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Transcriptional regulation of the presenilin-1 gene controls gamma-secretase activity

Sebum Lee[1], Hriday K. Das[1,2]

[1]Department of Pharmacology and Neuroscience, University of North Texas Health Science Center at Fort Worth, 3500 Camp Bowie Boulevard, Fort Worth, Texas 76107, [2]Department of Molecular Biology and Immunology, and Institute of Cancer Research, University of North Texas Health Science Center at Fort Worth, 3500 Camp Bowie Boulevard, Fort Worth, Texas 76107

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1. ABSTRACT

Inhibition of basal JNK activity by JNK inhibitor SP600125 or JNK1siRNA repressed presenilin-1 (PS1) expression in SK-N-SH cells by augmenting the level of p53, a repressor of the PS1 gene (1). We now showed that repression of PS1 transcription by JNK inhibitor SP600125 inhibited gamma-secretase mediated processing of amyloid precursor protein (APP) resulting in the accumulation of C99 fragment and the reduction of secreted Abeta$_{40}$ level without altering the expression of nicastrin (NCT). Co-treatment of cells with SP600125 and p53 inhibitor, pifithrin-alpha, partially nullified the suppressive effects of SP610025 on PS1 expression and secreted Abeta$_{40}$ level. Suppression of JNK1 by JNK1siRNA also decreased Abeta$_{40}$ level. Furthermore, overexpression of the repressors p53, ZNF237 and CHD3 of the PS1 gene also suppressed the processing of APP through repression of PS1 transcription by deacetylation of histone at the PS1 promoter. Transcriptional activator Ets2 increased PS1 protein and secreted Abeta$_{40}$ levels without affecting the expression of NCT by activating PS1 transcription via hyper-acetylation of histone at the PS1 promoter. Therefore, regulation of PS1 transcription modulates gamma-secretase activity.

2. INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia with progressive impairment in cognitive functions. Declined cognitive functions are due to the loss of neurons in the brain (2). The majority of AD cases are late onset sporadic form of AD and about 5% of AD is accounted for the inherited form of AD (3) which is caused by the mutations in the coding regions of amyloid precursor protein (APP) (4) or presenilins (5, 6). The underlying mechanisms of neuronal losses seen in sporadic AD and inherited familial AD (FAD) are not clear. However, deposition of amyloid plaques generated by amyloid beta (Abeta$_{1-40}$, Abeta$_{1-42}$) peptides is the common feature found in both types of AD. Amyloid plaques are believed to initiate and/or aggravate the loss of neurons in both cases (7, 8). Presenilin 1 (PS1) is a critical component of gamma-secretase (9) which is responsible for the generation of Abeta peptides (10). Amyloid precursor protein (APP) bears Abeta domain. To release Abeta domain, APP has to be processed by beta-secretase beforehand (10) and then the remaining C-terminal APP stub called C99 is processed by gamma-secretase. Gamma-secretase mediated cleavage of C99 causes a release of Abeta domain into the extracellular space (11). Later, Abeta peptides are

transformed from diffusible Abeta into aggregated Abeta fibrils on a sigmoid scale (12). In an attempt to reduce Abeta burden, gamma-secretase inhibitors are now being tested in human trials (13). Since, PS1 appears to be responsible for the aspartyl protease activity of gamma-secretase, another approach to reduce Abeta plaque burdens would be to reduce PS1 protein level by repressing PS1 transcription. In the latter case, upstream regulators of the PS1 gene transcription could be potential targets. Therefore, we have studied the regulation of transcription of the human PS1 gene. We have shown that Ets1/2 transcription factors upregulate PS1 transcription (14-17), whereas transcription factors p53, ZNF237, and CHD3 downregulate PS1 transcription (15, 18, 19). We have recently shown that inhibition of basal JNK activity by JNK inhibitor SP600125 represses PS1 transcription by a p53 dependent mechanism and also reduces PS1 protein level (1). Furthermore, the role of JNK on the regulation of PS1 expression was confirmed by JNK1 siRNA. In this report, we show that transcriptional regulation of the PS1 gene can modulate the PS1/gamma-secretase activity without altering the expression of other components (such as nicastrin) of the gamma-secretase enzyme.

3. MATERIALS AND METHODS

3.1. Cell culture

Human neuroblastoma SK-N-SH cell line was maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, 1% penicillin/streptomycin (Gibco, CA). The culture medium for SH-SY5Y cell line was the same except the addition of 1% glutamate (Gibco, CA). JNK inhibitor SP600125 and HDAC inhibitor trichostatin-A (TSA) were purchased from (Calbiochem, CA) and p53 inhibitor pifithrin-alpha (PFT-alpha) was from (Biomol international, PA).

3.2. Western blot analysis

Cells were treated with DMSO or 20uM SP600125 in DMSO as described before (1). Cells were also treated with DMSO or 7.5ug/ml or 15ug/ml of trichostatin-A (TSA) in DMSO as described previously (20). Protein expression levels were measured by Western blot analysis as mentioned previously. Briefly, cells were lysed with 1% protein lysis buffer (150mM NaCl, 25mM Tris-HCl pH 7.8, 1% NP-40, 10mM NaF, 1mM Na_3VO_4) and 50ug of total proteins were separated in 16% or 10% SDS PAGE. The blots were transferred to the PVDF membranes and then blocked with 5% BSA in 0.05% TBST for an hour. The membranes were incubated with primary antibodies against p53 and Ets2 (Santa Cruz biotechnology, CA), PS1 (Chemicon, CA), NCT (Abcam Inc., MA), Ac-H3 (Millipore, MA), Flag, APP, and actin (Sigma. MO) for overnight at 4°C. The blots were visualized using ECL detection system (Pierce, IL).

3.3. RT-PCR analysis

Total RNA was prepared using trizol reagent (Invitrogen, CA). cDNA was synthesized from 2ug RNA with random primers by AMV reverse transcriptase (Promega, CA). cDNA samples were amplified with primers for PS1 (Forward: 5'- GGA GCC TGC AAG TGA CAA CAG C-3' and Reverse: 5'- GTC ACA GGG ACA AAG AGC ATG ATCA-3;) and GAPDH (Forward: 5'- AGG TCG GAG TCA ACG GAT TTG GTC G-3' and Reverse: 5'- GCA GAG ATG ATG ACC CTT TTG-3') respectively using GoTaq Green Master Mix (Promega, CA). Expected product sizes are 382 bps for PS1 and 350 bps for GAPDH.

3.4. Transfection of APP, p53, ZNF237, CHD3, and Ets2 expression vectors

Constructions of pC1.Ets2, pCMV.p53, pCMV-Tag2-ZNF237, pCMV-Tag2-CHD3 were reported previously (15, 16, 18, 19). pcDNA3-APP751 was a kind gift from Dr. Rudolph Tanzi. SK-N-SH cells or SH-SY5Y cells were transfected with 10ug of the each control vector or expression construct using lipofectamine 2000 reagent (Invitrogen, CA) according to manufacturers' guide.

