirotaka, Smith, Miriam J., Heilig, Elizabeth, Beglopoulos, Vassilios, Kelleher, Raymond J. and Shen, Jie                                                                                                                                                      |

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# Indirect Regulation of Presenilins in CREB-mediated Transcription\*

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Hirotaka Watanabe<sup>‡</sup>, Miriam J. Smith<sup>‡</sup>, Elizabeth Heilig<sup>§</sup>, Vassilios Beglopoulos<sup>‡</sup>, Raymond J. Kelleher III<sup>§</sup>, and Jie Shen<sup>‡1</sup>

From the <sup>‡</sup>Center for Neurologic Diseases, Brigham & Women's Hospital, and <sup>§</sup>Center for Human Genetic Research, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02115

Presenilins are essential for synaptic function, memory formation, and neuronal survival. Previously, we reported that expression of cAMP response element-binding protein (CREB) target genes is reduced in the cerebral cortex of presenilin (PS) conditional double knock-out (cDKO) mice. To determine whether the reduced expression of the CREB target genes in these mutant mice is due to loss of presenilin directly or secondary to the impaired neuronal activity, we established a sensitive luciferase reporter system to assess direct transcriptional regulation in cultured cells. We first used immortalized PS-deficient mouse embryonic fibroblasts (MEFs), and found that both CREB-mediated transcription and Notch-mediated *HES1* transcription are decreased. However, the *ubiquitin-C* promoter-mediated transcription is also reduced, and among these three reporters, transfection of exogenous PS1 can rescue only the Notch-mediated *HES1* transcription. Further Northern analysis revealed transcriptional alterations of *Creb*, *ubiquitin-C*, and other housekeeping genes in PS-deficient MEFs, indicating transcriptional dysregulation in these cells. We then used the Cre/loxP system to develop a postnatal PS-deficient cortical neuronal culture. Surprisingly, in these PS-null neurons, CREB-mediated transcription is not significantly decreased, and levels of total and phosphorylated CREB proteins are unchanged as well. Notch-mediated *HES1* transcription is markedly reduced, and this reduction can be rescued by exogenous PS1. Together, our findings suggest that CREB-mediated transcription is regulated indirectly by PS in the adult cerebral cortex, and that attenuation of CREB target gene expression in PS cDKO mice is likely due to reduced neuronal activity in these mutant brains.

Over 150 mutations in the presenilin-1 (*PS1*)<sup>2</sup> gene have been identified in patients with early-onset familial Alzheimer disease (1). PS1 and its related paralogue, PS2, are membrane proteins with 9 transmembrane domains (2, 3). PS1 and PS2 are

broadly expressed and are present at high levels in the cerebral cortex, consistent with the involvement of its dysfunction in Alzheimer disease. PS is a critical component of the  $\gamma$ -secretase complex, which also includes other cofactors, nicastrin, Aph-1, and Pen-2 (4).

*PS1*-null mutant mice showed perinatal lethality and severe skeletal and neural developmental defects (5, 6), whereas PS2 deficiency results in no detectable phenotypes (7, 8). To investigate the physiological role of PS in the adult cerebral cortex, we previously generated viable PS conditional double knock-out (cDKO) mice, in which inactivation of PS is restricted to the postnatal forebrain. These PS cDKO mice initially exhibit synaptic impairment and later develop progressive neuronal degeneration (9). One of the molecular alterations we consistently identified in these mutant mice was reduced expression of the CREB target genes, including *c-fos*, *BDNF*, *egr-1*, and the CREB-binding protein (*CBP*) gene. The presence of a putative RBP-J $\kappa$  binding site in the *CBP* promoter region raised the possibility that PS may regulate transcription of CREB target genes through  $\gamma$ -secretase cleavage of Notch, which relieves transcriptional suppression of the *CBP* gene by RBP-J $\kappa$ .

CREB binds to the cAMP response element (CRE) in the promoter region of its target genes via a conserved basic leucine zipper motif (10). In response to a large array of physiological stimuli, CREB is rapidly phosphorylated at Ser-133 by several protein kinases, including PKA (11). Phosphorylated CREB then recruits several co-activator proteins, such as CBP, to the promoter regions of its target genes. As CBP has a specific activity of histone acyltransferase, it can change the chromatin state of the target genes into more relaxed ones, leading to initiation of transcription together with basic transcription factors, such as TAFII130 (11).

Among wide varieties of CREB functions, its activation in particular plays an essential role in neuronal cells, in which CREB target genes modulate neuronal survival, memory formation, synaptic refinement, and circadian rhythm (12, 13). Because long-term memory requires both gene transcription and protein synthesis, activation of CREB has been long considered as a critical molecular basis of learning and memory. Indeed, a vast array of its target genes has been reported to be induced in hippocampal slices following acute LTP induction, or in mouse brains after training for long-term memory formation (14, 15). Furthermore, several loss-of-function and gain-of-function studies of CREB in mice have provided additional experimental evidence for a crucial role of CREB in formation of several forms of memory (16, 17). But the relation between

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<sup>1</sup> To whom correspondence should be addressed: Harvard New Research Bldg., Rm. 636E, 77 Ave. Louis Pasteur, Boston, MA 02115. Fax: 617-525-5522; E-mail: jshen@rics.bwh.harvard.edu.

<sup>2</sup> The abbreviations used are: PS, presenilin; CREB, cAMP response element-binding protein; CBP, CREB-binding protein; CRE, cAMP response element; MEF, mouse embryonic fibroblast; cDKO, conditional double knock-out; Q-RT-PCR, quantitative reverse transcription PCR; PKA, cAMP-dependent protein kinase; DIV, days *in vitro*; Erk, extracellular signal-regulated kinase; WT, wild type; GFP, green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

PS function and CREB-dependent transcription remains elusive.

Here, we employed a highly sensitive luciferase reporter system to assess presenilin-dependent gene expression using both a widely used immortalized MEF cell line and our newly developed postnatal cortical neuronal cultures. Our findings indicate that the primary cortical neuronal culture system is more suitable for assessing PS-dependent gene expression, and that CREB-mediated transcription is not regulated by PS directly; rather, the decreased CREB target gene transcription observed in PS cDKO mice is likely secondary to reduced neuronal activity present in these mutant mice.