3.5. $Abeta_{1-40}$ Enzyme-Linked Immunosorbent Assay (ELISA)

Cells were grown to ~80% confluence in 60mm dishes. After the culture media were replaced with 1.5mL fresh culture media, the cells were further cultured. 24 to 36 hours later, the culture media were collected. Human Abeta1-40 was detected in the collected culture medium by ELISA using human beta-amyloid (1-40) kit (Biosource, KHB341). Briefly, 100uL medium placed in each well of a 96 well plate (Biosource, KHB341) coated with monoclonal antibody specific for the human Abeta NH2-terminus (Biosource, KHB341) was incubated for 24 hours at 4°C. Then the antibody specific for the human COOH-terminus of Abeta (Biosource, KHB341) was added and the samples were further incubated at 4°C for 24 hours with agitation. After removal of samples by extensive washings, the bound Abeta antibodies were detected by HRP labeled secondary antibody. Secondary antibodies were detected by stabilized chromogen substrate. The absorbance at 450nm was measured using a plate reader. The concentrations of the Abeta samples were compared with the results from the standard Abeta. Abeta samples were detected at concentrations from *3 to 6 pg/mL.*

3.6. Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation assay was performed as previously described (19). Briefly, the cells were fixed with 1% formaldehyde for 10 minutes. Fixing reaction was ended with 0.125M glycine. The fixed cells were lysed and sonicated in ChIP sonication buffer (1% Triton X-100, 0.1% Deoxycholate, 50mM Tris pH 8.1, 150mM NaCl, 5mM EDTA, 10ul/mL proteinase inhibitor cocktail) and 1mM phenylmethylsulfonyl fluoride (Sigma, MO). The whole cell lysates were then diluted in ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl pH 8.1, 167mM NaCl) and incubated with 2ug of anti Ac-H3 antibody (Millipore, MA) overnight at 4°C with agitation. The immune complex were then precipitated with 30uL protein agarose A/G and washed serially with low salt wash buffer (20mM Tris pH 8.0, 1% triton X-100, 2mM EDTA, 180mM NaCl), high salt wash buffer (20 mM Tris pH 8.0, 1% triton X-100, 2 mM EDTA, 500 mM NaCl), LiCl wash buffer (20mM Tris pH 8.0, 0.1% NP40, 2mM EDTA, and 250mM LiCl), 1X

TE (10mM Tris pH 8.0 and 1mM EDTA). The protein-DNA complex was eluted twice with Elution buffer (1% SDS, 0.1 M NaHCO3). Crosslinking was reversed in the presence of 200mM NaCl at 67°C overnight. Protein was removed with 0.2mg/mL proteinase K. DNA was then purified by phenol extraction method and analyzed by PCR with PS1 primers (Forward primer: 5'-CGACGCCAGAGCCGGAAATGAC-3'; Reverse primer: 5'–TTCCGATGTGAAACCGCGGACC-3') to detect PS1 promoter sequence from +25 to –66. 27 cycles of PCR were performed.

3.7. Statistical Analysis

Prism software (Graphpad software Inc.) was used for analyzing all the data. Comparison was made between groups by one-way ANOVA and students-Newman-Keuls (SNK) test. Probability (P value) was considered to be significant with less than 0.05.

4. RESULTS

4.1. JNK inhibitor SP600125 and JNK1 siRNA decrease the Abeta$_{40}$ level in human neuroblastoma SK-N-SH cells

In the previous report, we showed that inhibition of basal JNK activity by JNK specific inhibitor SP600125 or JNK1 siRNA in SK-N-SH cells represses p53-mediated PS1 suppression (1). We now show that JNK inhibitor SP600125 augments p53 protein level without enhancing the transcription of the p53 gene (Figures 1A –1D). SK-N-SH cells were treated with DMSO or 20uM SP600125 in DMSO for 24 hr. Total cellular protein and RNA were prepared for Western blot and RT-PCR analysis. Treatment of cells with SP600125 increased p53 protein level substantially (Figures 1A and 1B). On the other hand, treatment of cells with SP600125 has no detectable effect on p53mRNA level (Figures 1C and 1D). Therefore, SP600125 appears to augment the level of p53 protein and thus decreases PS1 transcription without enhancing transcription of p53. Furthermore, suppression of PS1 by SP600125 was antagonized by co-treatment with a p53 inhibitor, pifithrin-alpha (PFT-alpha) (1). p53 inhibitor PFT-alpha appears to modulate the nuclear import or export or both or decrease the stability of nuclear p53 (21). Knowing the suppressive effect of SP600125 and the SP600125 antagonizing effect of PFT-alpha on PS1 expression, we began to test if the changes in PS1 expression by SP600125 and PFT-alpha are correlated with the PS1/gamma-secretase activity. SK-N-SH cells were pretreated with DMSO or PFT-alpha for 2 hours before SP600125 treatment. The cells were then cultured in the presence of PFT-α and SP600125. 24 hours later, the Abeta$_{40}$ levels were measured by sandwich-ELISA and PS1 protein expression levels were determined by the Western blotting. As we reported previously (1), SP600125 reduced the PS1 protein level which was partially restored by PFT-alpha (Figure 1E). Consistent with the PS1 Western blot result, Abeta$_{40}$ level was decreased by ~50% with SP600125 treatment (Figure 1F). Co-treatment with PFT-alpha partially restored Abeta$_{40}$ level (Figure 1F). To further confirm that the PS1/gamma-secretase activity is inhibited by JNK inhibitor, SP600125, SK-N-SH cells were transiently transfected with APP expressing vector to detect C99 which is a substrate of the PS1/gamma-secretase. The level of C99 was substantially increased when APP-transfected cells were treated with SP600125 (Figures 1G and 1H). Co-treatment of cells with SP600125 and PFT-alpha restored C99 to the level observed in cells transfected with the APP expression vector (Figures 1G and 1H). The Western blotting data of C99 appears to be consistent with the sandwich-ELISA data of Abeta$_{40}$.

We have previously reported that transient transfection of JNK1-siRNA decreased JNK1 expression by ~40%, increased p53 protein level by ~40%, and suppressed PS1 protein expression by ~40% (1). Therefore, we tested if transfection of JNK1siRNA would decrease Abeta$_{40}$ secretion in SK-N-SH cells. Transient transfection of JNK1-siRNA into SK-N-SH cells decreased JNK1 expression (Figure 2A) (1) and reduced secreted Abeta$_{40}$ by ~30% relative to control siRNA transfected cells (Figure 2B). Therefore, the data with SP600125 and JNK1 siRNA suggest that the PS1/gamma-secretase activity can be downregulated by inhibiting basal JNK activity.

4.2. p53 inhibits PS1 transcription by deacetylation of histone at the PS1 promoter and, decreases PS1 protein and the Abeta$_{40}$ levels in human neuroblastoma SK-N-SH cells

We have shown previously that p53 inhibits the PS1-CAT reporter gene expression without binding to the PS1 promoter (1). Transcription factors Ets1/Ets2 bind to the -10 Ets site of the PS1 promoter and activate PS1 transcription (15). We recently showed that increased expression of p53 enhanced p53-Ets1/Ets2 interaction leading to the removal of Ets1/Ets2 from the -10 Ets site of the PS1 promoter and the subsequent repression of PS1 transcription (1). The acetylation of lysine residues on histone tails by histone acetyl transferase (HATs) facilitates transcription of the gene by transcriptional activator (22, 23). On the contrary, deacetylation of these lysine residues by histone deacetylase (HDACs) suppresses gene transcription by transcriptional repressor (24). To determine whether p53 overexpression in SK-N-SH cells also reduces PS1 protein level and thus PS1/gamma-secretase activity by inhibiting PS1 transcription by decreasing acetylation of histone at the PS1 promoter, cells were transiently transfected with empty pCMV vector or pCMV-p53 expressing vector for 48 hours. We tested by ChIP assay whether removal of Ets1/2 by p53-Ets1/2 interactions resulted in the decrease of histone acetylation at the PS1 promoter area around the main transcription initiation site (+1). Transfected cells were cross-linked and DNA-protein complexes were immuno-precipitated with anti-acetyl-H3. The DNA in the complexes was then analyzed by PCR for the presence of the PS1 promoter (Figure 3A). Transient transfection of pCMV-p53 decreased acetylation of histone H3 in the PS1 promoter (-25/+66) (Figures 3A and 3B). Total cellular mRNA and protein were extracted from the transfected cells for RT-PCR and Western blot analysis. To test if p53 represses PS1 expression at the level of transcription, we performed RT-PCR (Figure 3C). The RT-PCR results showed that p53

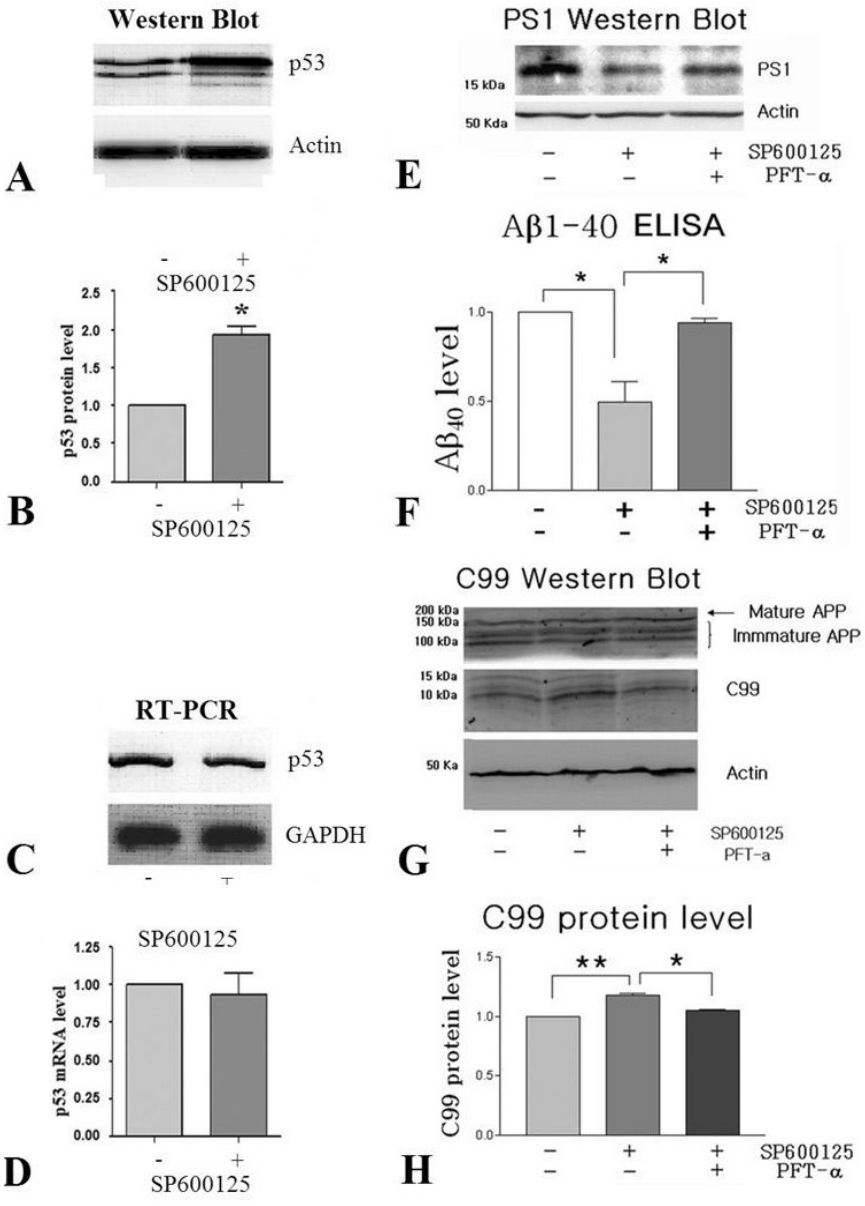

Figure 1. JNK inhibitor SP600125 and p53 inhibitor PFT-alpha control PS1/gamma-secretase activity in human neuroblastoma SK-N-SH cells. SK-N-SH cells were treated with chemical inhibitors at ~80% confluence. ***A.*** The changes in p53 protein expression with or without 20uM SP600125 treatment were detected by Western blotting method. ***B.*** p53 protein levels were normalized by actin protein levels and shown as means ± SEM. n=3. * P<0.05. ***C.*** The changes in p53 mRNA level were detected by RT-PCR after 12 hour treatment of 20uM SP600125. ***D.*** p53 mRNA levels were normalized by GAPDH mRNA levels and shown as means ± SEM. n=3. ***E.*** The effect of SP600125 on the PS1 protein expression was tested by Western blotting using PS1 antibody. The SK-N-SH cells were pretreated with DMSO or 50uM PFT-alpha for 2 hours before 20uM SP600125 was added. After 24 hour incubation with SP600125, PS1 protein was detected from 50ug total lysates. ***F.*** The levels of $Abeta_{40}$ peptide were measured by sandwich ELISA method according to the manufacturer's guide and presented as means ± SEM from 4 different determinations. $Abeta_{40}$ values from the cultures treated with SP600125 in the presence or absence of PFT-alpha are relative to the values with control cultures treated with DMSO which was set to "1" (Control $Abeta_{40}$ levels ranged from 3 to 6 pg/mL). * P<0.05. ***G.*** In order to detect C99 protein levels, SK-N-SH cells were transfected with pcDNA3-APP751 construct and then treated with SP600125 in the presence or absence of PFT-alpha for 24 hours. 50ug total proteins were used for the Western blotting using APP C- terminal antibody. APP blots show the similar transfection efficiency between the samples (Top panel). The protein bands were visualized using ECL system. ***H.*** The C99 protein levels were quantified using Labworks Image Analysis Software and presented as means ± SEM from 3 different determinations. * P<0.05 **P<0.01.

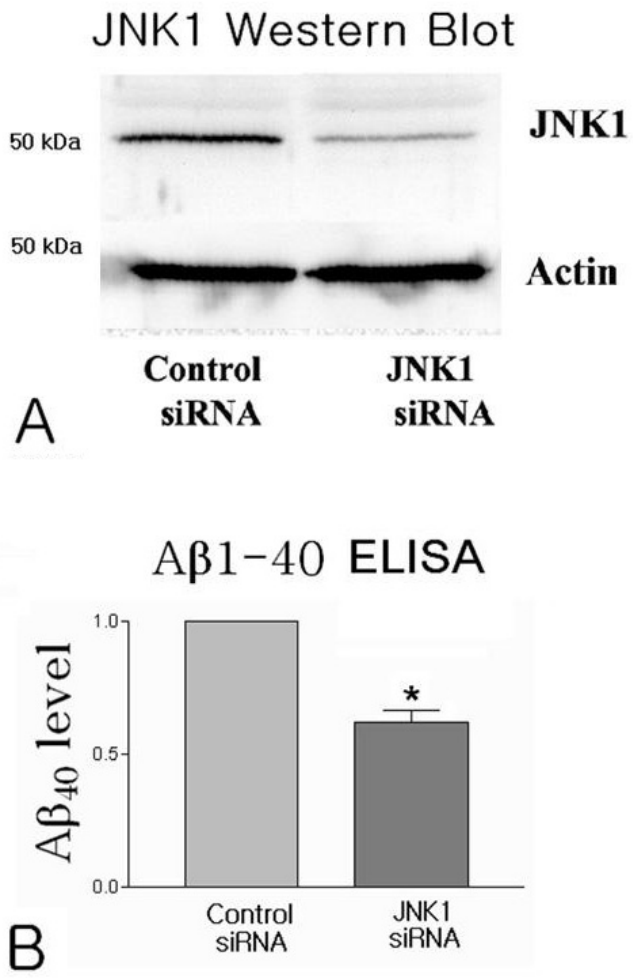

Figure 2. The effect of JNK1 siRNA on PS1/gamma-secretase activity. SK-N-SH cells were transfected with JNK1 siRNAs or control siRNAs (Dharmacon, CO) using Dharmafect siRNA transfection reagent. ***A.*** After 2 days of transfection, the suppression of JNK1 expression was tested by JNK1 Western blotting (1). 50ug total proteins were used for the Western blot analysis using antibodies against actin and JNK1. The blots were visualized by ECL detection system. ***B.*** The levels of $Abeta_{40}$ peptide from the conditioned media of SK-N-SH cells transfected with control siRNA or JNK1 siRNA were measured by ELISA method according to the manufacturer's guide and presented as means ± SEM from 3 different determinations. (Control $Abeta_{40}$ levels ranged from 3 to 6 pg/mL). * $P<0.05$.

represses PS1mRNA expression by ~70% (Figures 3C and 3D). Therefore, this data and our previous study (1) suggest that transcriptional repression of PS1 by p53 is mediated by chromatin remodeling via deacetylation of histone due to decreased binding of Ets 1/ 2 transcription factors to the PS1 promoter. Transient transfection of pCMV-p53 also increased p53 protein level substantially and decreased PS1 protein level by ~50% (Figures 3E and 3F).