## EXPERIMENTAL PROCEDURES

**Plasmids**—A 360-bp KpnI-BglII fragment of Hes-Luc, which contains the region from −194 to +160 of the mouse *Hes1* promoter (18), and a 1.28-kbp PacI-HindIII fragment of pFUGW-EGFP-NLS (19) were cloned into the multicloning site of pGL4.10 (Promega) to generate *HES1-luc* and *Ubc-luc*, respectively. *CRE-luc* was generated by inserting the oligonucleotide containing a 3× CRE sequence and a TATA-box promoter element, to the multicloning site of pGL4.10. Plasmids, pCS2-NΔE (20) and pCI-PS1<sup>3</sup> were previously described. pGL4.74, which encodes *Renilla* luciferase gene driven by thymidine kinase promoter, was purchased from Promega.

**Cell Cultures**—HEK293 (human embryonic kidney), wild-type mouse embryonic fibroblast (MEF) and PS DKO MEF (21) were maintained in high glucose Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum. Dissociated neocortical cultures were prepared from newborn *fPS1/fPS1*; *PS2*(−/−) mice as described previously (22). Briefly, neocortex was dissected from the brain of neonates. Neurons were dissociated by trypsin treatment (2.5 mg/ml for 10 min at 37 °C), were triturated with a siliconized Pasteur pipette, and then were plated at  $1.5 \times 10^5$  cells/cm<sup>2</sup> onto multiwell plates coated with Matrigel. Cultures were maintained in Minimal Essential Medium, 5 g/liter glucose, 0.1 g/liter transferrin, 0.25 g/liter insulin, 0.3 g/liter glutamine, 5% fetal bovine serum, 2% B-27 supplement, and 2–4 μM cytosine arabinoside. Cultures were maintained at 37 °C in a humidified incubator gassed with 95% air and 5% CO<sub>2</sub>. HEK293 cells were transfected with FuGENE6 reagent (Roche Applied Science). MEF cells were transfected with Lipofectamine2000 (Invitrogen). Cortical neuronal culture was transfected with calcium phosphate (Clontech) at DIV8. For Fig. 5, A and B, neuronal cultures were treated with 10 μM forskolin (Sigma), 40 mM KCl, and 100 nM PMA (Sigma) for 4 h.

**Lentivirus Production and Infection**—Production of recombinant lentiviruses is achieved by transfecting HEK293T cells with three plasmids. VSVg and Δ8.9 are plasmids encoding the envelope and the gag/pol/tat proteins of lentivirus, respectively. pFUGW-EGFP-NLS-Cre and pFUGW-EGFP-NLS were previously described (19). Viruses were harvested 48 h after transfection by collecting the medium from transfected cells, and filtrated. Titer of the lentivirus was estimated by measuring the GFP-positive cells with flow cytometry, following the infection

of diluted lentivirus to HEK293 cells. Neurons were infected with each lentivirus at 3–4 of multiplicity of infection (moi). To determine infection efficiency of the lentivirus, separate cultures were prepared besides reporter assay, and stained with Hoechst 33258 at DIV10. After counting the signals of GFP-expressing cell (infected cell number) and Hoechst 33258 (total cell number) under fluorescent microscope, we calculated the infection efficiency by ratio of the number of GFP-positive cells and Hoechst 33258-positive cells. Neurons were maintained until DIV 9–11 for biochemical analyses.

**Western Blot**—Neuronal culture was harvested with Laemmli buffer at the designated day. Proteins were separated in NuPAGE Novex 4–12% Bis-Tris Gel (Invitrogen), and transferred to nitrocellulose membrane. Primary antibodies were rabbit anti-PS1 polyclonal (Calbiochem), mouse anti-PS1 monoclonal (Millipore), mouse anti-α-tubulin monoclonal (Sigma), rabbit anti-phospho-CREB (Ser-133) monoclonal (Upstate), rabbit anti-CREB monoclonal (Upstate), rabbit anti-phospho-ERK1/2 (Thr-202/Tyr-204) polyclonal (Cell Signal Tech.), and rabbit anti-ERK1/2 monoclonal (Sigma). The membrane was then incubated with IRDye 800CW or IRDye 680-labeled second antibodies. Signals were developed with Odyssey Infrared Imaging System (LI-COR Bioscience).

**Quantitative Reverse Transcription-PCR (Q-RT-PCR) and Northern Blot**—RNA extraction and Q-RT-PCR were performed as described previously (23). Briefly, total RNA was treated with DNase I and reverse-transcribed in the presence of random hexamers. PCR reactions were performed using SYBR Green PCR Master Mix in 7500 Fast Real-Time PCR System (Applied Biosystems) with cDNA and gene-specific primers. Reactions were performed in triplicate, and threshold cycle values were normalized to 18 S rRNA (Ambion). Statistic significance was calculated using the Student's *t* test. The primer pairs used in this study are as follows: 5'-CTTGGAGGAGAACACATGAG-3' and 5'-TCTGGGCATTCTGGAAGTAG-3' for *PS1*, 5'-GATGTTCTCGGGTTTCAACG-3' and 5'-GGAGAGGAGTCGGCTGG-3' for *c-fos*, 5'-GAAGGCGATGGTGAGACG-3' and 5'-CACTGACTAGGCTGAAAAGG-3' for *egr-1*, 5'-TGTCAGCACTACGGTGTTC-3' and 5'-AACCGACAGTACTGACAAC-3' for *Nurr*, 5'-TTGTCTCCTGCGAC TTCA-3' and 5'-TCCACCACCCTGTTGCTGTA-3' for *GAPDH*. For Northern blot, 10 μg of total RNA were separated in formaldehyde agarose gels, and transferred into nylon membrane. Hybridization was performed using [α-<sup>32</sup>P]dCTP-labeled probes.

**Luciferase Assay**—Firefly luciferase plasmid and pGL4.74 (Tk promoter-driven *Renilla* luciferase) were transduced to MEF or DIV8 neuronal culture. 1 or 2 days after transfection, cell lysates were collected with 1× Passive lysis buffer (Promega). Luciferase activities were measured with Dual-Luciferase Reporter Assay system (Promega), and each firefly luciferase activity was divided by *Renilla*'s one to normalize luciferase activity toward transfection efficiency. As for measuring *HES1-luc* activity, NΔE-expressing plasmid was also cotransfected to augment the effect of Notch cleavage, except Fig. 1B. The values showed mean ± S.E.