Next, we asked if the reduction of PS1 protein expression causes the downregulation of the PS1/gamma-secretase activity. To measure the PS1/gamma-secretase activity, we measured $Abeta_{40}$ levels in conditioned medium of the pCMV or pCMV-p53 transfected cells by sandwich ELISA method. $Abeta_{40}$ level was significantly decreased in cells transfected with pCMV-p53 (Figure 3G). Therefore, this data supports the hypothesis that suppression of PS1 expression level reduces PS1/gamma-secretase activity.

4.3. ZNF237 and CHD3 decrease PS1 protein and $Abeta_{40}$ levels in human neuroblastoma SH-SY5Y cells by suppressing PS1 transcription via deacetylation of histone at the PS1 promoter

We used neuroblastoma SH-SY5Y cell line in these experiments instead of SK-N-SH cell line, because SH-SY5Y cell line does not express ZNF237 whereas SK-N-SH cell line does (18). In previous report, we showed that ZNF237 and CHD3 interacted with the Ets transcription factor ERM, an activator of the PS1 gene, to decrease PS1 promoter activity in human neuroblastoma SH-SY5Y cells (18, 19) suggesting that ZNF237 and CHD3 are the repressors of the PS1 gene. We dissected the mechanism by which ZNF237 and CHD3 inhibit PS1

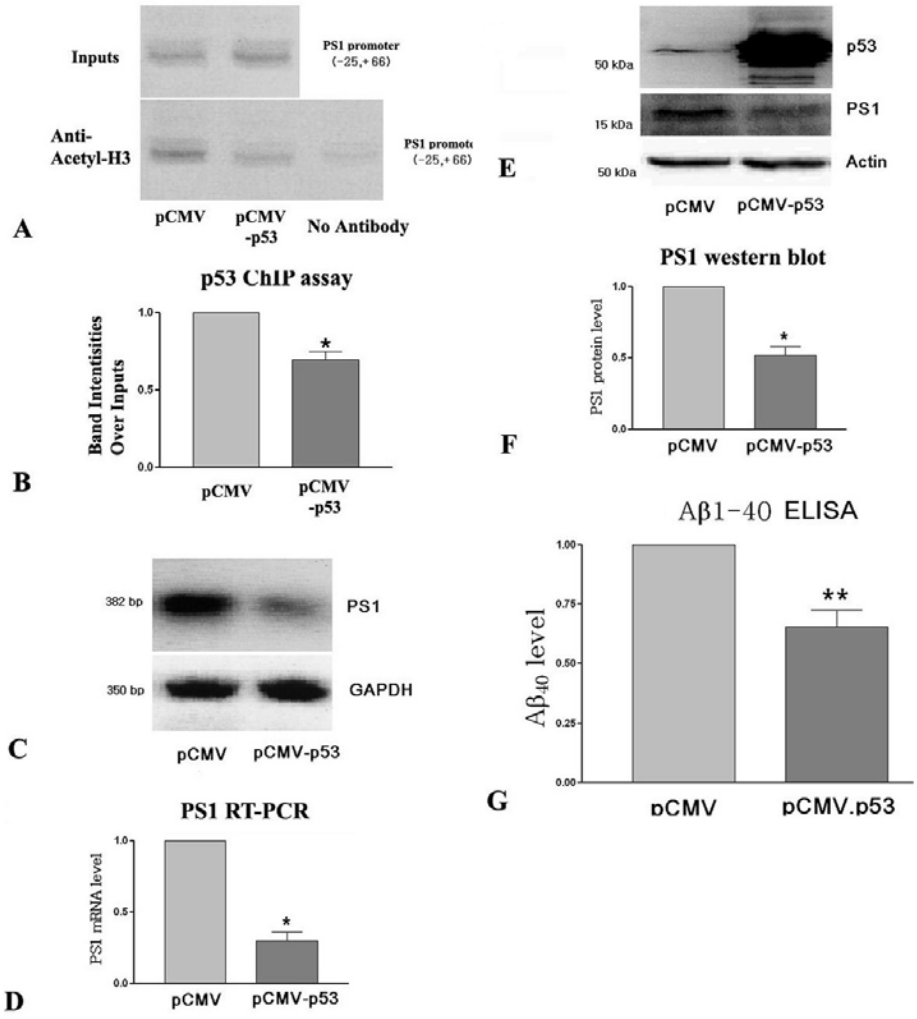

Figure 3. p53 represses PS1 expression and PS1/gamma-secretase activity in human neuroblastoma SK-N-SH cells. A. Acetylated histone H3 was detected as a marker for the transcriptional activity on the PS1 promoter by ChIP assay. SK-N-SH cells were transfected with 10ug of pCMV vectors or pCMV-p53 expression vectors using lipofectamine 2000. After 48 hours of transfection, cross-linked DNA-protein complexes were precipitated using antibody against acetylated histone H3. The precipitated DNA was analyzed by PCR to detect the PS1 promoter sequence from -25 to +66. 5% total lysates without immunoprecipitation were used as input controls (upper panel). The cell lysates after immunoprecipitation with anti-Ac-H3 antibody were used for PCR amplification of the PS1 promoter sequence (lower panel). B. Bar graphs represent the fold changes in the levels of acetylated histone H3 on the cellular PS1 promoter after p53 transfection normalized over the input controls and shown as means ± SEM. *P<0.05. C. The effect of pCMV-p53 transfection on PS1 mRNA expression level was tested by RT-PCR. SK-N-SH cells were transfected with 10ug of pCMV vectors or pCMV-53 expression vectors for 24 hours and lysed with Trizol reagent. cDNA was generated from 2μg total RNA with random primers and PCR-amplified with 35 and 27 cycles for PS1 and GAPDH respectively. PCR products (382 bps for PS1 and 350 bps for GAPDH) were run on 1% agarose gel and visualized by ethidium staining. D. Band intensities of the RT-PCR products were quantified using Labworks Image Analysis Software. PS1 mRNA levels were normalized against GAPDH mRNA levels and presented as means ± SEM. n=3. * P<0.05. E. In order to test the direct role of p53 on PS1 expression and PS1/gamma-secretase activity, SK-N-SH cells were transfected with 10ug of pCMV vectors or p53 expression vectors using lipofectamine 2000. The transfected cells were cultured 2 more days and lysed for Western blotting with antibodies specific to p53, PS1, and actin. The protein bands were visualized using ECL system. F. The PS1 protein levels quantified using Labworks Image Analysis Software were normalized against actin and presented as means ± SEM from 4 different determinations. * P<0.05. G. The culture media from pCMV or pCMV-p53 transfected SK-N-SH cells were collected and 100uL of conditioned medium was used for $Abeta_{40}$ detection according to the manufacturer's instructions. The levels of $Abeta_{1-40}$ peptide were presented as means ± SEM from 4 different determinations. $Abeta_{40}$ values from the pCMV-p53 transfected cultures are relative to the values with the pCMV control transfection which was set to "1" (Control $Abeta_{40}$ levels ranged from 3 to 6 pg/mL). ** P<0.01.