**Immunocytochemistry**—Neuronal cultures on coverglasses from around DIV10 were fixed with methanol, blocked with a

<sup>3</sup> E. Heilig and R. J. Kelleher, unpublished data.

solution containing 3% nonfat dry milk and 0.1% saponin for 1 h at room temperature, and incubated with the indicated primary antibodies overnight at 4 °C. The following primary antibodies were used: anti-MAP2 rabbit polyclonal IgG (1: 2000, Covance), and anti-synaptophysin mouse monoclonal antibody (1: 2000, Sigma). Cultures were then washed in phosphate-buffered saline and incubated with appropriate Alexa Fluor (546 or 680)-conjugated secondary antibodies (1: 500, Molecular Probes) for 1 h at room temperature. Signals were detected by LSM 510 confocal microscope (Zeiss).

## RESULTS

**Transcriptional Alterations in PS DKO MEFs**—Although we previously observed decreases in levels of CREB target gene transcripts at multiple ages in the cerebral cortex of PS cDKO mice (9), the concomitant presence of a number of synaptic functional defects makes it difficult to conclude whether PS regulates CREB-mediated gene expression directly or indirectly due to impaired synaptic function in these mutant mice. To address this important question directly, we developed a sensitive luciferase-based transcription reporter system and then used it in immortalized wild-type (WT) and PS-deficient (PS DKO) mouse embryonic fibroblasts (MEFs) (21) to determine if inactivation of PS in non-neuronal cells similarly results in reduction of CREB-mediated transcription.

We first constructed a reporter plasmid (*CRE-luc*), which contains three repeats of CRE sequences upstream of a minimal promoter, followed by the firefly luciferase cDNA (Fig. 1A). To test the validity of this reporter system, we examined the effect of forskolin on the induction of luciferase activity, since forskolin is well known to increase CREB transcription by activating cAMP-dependent protein kinase A (PKA) (24). After transfecting the *CRE-luc* construct into WT MEFs, we could detect ~2.5-fold luciferase activity compared with the control reporter, *minP-luc* (Fig. 1B), indicating that three repeats of CRE sequences promotes transcription. Furthermore, the *CRE-luc* construct yielded more than 7-fold induction of the luciferase activity in the presence of forskolin when compared with basal conditions (Fig. 1B), whereas the *minP-luc* did not respond to forskolin at all (data not shown). These results confirmed that the *CRE-luc* construct could be used as a reporter for CREB-mediated transcriptional activity. Next, to measure PS-dependent CREB activity, we transfected the *CRE-luc* reporter construct into WT and PS DKO MEFs. The luciferase activity was much lower in PS DKO MEFs than in WT MEFs (Fig. 1C), suggesting an attenuation of CREB-mediated transcription in PS-deficient MEFs.

Because normal transcription of a Notch target gene, *Hes1*, is dependent upon PS-mediated  $\gamma$ -secretase cleavage of Notch (25–27), we next tested whether Notch-mediated *Hes1* expression is indeed decreased in PS DKO MEFs. For this purpose, we generated a similar reporter system, in which *Hes1* transcription can be measured by activities produced from the *HES1-luc* construct (Fig. 1A). To validate this reporter, the *HES1-luc* plasmid was transfected in WT MEFs along with an N-terminal-truncated Notch1 construct (NΔE), which lacks the large extracellular domain and is a direct substrate of PS-mediated  $\gamma$ -secretase activity. Drastic increases of luciferase activity were

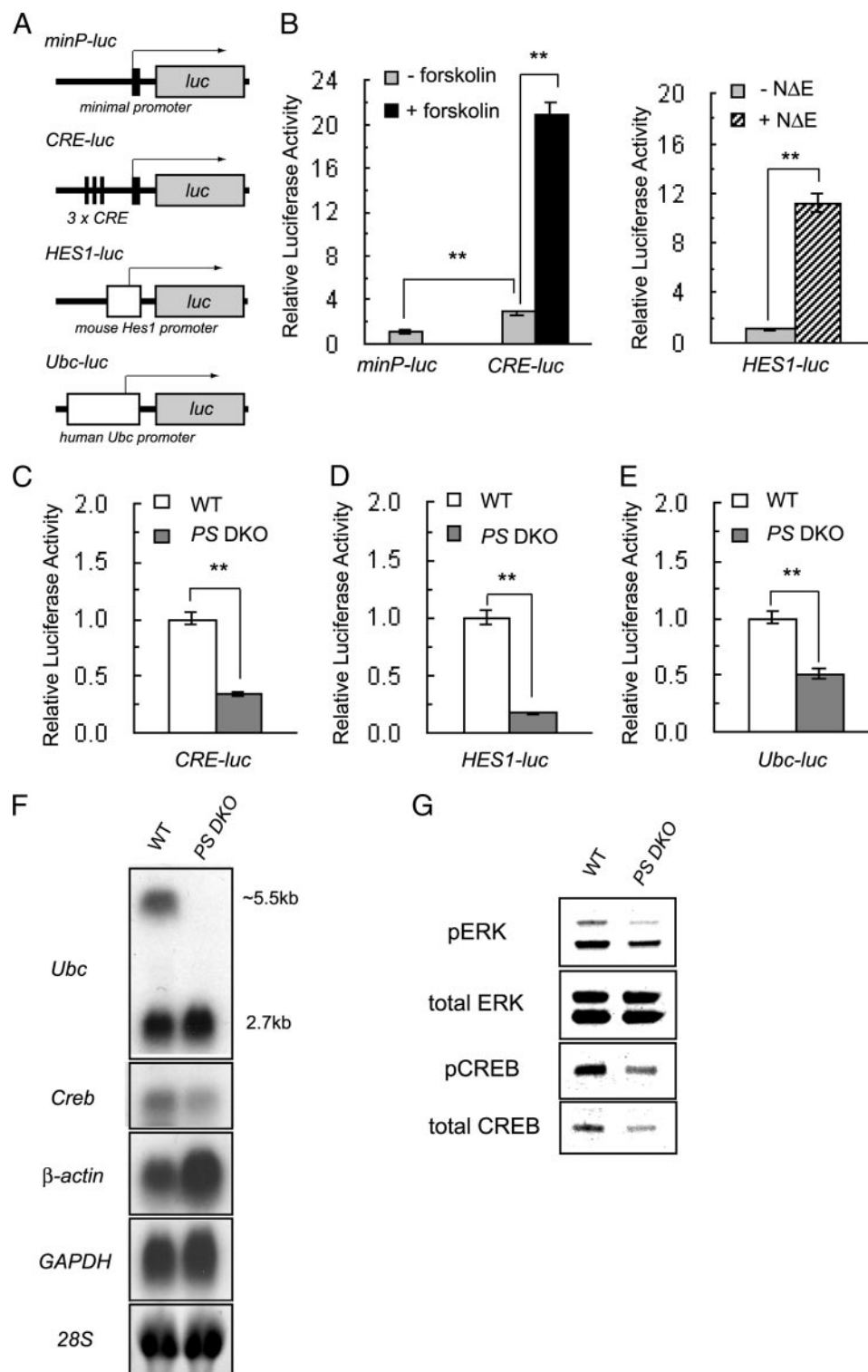
observed in the presence of NΔE (Fig. 1B), verifying that the *HES1-luc* construct can measure PS-mediated  $\gamma$ -secretase cleavage of Notch. When WT and PS DKO MEFs were compared, similar to the case of *CRE-luc*, significant reduction of the luciferase activity was observed in PS DKO MEFs (Fig. 1D), confirming the requirement of PS for the intramembrane cleavage of Notch to release the active intracellular domain (NICD), which then activates Notch target genes such as *Hes1* (25).

To exclude the possibility of a general transcriptional defect, we constructed another reporter plasmid, which contained the promoter region of the human ubiquitin-C (*Ubc*) gene, to assess integrity of general transcription machinery (Fig. 1A). Although the *Ubc* promoter contains several putative transcription factor binding sites including AP-1, Sp-1, and NF- $\kappa$ B (28), it was reported to have consistent expression among many tissues in transgenic mice, in which transgenes were driven by this promoter (29). When we compared *Ubc-luc* activities between WT and PS DKO MEFs, unexpectedly, its activity was also significantly decreased in PS DKO MEFs (Fig. 1E), raising the possibility that there is a general transcriptional defect.

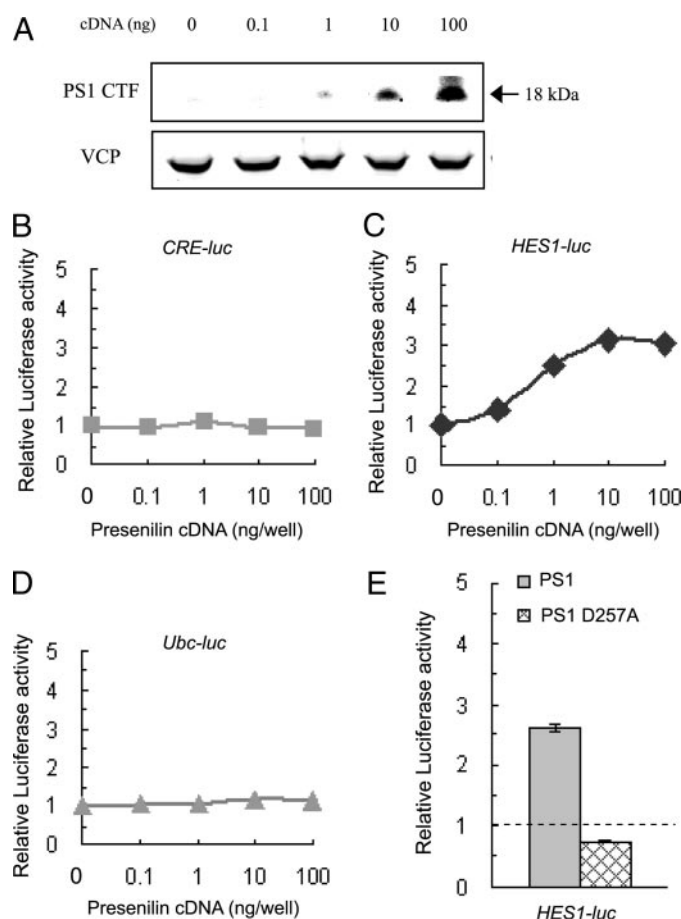
We then performed Northern analysis to assess transcription of *Ubc* and two other housekeeping genes,  $\beta$ -actin, and *GAPDH*. We found that only one *Ubc* mRNA species was detected in PS DKO MEFs, whereas two *Ubc* mRNA species were detected in WT MEFs, as previously reported (30), likely also due to allelic variation of transcription (31). We utilized two different probes specific for 5'-untranslated region and 3'-unique sequence, excluding ubiquitin repeat sequences. In addition, there is an approximate 20% decrease of total *Ubc* mRNAs in PS DKO MEFs compared with that in control MEFs (Fig. 1F). In contrast to *Ubc*, our Northern analysis also revealed marked increases of  $\beta$ -actin mRNAs and a smaller increase in *GAPDH* mRNAs in PS DKO MEFs (Fig. 1F). Together, these results indicate a complex transcriptional dysregulation in PS DKO MEFs.

To test further whether the reduced *CRE-luc* activity in PS DKO MEFs was caused by intrinsic upstream changes in activation of CREB signaling, we next examined phosphorylation of extracellular signal-regulated kinase1/2 (ERK1/2) and CREB, which are well known to regulate CREB-mediated transcription through phosphorylation at specific amino acid residues (11, 32, 33). Similar to other groups' reports (34, 35), an approximate 50% decrease of phosphorylated ERK1/2 (Thr-202/Tyr-204) was observed in PS DKO MEFs (Fig. 1G). Surprisingly, the levels of total CREB proteins (Fig. 1G) and *Creb* mRNAs (Fig. 1F) were also significantly reduced in PS DKO MEFs, so were Ser-133-phosphorylated CREB (Fig. 1G). Collectively, these results indicate that the reduction of the *CRE-luc* activity in PS DKO MEFs could result from both reduced expression and decreased activity of CREB.