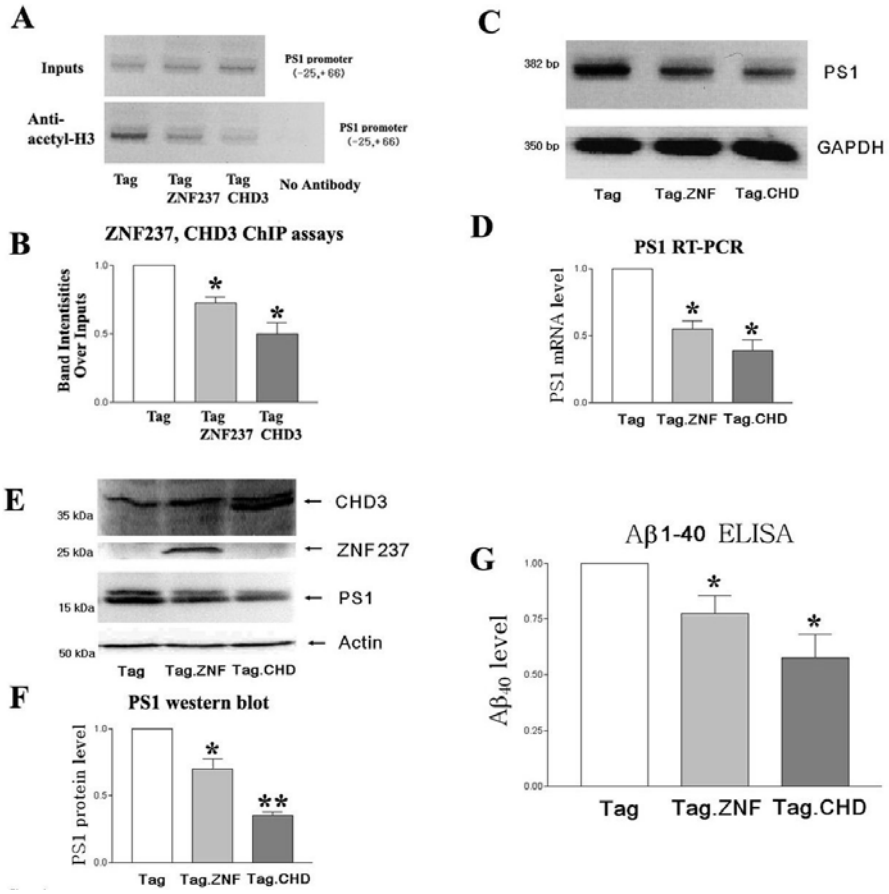

Figure 4. Transfection of ZNF237 and CHD3 decreases the PS1 expression and PS1/gamma-secretase activity in human neuroblastoma SH-SY5Y cells. A. Transcriptional activity on the PS1 promoter was measured by acetylated histone H3 associated with the PS1 promoter. PS1 promoter sequence was precipitated with anti-Ac-H3 as described in materials and methods and detected by PCR from SH-SY5Y cells transfected with 10ug of pCMV-Tag2 or pCMV-Tag2-ZNF237 (208 amino acid form) or pCMV-Tag2-CHD3 (amino acids 1676-2000). 5% total lysates were used for input controls (upper panel). After immunoprecipitation, PS1 promoter sequence was detected by PCR. B. The fold changes in the acetylated histone H3 on the PS1 promoter after transfection with ZNF237 or CHD3 were normalized over the input controls and shown as means ± SEM. *P<0.05. C. PS1 mRNA levels from the Tag or Tag-ZNF237 or Tag-CHD3 expressing SH-SY5Y cells were measured by PS1 RT-PCR. Total cDNAs were prepared from 2ug RNA using AMV RTase with random primers. 35 cycles of PCR was performed with the primers for PS1 and 27 cycles of PCR was performed with the primers for GAPDH. D. The PS1 mRNA levels were quantified and normalized against the levels of GAPDH mRNA. The levels of PS1 mRNA were presented as means ± SEM from 3 independent determinations. * P<0.05. E. 50ug of total proteins from Tag or Tag-ZNF237 or Tag-CHD3 expressing SH-SY5Y cells were analyzed for the Western blotting using antibodies against anti-Flag (Sigma, F1804), anti-PS1 (Chemicon, AB5232) and anti-actin (Sigma, A5441). A protein band unrelated to CHD3 appears in all samples. The nature of this non-specific protein band is unknown. F. The intensities of the PS1 protein bands were quantified using Labworks Image Software and presented as means ± SEM from 3 independent determinations. * P<0.05 **P<0.01. G. $Abeta_{40}$ levels from the cell culture media of SH-SY5Y cells transfected with Tag or Tag-ZNF237 or Tag-CHD3 were measured using beta-amyloid 1-40 sandwich ELISA kit (Biosource, KHB341) as instructed in manufacturer's guide. 100uL conditioned media were applied to each well and the absorbance was read at 450nm. The $Abeta_{40}$ levels are represented as means ± SEM from 4 different determinations. $Abeta_{40}$ values from the ZNF237 or CHD3 transfected cultures are relative to the values with the pCMV-Tag2 control transfection which was set to "1" (Control $Abeta_{40}$ levels ranged from 3 to 6 pg/mL). * P<0.05.

transcription by deacetylation of histone at the PS1 promoter. We tested if acetylation of histone at the PS1 promoter, PS1 mRNA, and protein levels are suppressed by the transfection of ZNF237 and CHD3 genes into SH-SY5Y cells. SH-SY5Y cells were transiently transfected with empty pCMV-Tag2 vector or pCMV-Tag2-ZNF237 or pCMV-Tag2-CHD3 for 2 days. Transient transfection with pCMV-Tag2-ZNF237 and pCMV-Tag2-CHD3 decreased acetylation of histone at the PS1 promoter as detected by ChIP assay (Figures 4A and 4B). Transient transfection with pCMV-Tag2-ZNF237 and pCMV-Tag2-CHD3 also decreased PS1mRNA levels by ~50% and ~60% respectively (Figures 4C and 4D). Transient transfection with pCMV-Tag2-ZNF237 and pCMV-Tag2-CHD3 also increased the expression of Tag-ZNF237 and Tag-CHD3 proteins (Figure 4E, lanes 2 and 3), and decreased PS1

protein level by ~30% and ~60% (Figures 4E and 4F) respectively in SH-SY5Y cells. Furthermore, the $Abeta_{40}$ levels in conditioned media of the cells transiently transfected with pCMV-Tag2-ZNF237 and pCMV-Tag2-CHD3 were reduced by ~25% and ~40% respectively compared to vector transfected cells (Figure 4G). Therefore, these data suggest that the repressive effects of ZNF237 and CHD3 on PS1 protein expression are directly correlated with the decrease of PS1/gamma-secretase activity.

4.4. Ets2 transcription factor enhances PS1 protein and $Abeta_{40}$ levels in human neuroblastoma SK-N-SH cells by activation of PS1 transcription via increased acetylation of histone at the PS1 promoter

We reported previously that Ets2 transcription factor recognizes -10 Ets motif on the PS1 promoter and increases PS1 promoter activity by CAT-reporter assay in human neuroblastoma SK-N-SH cells (15). In this study we asked whether the increased PS1 promoter activity by Ets2 is translated into the increased acetylation of histone at the PS1 promoter as well as increased expressions of PS1 mRNA and protein levels. We performed ChIP assay, RT-PCR, and Western blotting to evaluate the effect of Ets2 on histone acetylation at the PS1 promoter, and the PS1 mRNA and protein expression in SK-N-SH cells transfected with empty pC1 vector or pC1.Ets2 expression vectors. Transient transfection with pC1.Ets2 increased acetylation of histone at the PS1 promoter (Figures 5A and 5B). Transient transfection with pC1.Ets2 increased PS1 mRNA level (Figures 5C and 5D) by ~2 fold and PS1 protein level by ~2 fold (Figures 5E and 5F). Therefore, these data show that transcriptional activation of the PS1 gene by Ets2 increases PS1 protein level. To determine if the increased PS1 expression is associated with the enhanced PS1/gamma-secretase activity, we also measured $Abeta_{40}$ peptides from the culture media of pC1 or pC1.Ets2 expressing SK-N-SH cells. Our $Abeta_{1-40}$ ELISA data showed that secreted $Abeta_{40}$ level was increased by ~2 fold in pC1.Ets2 transfected cells relative to pC1 transfected cells (Figure 5G). This data suggests that the upregulation of PS1 gene expression by Ets2 transcription factor increases the PS1/gamma-secretase activity. It is also to be noted that detection of $Abeta_{1-40}$ in our experiments was carried out without overexpressing APP or C99. This allows us to get more physiological values of secreted $Abeta_{1-40}$.

4.5. JNK inhibitor SP600125 and Ets2 transcription factor do not alter the expression of another PS1/gamma-secretase component nicastrin (NCT)

The gamma-secretase is an equimolecular complex of four noncovalently associated integral membrane proteins, PS1, nicastrin (NCT), Aph-1, and Pen-2 (9). PS1 acts as the catalytic subunit of the gamma-secretase enzyme (10). We have shown that suppression of PS1 transcription by JNK inhibitor SP600125 or p53 or ZNF237 or CHD3 reduces PS1 protein expression and gamma-secretase enzyme activity. On the contrary, activation of PS1 transcription by Ets2 enhances PS1 protein expression and gamma-secretase enzyme activity. In order to determine whether regulation of PS1 expression also controls the expression of any other components (NCT, Aph-1, and Pen-2) of the gamma-secretase enzyme, we treated SK-N-SH cells with or without JNK inhibitor. As shown in Figure 6A, treatment of SK-N-SH cells with 20uM SP600125 decreased PS1 expression (Figure 1E) but did not have any detectable effect on the expression of both mature (120 kDa) and immature (110 kDa) forms of NCT. Similarly, transient transfection of SK-N-SH cells with pC1.Ets2 increased Ets2 and PS1 expression (Figure 5E) but did not alter the expression of NCT (Figure 6B). These results appear to suggest that regulation of PS1 expression has no effect on the expression of other components of the PS1/gamma-secretase enzyme.

4. 6. HDAC inhibitor trichostatin-A (TSA) increases histone acetylation and augments PS1 expression in SK-N-SH cells

We showed before that inhibition of PS1 expression by SP600125, p53, ZNF237, and CHD3 is mediated by deacetylation of histone at the PS1 promoter. On the contrary, activation of PS1 expression by Ets2 is mediated by hyper-acetylation of histone at the PS1 promoter. Deacetylation of histone is mediated by histone deacetylase (HDACs). Therefore, HDAC inhibitor would prevent deacetylation of histone and increase acetylation of histone to activate PS1 expression. We now showed that HDAC inhibitor TSA augmented PS1 protein level and also increased acetylation of histone. SK-N-SH cells were treated with 7.5ug/ml or 15ug/ml of TSA in DMSO for 24 hr . Total cellular protein was prepared for Western blot analysis. We could not detect acetylation of H3 but detect PS1 protein expression in cellular extract prepared from DMSO treated cells (Figure 7A, lane 1). On the contrary, treatment of cells with TSA increased acetylation of histone H3 and also PS1 protein level substantially (Figure 7). These results suggest that although the amount of Ac-H3 in the chromatin in very low in the normal cells, the level of Ac-H3 at the PS1 promoter is high enough (Figure 4A, lane 1) to mediate PS1 transcription. Inhibition of HDACs by TSA augments the amount of Ac-H3 both in the chromatin and in the PS1 promoter resulting in the activation of PS1 expression. These data also confirm the hypothesis that modulation of the acetylation of histone controls PS1 expression.

5. DISCUSSION

The data presented here indicate that the transcriptional regulation of PS1 expression is mediated by acetylation and deacetylation of histone at the PS1 promoter. Our data also suggest that regulation of PS1 transcription controls PS1/gamma-secretase activity as shown by measuring the level of secreted $Abeta_{40}$ peptide, a major Abeta species of the PS1/gamma-secretase mediated metabolism of APP, in conditioned medium of neuroblastoma cells. Various PS1 mutations and PS1 deficiency are known to alter the PS1/gamma-secretase activity and thus the production of Abeta peptides (25-32). In addition, PS1 contains two aspartate residues responsible for the aspartyl protease activity of gamma-secretase (33, 10).