**Transfection of Exogenous PS1 to DKO MEFs Failed to Rescue the Reduced Activity of the CRE-luc Reporter**—To determine whether the reduction of luciferase activity from the *CRE-luc* reporter in PS DKO MEFs is intrinsic to the loss of PS, we next examined whether introduction of exogenous PS1 can rescue this transcriptional defect. For this purpose, we transfected varying amounts of wild-type human PS1 cDNAs into PS DKO MEFs. The presence of the exogenous human PS1 was con-



**FIGURE 1. Reduced CREB-mediated transcriptional activities in immortalized PS-deficient MEFs.** *A*, schematic diagrams of each firefly luciferase constructs. The bold vertical line represents minimal promoter containing a TATA-box. Specific promoters are indicated as white open squares. *B*, validation of CRE-luc and HES1-luc constructs. Left, CRE-luc activities were measured using the lysates of WT MEFs treated in the absence (–forskolin) or presence (+forskolin) of forskolin. The value of minP-luc activity was set as 1. Right, HES1-luc reporter was transfected into WT MEFs together with either control (–NΔE) or NΔE-expressing plasmid (+NΔE). The value of cultures transfected with the control plasmid was set as 1. *C–E*, WT and PS DKO MEFs were transfected with each firefly luciferase reporter (described in the bottom of each graph; CRE, *C*; HES1, *D*; Ubc, *E*), and luciferase activities were measured. The value of WT MEF cultures was set as 1. Data were analyzed using Student's *t* test ( $n = 4\sim5$ ). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ . *F*, alterations in mRNA levels of control genes between immortalized WT and PS DKO MEFs. Northern blot was performed using 10  $\mu$ g of total RNAs from both cells. Specific probes used here for assessing the amount of mRNA expression were as follows: Ubc, Creb,  $\beta$ -actin, and GAPDH. The level of 28 S rRNA was used for normalization of RNA amounts. *G*, phosphorylation levels of both CREB and Erk1/2 proteins between WT and PS DKO MEFs. The protein lysates from WT and PS DKO MEFs were analyzed by Western blot using the antibodies against phosphorylated (Ser-133)-specific CREB (pCREB), total CREB (total CREB), phosphorylated (Thr-202/Tyr-204)-specific Erk1/2 (pErk), or total Erk1/2 (total Erk).



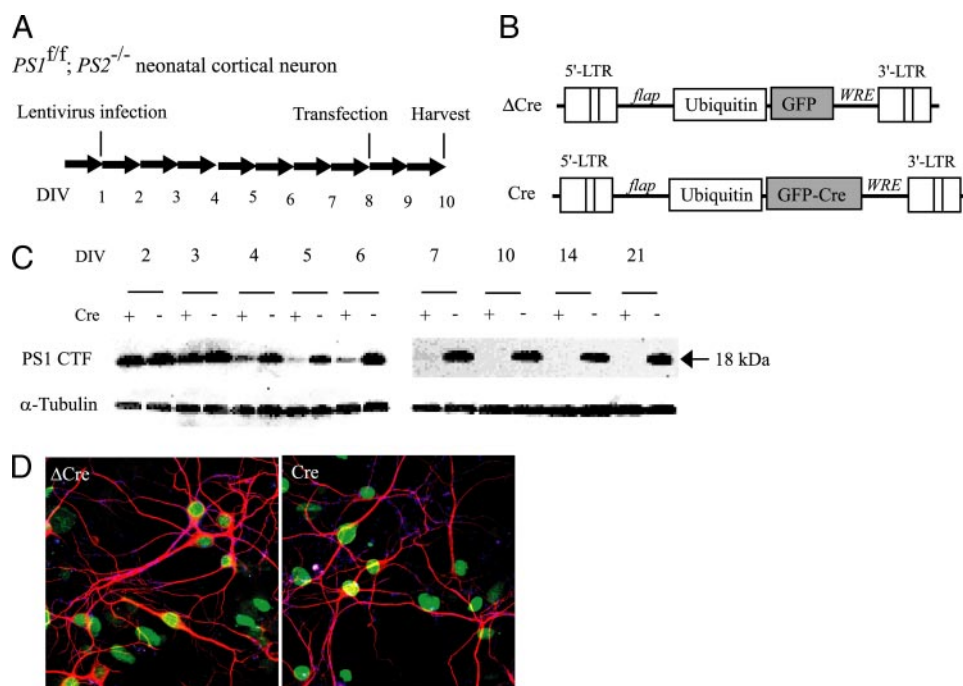
**FIGURE 2. Failure to rescue the reduced CREB transcriptional activity by exogenous PS1 expression in PS DKO MEFs.** A, Western blot was performed using cell lysates from PS DKO MEFs transfected with an increasing amount of human PS1 expression plasmid. Valosin-containing protein (VCP) was used here as a loading control. B–D, the effect of exogenous expression of human PS1 on transcriptional activity in PS DKO MEFs. Increasing amounts of PS1-expressing plasmid was transfected into PS DKO MEF together with *CRE-luc* (B), *HES1-luc* (C), or *Ubc-luc* (D). Luciferase activities from no PS1 expression plasmid were set as 1. E, importance of  $\gamma$ -secretase activity within PS protein. *HES1-luc* reporter was transfected into PS DKO MEF together with control, PS1-expressing, or PS1 D257A-expressing plasmid. Relative value of PS1 expression plasmids to control plasmid is depicted in the graph ( $n = 3$ ).

firmed by Western blotting (Fig. 2A). Surprisingly, when measuring the luciferase activity, we found that exogenous PS expression does not affect the *CRE-luc* activity under basal (Fig. 2B) or forskolin-stimulated conditions (data not shown).

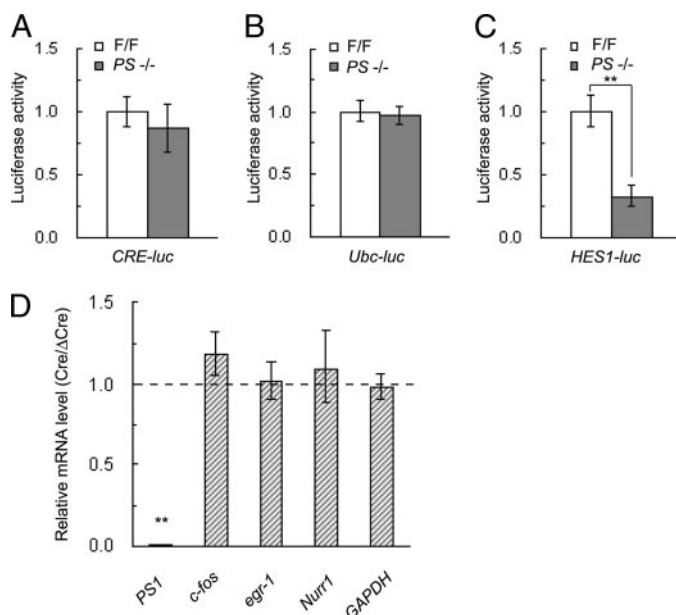
We next tested whether exogenous PS1 can rescue transcriptional defects of *Hes1-luc* in PS DKO MEFs. The *HES1-luc* activities were higher with increasing amounts of the exogenous PS1 cDNA, though the assay was saturated with more than 10 ng of cDNAs (Fig. 2C). Co-transfection of PS1 D257A mutant, a mutant defective of  $\gamma$ -secretase cleavage activity, did not lead to increase in *HES1-luc* activity (Fig. 2E). These results indicate that exogenous human PS1 can rescue the deficiency of  $\gamma$ -secretase-mediated activation of *Hes1-luc* but not CRE-dependent transcription in PS DKO MEFs. In addition, no rescue was observed for the *Ubc-luc* reporter when exogenous PS1 was introduced into the PS DKO MEFs (Fig. 2D). Collectively, these results suggest that PS does not play a direct role in CRE-mediated transcription, at least in these MEFs.

**Establishment of Neonatal PS-null Neuronal Culture**—Because CRE-dependent gene expression is impaired in the postnatal forebrain-restricted PS cDKO mice, in which PS inactivation is restricted to mature neurons (9), we next tested whether *CRE-luc* activity is decreased in primary neuronal cultures. We first developed primary PS-null cortical neuronal cultures from neonates carrying homozygous floxed *PS1* and *PS2*-null alleles (*fPS1/fPS1*; *PS2*–/–), in which PS1 can be inactivated upon introduction of the Cre recombinase (Fig. 3A), thus circumventing the requirement of PS in neural development (5, 6, 8, 26). One day after culture preparations (DIV1), we infected a lentivirus carrying the cDNA encoding Cre recombinase (Fig. 3B) into neuronal cultures to inactivate PS1 expression. When we checked viral infection efficiency by GFP fluorescence, which is readily detectable due to the fusion of GFP to the Cre recombinase, the infection rate was estimated at more than 95% (Fig. 3D). To evaluate the loss of PS1 protein by Cre-mediated recombination, we collected cell lysates at each day after viral infection, and performed Western analysis. Levels of PS1 protein were unchanged in culture infected with the control lentivirus (Fig. 3C). In contrast, PS1 expression began to be decreased in the Cre-infected neuronal culture at DIV3 (2 days after infection of the Cre virus) and was completely absent by DIV7 (Fig. 3C). To determine the impact of PS inactivation on neuronal morphology in cultures, we performed immunostaining in neuronal cultures at DIV10 with antibodies for synaptophysin and MAP2, markers of presynaptic terminals and neurites, respectively. No difference was detected between control ( $\Delta$ Cre) and Cre-infected neurons (Fig. 3D), suggesting that PS inactivation does not cause gross morphology changes of postnatal neurons in culture under these conditions.

**CRE-mediated Transcription Is Not Altered in PS-deficient Cortical Neuronal Cultures**—Given our previous observation of reduced CREB target gene expression in the adult mouse cerebral cortex lacking PS at multiple ages (9), we would expect to see robust decreases of CREB target genes in PS-deficient neuronal cultures. Therefore, we next used the above primary neuronal culture system to examine the effect of neuronal PS inactivation by transfecting the *CRE-luc* reporter plasmids around DIV8 (Fig. 3A). A slight, but not statistically significant decrease of luciferase activity was detected in PS-deficient cultures compared with control lentivirus-infected cultures (Fig. 4A). Furthermore, co-transfection of exogenous PS1 cDNAs with this reporter did not affect the *CRE-luc* activity in PS-deficient neurons (data not shown). In addition, there was no difference in *Ubc-luc* activity between control and PS-null cultures, indicating that general transcription is unaltered in these cells (Fig. 4B). We further tested the *HES1-luc* reporter. When we transfected the culture with the *HES1-luc* reporter, luciferase activity was significantly decreased in PS-deficient neurons, compared with control cultures (Fig. 4C). In addition, this decrease in *HES1-luc* activity was rescued by exogenously expressing human PS1 to PS-deficient neuron; the activity was rescued from  $36.3 \pm 4.9\%$  (vector alone) to  $91.4 \pm 10.4\%$  (PS1 construct), compared with control neuronal cultures ( $n = 3$  experiments). These results indicate that CREB-mediated transcription is not affected by loss of PS function in primary neuronal cultures.



**FIGURE 3. Establishment of neonatal cortical neuronal cultures using *fPS1/fPS1; PS2(-/-)* newborn pups.** A, time scale of cortical neuronal culture used here. B, diagram of lentivirus DNA structures. The lentivirus DNAs contain self-inactivating long terminal repeat (LTR), flap sequence, ubiquitin C promoter-driven GFP, or GFP-Cre, and woodchuck regulatory element (WRE). C, time course of PS1 inactivation in neuronal culture. The lentivirus containing either GFP alone ( $\Delta$ Cre) or GFP/Cre fusion protein (Cre) was infected into *fPS1/fPS1; PS2(-/-)* neuronal cultures, and Western blot was performed using cell lysates collected from the culture at the designated day.  $\alpha$ -Tubulin was used here as a loading control. D, immunocytochemistry of neuronal markers.  $\Delta$ Cre (left) and Cre (right) lentivirus-infected cortical cultures were stained with either a MAP2 (red) or a synaptophysin (blue) antibody. Green fluorescences indicate GFP expression in nucleus of the cells that were successfully infected with the lentivirus.