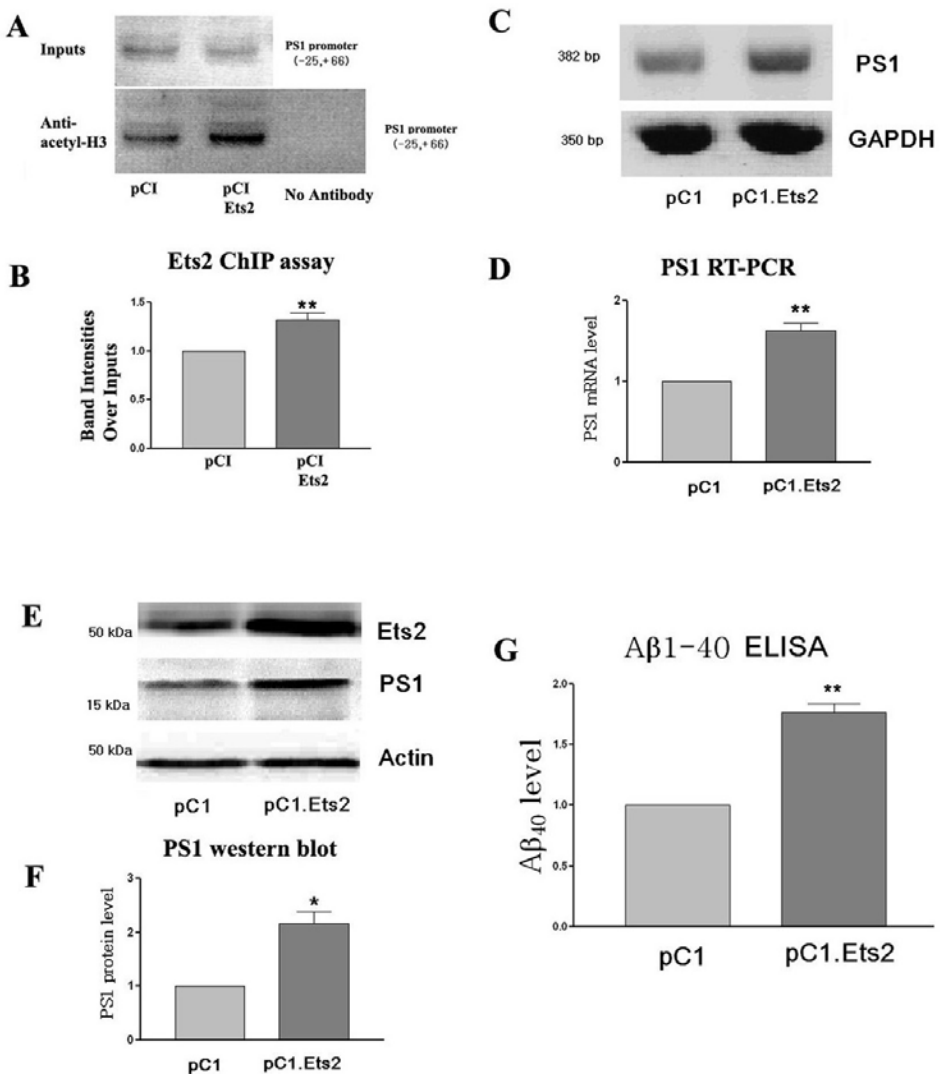

Figure 5. Ets2 enhances PS1 expression and PS1/gamma-secretase activity in human neuroblastoma SK-N-SH cells. A. The level of acetylated histone H3 on the PS1 promoter was measured by Chromatin immunoprecipitation assay. SK-N-SH cells were transfected with 10ug of empty pC1 or pC1.Ets2 expressing vector by lipofectamine 2000 for 48 hours before protein-DNA complex was fixed with 1% formaldehyde. 5% total lysates were used as input controls (upper panel). PS1 promoter sequence from -25 to +66 in the DNA-protein complex precipitated with anti-Ac-H3 was detected by PCR. B. The changes in the acetylated histone H3 on the PS1 promoter was normalized over input controls and presented as means ± SEM. ** P<001. C. The effect of Ets2 transcription factor on the PS1 mRNA expression was tested by RT-PCR. Total RNA from SK-N-SH cells transfected with empty vectors or Ets2 expressing vectors was reverse transcribed using random primers and resulting cDNA was amplified with primers for PS1 and GAPDH. PCR products were run on 1% agarose gel and visualized using Ethidium staining. D. The DNA gel bands were quantified using Labworks Image Analysis Software. PS1 mRNA levels were normalized against GAPDH mRNA levels and presented as means ± SEM (n=3). ** P<001. E. The role of Ets2 transcription factor on the PS1 protein expression was tested by Western blotting. The blots were visualized using ECL detection system. F. The band intensities for PS1 protein were quantified using Labworks Image Analysis Software. The levels of PS1 protein were normalized against actin protein levels and presented as means ± SEM from 3 independent determinations. * P<0.05. G. The effect of Ets2 transcription factor on the PS1/gamma-secretase activity was tested by Abeta1-40 sandwich ELISA. SK-N-SH cells were transfected with 10ug of empty vectors or Ets2 expressing vectors in 60mm culture dishes. The culture media were replaced with 1.5mL fresh media and the cells were further cultured for 24 hours. $Abeta_{40}$ levels from the conditioned media from the pC1 or pC1.Ets2 transfected cells were measured using beta-Amyloid 1-40 sandwich ELISA kit (Biosource, KHB341) as instructed in manufacturer's guide. 100uL conditioned media were applied to each well and the absorbance was read at 450nm. The $Abeta_{40}$ levels are represented as means ± SEM from 4 different determinations. $Abeta_{40}$ values from the pCI.Ets2 transfected cultures are relative to the values with control transfection which was set to "1" (Control $Abeta_{40}$ levels ranged from 3to 6 pg/mL), ** P<0.01.

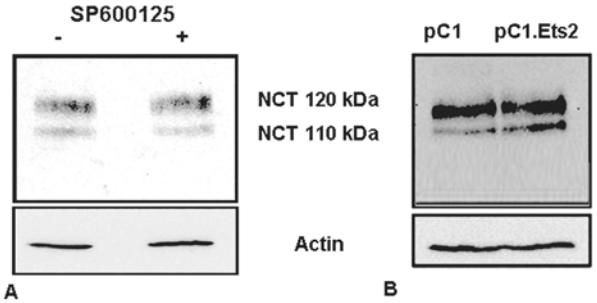

Figure 6. JNK inhibitor SP600125 and Ets2 have no effect on the expression of nicatrin (NCT) in human neuroblastoma SK-N-SH cells. A. The effect of SP600125 on NCT protein expression was tested by Western blotting using actin, and nicastrin antibodies. The SK-N-SH cells were treated with DMSO or 20uM SP600125 in DMSO. After 24 hour incubation with SP600125, NCT protein was detected from 50ug total lysates. The positions of actin, mature (120 kDa) and immature (110 kDa) forms of NCT are marked (n=3). B. SK-N-SH cells were transfected with 10ug of empty pC1 or pC1.Ets2 expressing vector by lipofectamine 2000 for 48 hours. The role of Ets2 transcription factor on NCT protein expression was tested by Western blotting. The blots were visualized using ECL detection system. Positions of protein bands are marked (n=3).

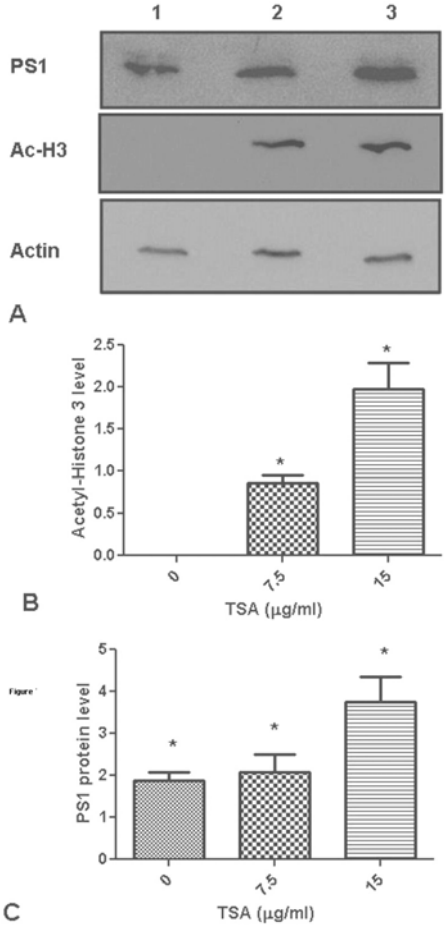

Figure 7. HDAC inhibitor trichostatin-A increases histone acetylation and augments PS1 expression in SK-N-SH cells. SK-N-SH cells were treated with DMSO or 7.5ug/ml or 15ug/ml of TSA in DMSO at ~80% confluence. A. The effect of TSA on the PS1 protein expression and acetylation of H3 were tested by Western blotting using actin, PS1 and Ac-H3 antibodies. 20ug of protein were used for Western blot analysis. The blots were visualized using ECL detection system. Positions of Actin (42 kDa), PS1 (20 kDa-CTF) and Ac-H3 (17 kDa) protein bands are marked. B. The band intensities for Ac-H3 protein were quantified using Labworks Image Analysis Software. The levels of Ac-H3 protein were normalized against actin protein levels and presented as means ± SEM from 3 independent determinations. * $P<0.05$. C. The band intensities for PS1 protein were quantified using Labworks Image Analysis Software. The levels of PS1 protein were normalized against actin protein levels and presented as means ± SEM from 3 independent determinations. * $P<0.05$.

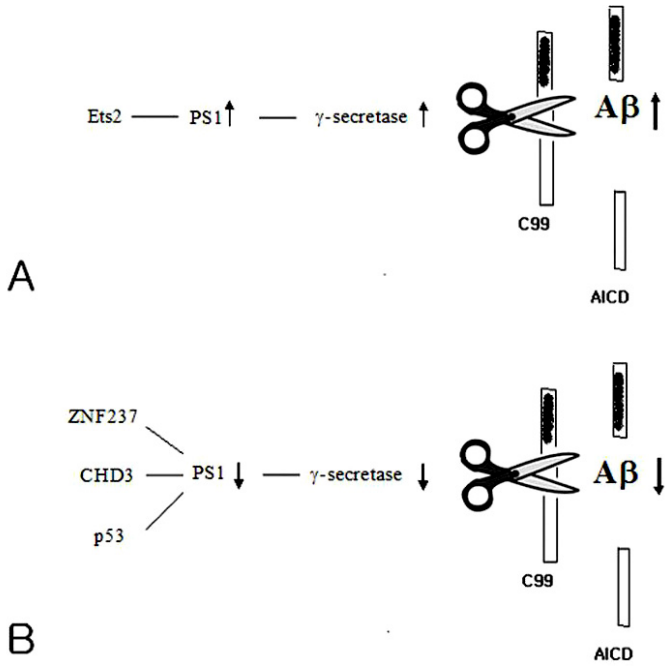

Figure 8. Hypothetical scheme of the regulation of PS1/gamma-secretase activity. PS1 is a critical regulator of gamma-secretase activity. A. Transcriptional upregulation of PS1 by Ets2 increases PS1 expression. As a result, PS1 mediated gamma-secretase activity and the production of $Abeta_{40}$ are increased. B. Transcriptional downregulation of PS1 by ZNF237, CHD3, and p53 decreases PS1 expression. Hence PS1 mediated gamma-secretase activity and the production of $Abeta_{40}$ are decreased.

To gain insight into how transcriptional regulation of the PS1 gene may potentially control PS1/gamma-secretase activity, we have previously identified several transcription factors that regulate PS1 transcription (14-19). Several Ets factors that recognize specifically the -10 Ets motif of the PS1 promoter have been identified by yeast one-hybrid selection (16, 17). Ets transcription factors Ets2 and ERM specifically bind to the -10 Ets element and transactivate PS1 transcription in transient transfection assay (16, 17). Using the C-terminal 415 amino acid of ERM as bait for yeast two hybrid selection in a human brain cDNA library two proteins were identified, which interact with Ets transcription factor ERM (18, 19). One ERM-interacting protein was a zinc finger protein ZNF237 (18) and the other protein was a chromatin remodeling factor CHD3/ZFH (19). ZNF237 is a member of the myeloproliferative and mental retardation motif (MYM) gene family (34). ZNF237 is widely expressed in different tissues in eukaryotes under several forms derived by alternative splicing, including a large 382 amino acid form containing a single MYM domain, and 2 shorter forms of 208 and 213 amino acids respectively that do not contain MYM domain (34). Both the 382 as well as the 208 amino acid forms have been shown to repress PS1 transcription in neuroblastoma cells (18). The C-terminal fragment (amino acids 1676 -2000) of CHD3, a component of the histone deacetylase (HDACs) complex (35), has been shown to interact with ERM and repress the transcription of the PS1 gene (19). Transfection of SH-SY5Y cells with CHD3 expression vector inhibited PS1 transcription and PS1 protein expression as well as increased recruitment of CHD3 into the PS1 promoter (19). Conversely, treatment of cells with HDAC inhibitor TSA enhanced acetylation of histone and augmented PS1 expression (Figure 7). These data suggest that chromatin remodeling and deacetylation of histones by CHD3-containing HDAC may play a crucial role in the repression of PS1 transcription.

In this report, we extend our previous studies to correlate the transcriptional regulation of the PS1 gene with the PS1/gamma-secretase activity. We now show that activation of PS1 transcription by Ets2 increased PS1 protein and secreted $Abeta_{40}$ levels by augmenting acetylation of histone at the PS1 promoter. On the contrary, repression of PS1 transcription by p53, ZNF237 and CHD3 reduced PS1 protein and secreted $Abeta_{40}$ levels by decreasing acetylation of histone at the PS1 promoter. Therefore, our studies document that regulation of PS1 transcription leads to the changes in PS1/gamma-secretase activity (Figure 8). Our studies also demonstrate that it is potentially possible to reduce APP-processing and production of Abeta peptide by inhibiting histone acetyl transferase (HATs) activity or augmenting histone deacetylase (HDACs) activity.

JNK inhibitor SP600125 was shown to suppress PS1 expression by upregulating p53 (1). We have previously reported that JNK inhibitor SP600125 enhances p53-Ets1/2 interaction and thus interferes with the DNA binding of Ets1/2 onto the PS1 promoter (1) leading to the repression of PS1 transcription and PS1 protein level. In

this report, we tested the effects of JNK inhibitor and p53 inhibitor on the PS1/gamma-secretase activity. Treatment of cells with SP600125 decreased secreted $Abeta_{40}$ level which is partially recovered by a p53 inhibitor, PFT-alpha. We also verified ELISA data by measuring the protein level of C99, a substrate of PS1/gamma-secretase proteolysis. Furthermore, the role of JNK on the regulation of the PS1/gamma-secretase activity was confirmed by JNK1 siRNA. Since, JNK inhibitor and JNK1 siRNA represses PS1 expression through increasing p53 protein level (1), we tested the direct role of p53 on the PS1/gamma-secretase activity. The transient transfection of p53 also decreased PS1 transcription, PS1 protein level, and secreted $Abeta_{40}$ in neuroblastoma cells. Therefore, we have demonstrated that inhibition of JNK pathway represses PS1 transcription and the PS1/gamma-secretase activity via upregulation of p53 protein expression. In conclusion, our studies point to a potential new approach to use JNK-siRNA or JNK inhibitor SP600125 or activator of HDACs, and inhibitor of HATs to target PS1 transcription for therapy to prevent or treat Alzheimer's disease.

Mutations of the PS1 gene (FADPS1) that cause familial AD also increase p53 activity by augmenting p53 transcription (36). Enhanced PS1/gamma-secretase activity by FADPS1 generates higher level of AICD by proteolytic processing of APP, which subsequently activates p53 transcription and p53 protein level triggering p53-mediated apoptosis in FAD brains (36). On the contrary our results (1) suggest that increased p53 level downregulates PS1 expression and PS1/gamma-secretase activity suggesting a feed back mechanism of transcriptional regulation of PS1 and p53 genes. Therefore, inhibition of FADPS1 transcription by SP600125 in FAD brains would not only inhibit the production of amyloidogenic $Abeta_{40//42}$ peptide to reduce p53-independent apoptosis but also decrease the amount of AICD resulting in the inhibition of p53 transcription and p53 protein level to reduce p53-dependent apoptosis. Therefore, JNK inhibitor SP600125 may have potential therapeutic value to treat both sporadic and familial forms of Alzheimer's disease by reducing both p53-independent and p53-dependent apoptosis of neurons.

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Abbreviations: AD: Alzheimer's disease, Aph-1: Anterior pharynx-1, APP: Amyloid precursor protein, AICD: APP intra-cellular domain, CHD3: Chromodomain helicase DNA binding protein 3, Ets: Avian erythroblastosis virus E26 oncogene homologue, FAD: Familial Alzheimer's disease or Early onset Alzheimer's disease, HDAC: Histone deacetylase, JNK: c-jun-NH2-terminal kinase, PCR: Polymerase chain reaction, Pen-2: Presenilin enhancer-2, NCT: Nicastrin, PS1: Presenilin-1, RT-PCR: Reverse transcription and polymerase chain reaction, TSA: trichostatin-A, ZNF237: Zinc finger protein 237

Key Words: Presenilin-1, Transcription, Gamma-Secretase, $Abeta_{40}$

Send correspondence to: Hriday K. Das, Department of Pharmacology and Neuroscience, University of North Texas Health Science Center at Fort Worth, 3500 Camp Bowie Boulevard, Fort Worth, Texas 76107, Tel: 817-735-5448, Fax: 817-735-2091, E-mail: hdas@hsc.unt.edu

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