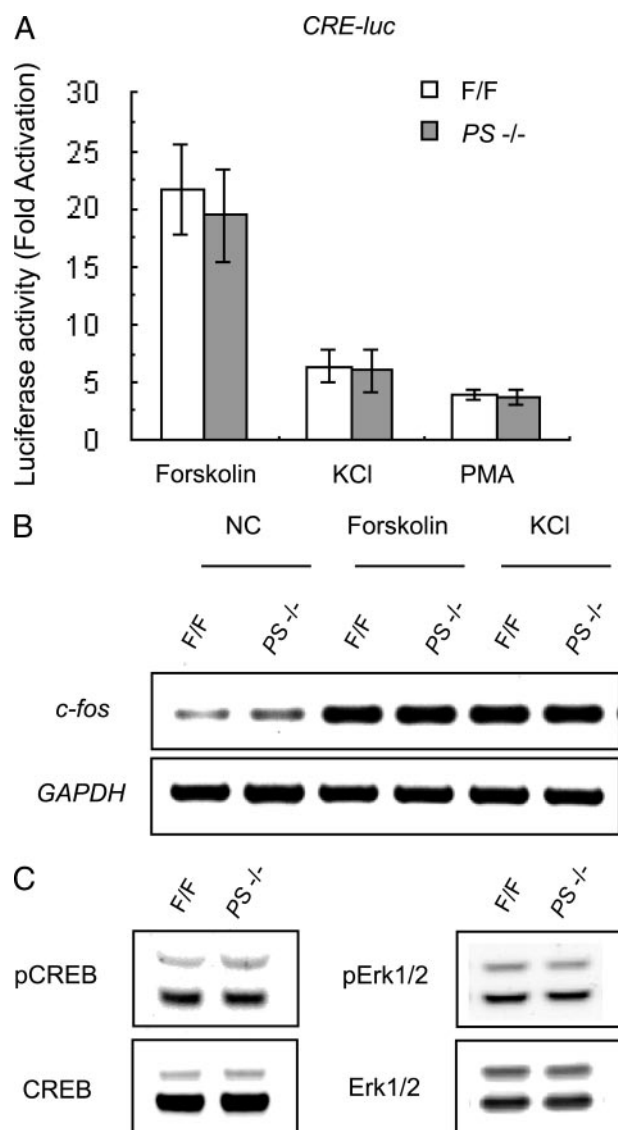
**FIGURE 4. Normal CREB-mediated transcription in *PS*-null cortical cultures.** A–C, luciferase reporter experiments in lentivirus-infected neuronal cultures.  $\Delta$ Cre- (F/F) or Cre- (*PS*<sup>-/-</sup>) infected cultures were transfected with *CRE-luc* (A), *Ubc-luc* (B), or *HES1-luc* (C) reporter plasmids on DIV8. Luciferase activities were measured, and the value of  $\Delta$ Cre-infected (F/F) culture was set as 1. Data are shown as average values among three independent experiments. \*\*,  $p < 0.01$ . D, quantitative RT-PCR analysis of CREB target genes in  $\Delta$ Cre- or Cre-infected cultures. Quantitative RT-PCR was performed using primer pairs for *PS1*, *c-fos*, *egr-1*, *Nurr1*, *GAPDH*, and *S18 rRNA*. mRNA expression is shown as a percentage values of the control ( $n = 3$ ). \*\*,  $p < 0.01$ .

We next performed quantitative RT-PCR using primers specific for a few CREB target genes. *PS1* mRNAs were almost eliminated in *PS*-deficient cultures at DIV10, whereas levels of *GAPDH* mRNA were unchanged (Fig. 4D). Consistent with the results obtained from analysis using the *CRE-luc* reporter results, expression of three CREB target genes, *c-fos*, *egr-1*, and *Nurr1*, was unchanged in *PS*-null neuronal cultures (Fig. 4D), providing further support for the absence of a direct involvement of PS in the regulation of CREB-mediated transcription.

**Activation of CREB Pathway Does Not Lead to an Alteration in CRE-mediated Transcription by Neuronal PS Deficiency**—In the experiments described thus far, we could not detect any alteration in CREB transcriptional activity in *PS*-deficient cortical neurons under basal condition. However, we cannot exclude the possibility that the absence of transcriptional changes may be due to a lack of positive regulatory effects under basal conditions. Therefore, we next examined *CRE-luc* activity under stimulated

conditions. Forskolin, phorbol ester (PMA), and high potassium are known to increase CREB transcriptional activity by PKA activation, cAMP production, and  $Ca^{2+}$  signaling, respectively (11). When we applied these stimuli in control (F/F) neuronal cultures with the *CRE-luc* reporter, significant inductions of activity were obtained, especially in the case of forskolin (Fig. 5A). However, we could not detect any difference between control and *PS*-deficient cultures (Fig. 5A). We also measured levels of *c-fos* mRNAs using semi-quantitative RT-PCR, after either forskolin or KCl stimulation. Similar to the results of the reporter assay, we observed a strong induction compared with basal levels (NC), but no significant difference between control and *PS*-deficient cultures (Fig. 5B). Collectively, these results further suggest that PS is not directly involved in CREB-mediated transcription.

Other groups and this study (Fig. 1G) have reported that the ERK activity is lowered in *PS1*- or *PS1/PS2*-deficient MEF cells (34–36). Furthermore, ERK activation has been shown to play important roles in the regulation of CREB activity in both brains and neuronal cultures (32, 33). To determine whether there is any effect of PS deficiency on these signaling pathways, we next investigated by Western analysis the phosphorylation status of both CREB and ERK. We found no difference in their phosphorylation levels between control and *PS*-deficient neurons (Fig. 5C). Together with our previous results showing that levels of



**FIGURE 5. No difference in signaling pathways involved in CREB activation in PS-null cortical cultures.** A, luciferase reporter experiment of CREB-mediated transcriptional activity following stimuli. Luciferase activity was calculated as its fold activation of the value from unstimulated cultures ( $n = 3$ ). B, semi-quantitative RT-PCR of *c-fos* mRNA. Total RNAs were purified from control (F/F) and PS-deficient (PS<sup>-/-</sup>) culture, which were treated with vehicle (NC), forskolin, or KCl. C, detection of CREB (Ser-133) and Erk1/2 (Thr-202/Tyr-204) phosphorylation in neuronal cultures. There is no difference in the level of phosphorylated CREB in PS-deficient ( $98.3 \pm 4.8\%$ ) and control neuronal cultures ( $p > 0.05$ ;  $n = 3$ ). Similarly, levels of phosphorylated Erk1 ( $94.0 \pm 15.0\%$ ) and Erk2 ( $94.5 \pm 7.7\%$ ) in PS-deficient neuronal cultures are also not significantly changed. Representative photos are shown here among three independent experiments.

phosphorylated CREB (9) and ERK<sup>4</sup> were not changed in the cerebral cortices of PS cDKO mice, these results suggest that PS deficiency does not directly affect CREB and ERK phosphorylation.

## DISCUSSION

We previously reported that postnatal forebrain-restricted conditional inactivation of both *PS1* and *PS2* resulted in synaptic and memory impairment and progressive neurodegenera-

tion (9). Reduction of CREB-mediated transcription observed in the cerebral cortex of PS cDKO mice at multiple ages was thought to be a possible key molecular pathway underlying neurodegeneration and synaptic impairment (9, 37). In addition, we identified a putative RBP-J $\kappa$  binding site in the mouse *cbp* promoter. Thus, we proposed that PS might regulate CRE-dependent gene expression through its  $\gamma$ -secretase-mediated cleavage of Notch. However, due to the presence of multiple neuronal defects in PS cDKO mice, it was difficult to determine whether PS regulates the gene expression directly or indirectly due to impaired synaptic function in these mice. In the current study, we used sensitive luciferase reporter systems to address whether presenilins regulate CREB target gene expression directly in two different cell culture systems. We found that CREB-mediated transcriptional activity is reduced in immortalized PS DKO MEFs (Fig. 1C) but is unchanged in PS-null primary cultured neurons (Fig. 4A).

How can we reconcile this apparent discrepancy between these two experimental systems? We performed a number of control experiments to resolve this issue. First, we tested a control reporter, *Ubc-luc*, the luciferase activity of which should not be affected by PS inactivation. Surprisingly, the luciferase activity derived from *Ubc-luc* is decreased in PS DKO MEFs (Fig. 1E), though its activity is unchanged in PS-null cultured neurons (Fig. 4B), as expected. Second, we transfected *PS1* cDNAs into the MEFs to determine if introduction of exogenous PS1 can rescue the phenotype in PS DKO MEFs. We found that expression of PS1 in PS DKO MEFs was able to rescue the reduction of *HES1-luc* activity but not the decreased activities of *CRE-luc* or *Ubc-luc* (Fig. 2), suggesting that the decreased activities of *CRE-luc* or *Ubc-luc* in PS DKO MEFs are not due to loss of PS in these cells. Introduction of PS1 into PS-null neuronal cultures also rescued the reduced *HES1-luc* activity but had no effect on activities of *CRE-luc* or *Ubc-luc*. Third, we performed Northern analysis to determine levels of *Ubc* mRNAs and transcripts of two other housekeeping genes,  $\beta$ -actin and *GAPDH*. To our great surprise, two *Ubc* mRNA species were found in WT MEFs as previously reported (30), whereas only one transcript was expressed in PS DKO MEFs (Fig. 1F). The level of total *Ubc* mRNAs was lower in PS DKO MEFs, compared with the control (Fig. 1F). Moreover, levels of  $\beta$ -actin and *GAPDH* transcripts were increased in these MEFs (Fig. 1F). Lastly, while levels of CREB mRNAs and total and phosphorylated proteins were decreased in PS DKO MEFs (Fig. 1, F and G), levels of total and phosphorylated CREB were unchanged in PS-null cultured neurons (Fig. 5C), consistent with our earlier findings in PS cDKO mice (9). The results of these control experiments suggest that these immortalized PS DKO MEFs have undergone transcriptional dysregulation that is independent of PS, perhaps during extended passages. Thus, it would be prudent to always perform rescue experiments when using these PS DKO MEFs to uncover PS-dependent transcription and signaling. Furthermore, the primary PS-null neuronal cultures, though more cumbersome to generate and maintain, would be more suitable for such studies. Compared with other neuronal culture systems that have been reported so far (38, 39), in which neurodevelopmental requirements of PS are not circumvented and PS2 still remains, our newly developed post-

<sup>4</sup> H. Watanabe and J. Shen, unpublished data.

natal neuronal cultures lack both presenilins and do not exhibit any detectable neurodevelopmental abnormalities.

As for our central question of whether PS regulates CREB target gene expression directly through Notch-mediated signaling, our results in the current study argue against such a direct regulation. Unlike in the *PS* cDKO cerebral cortex, we observed no disturbance in CREB target gene expression in *PS*-deficient neuronal cultures (Fig. 4). And the sensitive *CRE-luc* reporter system failed to uncover significant reductions in luciferase activities in these *PS*-null primary neurons, whereas transcriptional activities mediated by the canonical Notch target *Hes1* was dramatically reduced (Fig. 4), indicating that transcription of CREB target genes is not regulated directly by PS through  $\gamma$ -secretase dependent Notch signaling.

The difference in the CREB target gene expression observed between *PS* cDKO mice and *PS*-null cultured neurons may be further explained as follows. In *PS* cDKO mice, loss of presenilins begins at the third postnatal week, and a number of cellular (e.g. short-term and long-term synaptic plasticity impairment) and molecular (e.g. reduced CREB target gene expression) defects were identified at 2 months of age (9). In contrast, the *PS*-null neuronal culture, in which loss of PS proteins is almost complete in all cells 4–6 days after introduction of the *Cre* cDNA by lentivirus, is much more likely to yield direct effects of PS inactivation. Indeed, expression of a Notch target gene, *Hes1*, which is mediated directly through  $\gamma$ -secretase activation of Notch, is markedly reduced in *PS*-null neuronal cultures (Fig. 4C), while the *CRE-luc* and the *Ubc-luc* activities were not altered (Fig. 4, A and B). Based on these findings, it is unlikely that reduction of CREB target gene expression observed in the *PS* cDKO cortex is regulated directly through the Notch pathway. *PS* cDKO mice, in which synaptic defects are followed by progressive neurodegeneration, are better suited to uncover both direct and indirect effects of loss of PS function over an extended period of time during the aging process. Thus, the decrease of CREB target gene expression in the forebrain of mutant mice could result indirectly from neuronal dysfunctions. Furthermore, in a related but independent study, we similarly generated a forebrain-specific *CBP* cKO mouse to address whether *CBP* is indeed a key downstream regulator of PS in promoting neuronal survival. No neurodegeneration was identified in *CBP* cKO up to 9 months of age,<sup>5</sup> whereas 24% of cortical neurons and 35% of cortical volume were lost in *PS* cDKO mice at this age (9).

Despite the fact that PS regulates CREB target gene expression indirectly, it remains possible that the down-regulation of the CREB pathway by loss of PS function plays an important role in exacerbating neurodegeneration. The possibility of presenilin mutations may cause the disease through a partial loss of function mechanism has been elaborated extensively (40). Recent reports have also shown that reduction of CREB activity has been seen in the brain of Alzheimer disease patients (41, 42) or in neuronal cells treated with A $\beta$  peptides (43, 44). Thus, both loss of PS function and accumulation of A $\beta$  peptides may impair the CREB downstream pathways, which could lead to

further deterioration of normal neuronal function and survival. Furthermore, *PS1* has been shown as a CREB target gene (45), indicating that reduced CREB transcriptional activity can decrease the production of *PS1* mRNAs in both familial and sporadic Alzheimer brains. Modulation of this pathway, therefore, will likely have therapeutic benefits to slow neurodegeneration processes occurring in Alzheimer disease (37).

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**Indirect Regulation of Presenilins in CREB-mediated Transcription**  
Hiroataka Watanabe, Miriam J. Smith, Elizabeth Heilig, Vassilios Beglopoulos,  
Raymond J. Kelleher III and Jie Shen

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