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ORIGINAL ARTICLE

Role and regulation of p27 in neuronal apoptosis

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Abstract

It is necessary for the cell-cycle machinery of neurons to be suppressed to promote differentiation and maintenance of their terminally differentiated state. Reactivation of the cell cycle in response to neurotoxic insults leads to neuronal cell death and some cell-cycle-related proteins contribute to the process. p27 kip1 (p27), an inhibitor of cyclin-dependent kinases, prevents unwarranted cyclin-dependent kinase activation. In this study, we have elucidated a novel mechanism via which p27 promotes apoptosis of neurons stimulated by neurotoxic amyloid peptide $A\beta_{42}$ (Amyloid $\beta_{1\text{-}42}$ peptide). Co-immunoprecipitation analysis revealed that p27 promotes interaction between Cyclin-dependent kinase 5 (Cdk5) and cyclin D1, which is induced by $A\beta_{42}$ in cortical neurons. As a result, Cdk5 is sequestered from its neuronal activator p35 resulting in kinase deactivation. The depletion of p27, which was achieved by specific siRNA, restored Cdk5/p35 interaction by preventing association between Cdk5 and cyclin D1 and also abrogated $A\beta_{42}$ induced apoptosis of cortical neurons. Furthermore, analysis of cell cycle markers suggested that p27 may play a role in $A\beta_{42}$ induced aberrant cell cycle progression of neurons, which may result in apoptosis. These findings provide novel insights into how p27, which otherwise performs important neuronal functions, may become deleterious to neurons under neurotoxic conditions.

Keywords: Apoptosis, Cdk5, Cyclin, neurons, p27.

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Neuronal development is accompanied by a loss of neurons. Such death of neurons occurs as a result of the competition for trophic factor support or their inability to innervate the target tissue. Neuronal apoptosis is also a major cause that contributes to neurodegenerative disorders like Alzheimer's disease (AD), Parkinson's disease, Amylotrophic Lateral sclerosis etc. (Becker and Bonni 2004). Deregulation of the neuronal cell cycle machinery strikingly contributes to the apoptosis of a subset of neurons when they face insult *in vitro* or *in vivo* (Herrup and Busser 1995; Nagy *et al.* 1997; Busser *et al.* 1998; Park *et al.* 1998; Chong *et al.* 2006; Modi *et al.* 2012). Despite various efforts in this direction, molecular mechanisms that underlie cell-cycle-related neuronal apoptosis (CRNA) are poorly understood.

Levels of cell-cycle proteins are elevated in apoptotic neurons from the patients of AD (Busser *et al.* 1998; Yang *et al.* 2003). The up-regulation of the intermediates of cell-cycle machinery was demonstrated in *in vitro* and animal models of AD (Herrup and Busser 1995; Giovanni *et al.* 1999; Yang *et al.* 2003; Modi *et al.* 2012). The aberrant activation of cell cycle components upon neurotoxic stimulus is mediated via signaling pathways, which otherwise have been implicated in neuronal survival. Recently, we demonstrated that aberrant activation of the mitogen activated protein kinase kinase-extracellular signal-regulated kinase (MEK-ERK) pathway, which typically promotes neuronal differentiation and survival, is hyperactivated upon neurotoxic insult by amyloid peptide $A\beta_{42}$ (Modi *et al.* 2012). As a result, cyclin D1 levels are elevated resulting in CRNA (Modi *et al.* 2012). In order to maintain the differentiated state of neurons various factors like cyclin-dependent kinase (CDK) inhibitors, which are elevated during neuronal differentiation and other regulators of the cell cycle suppression, act synergistically in a tightly regulated fashion. Cyclin-dependent kinase inhibitors (CKIs) of Cip/Kip family and

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Abbreviations used: AD, Alzheimer's disease; APP, amyloid precursor protein; $A\beta_{42}$, amyloid $\beta_{1\text{-}42}$ peptide; BrdU, 5′ Bromo- 2′-deoxy Uridine; BSA, bovine serum albumin; Cdk5, cyclin-dependent kinase 5; CDK, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; CRNA, cell-cycle-related neuronal apoptosis; IP, immunoprecipitate; MEK-ERK, mitogen-activated protein kinase kinase-extracellular signal-regulated kinase; PBS, phosphate-buffered saline; PC 12, rat pheochromocytoma; PCNA, proliferating cell nuclear antigen; PI, propidium iodide; PS1, presenillin 1; shRNA, short hairpin RNA; siRNA, small interfering RNA; Tg, transgenic; TUNEL, terminal deoxy nucleotidyl transferase dUTP nick end labeling.

INK4 family member play an important role in suppressing the neuronal cell cycle (Zindy *et al.* 1997; Erhardt and Pittman 1998; Cunningham *et al.* 2002). Besides their role in cell-cycle suppression, CKIs play an important role in neurogenesis, neuronal differentiation and migration (Parker *et al.* 1995; Erhardt and Pittman 1998; Zindy *et al.* 1999; Zheng *et al.* 2010). It has been previously demonstrated that Cdk5, a neuron-specific Cdk, in complex with p35/p39 remains active despite the presence of abundant cell-cycle inhibitors (Lee *et al.* 1996). In this study, we have identified a novel function for p27 in promoting $A\beta_{42}$ induced neuronal apoptosis. p27 (also known as KIP1, and encoded by *CDKN1B*) is a tumor suppressor protein (Fero *et al.* 1998; Kossatz and Malek 2007) and it is expressed highly in quiescent cells (Zindy *et al.* 1999; Dyer and Cepko 2001; Cunningham *et al.* 2002; Qiu *et al.* 2009). It binds to and suppresses the activity of Cdks. p27 has Cdk inhibitory region which includes cyclin-binding domain and Cdk binding domain (Zeng *et al.* 2000; Jakel *et al.* 2012). The existence of ternary complex between Cdk, cyclin and p27 is thermodynamically more stable than the binary conformation (Bowman *et al.* 2006). p27 depletion elevates cell-cycle activity and death in cortical neurons (Akashiba *et al.* 2006). Besides their role in cell-cycle regulation Cip/Kip proteins have other important neuronal functions such as neurogenesis (Zheng *et al.* 2010; Andreu *et al.* 2015), neuronal differentiation (Lee *et al.* 1996; Hengst and Reed 1998; Baldassarre *et al.* 2000; Zheng *et al.* 2010; Andreu *et al.* 2015) and neuronal migration (Kawauchi *et al.* 2006; Nguyen *et al.* 2006; Itoh *et al.* 2007; Godin *et al.* 2012). During CNS (central nervous system) development, it may promote neuronal differentiation by stabilizing neurogenin 2 (Nguyen *et al.* 2006; Itoh *et al.* 2007; Godin *et al.* 2012). It also promotes migration of neurons in the cortical plate (Nguyen *et al.* 2006; Itoh *et al.* 2007; Godin *et al.* 2012). Therefore, p27 contributes significantly to the development of neurons by regulating diverse processes. Here, we demonstrate that p27 promotes neuronal apoptosis in response to neurotoxic amyloid peptide $A\beta_{42}$ by virtue of its ability to stabilize Cdk5/cyclin D1 complex, which results in dissociation of Cdk5 and p35. As a result, neurons undergo cell cycle reactivation that leads to cell death.

Materials and methods

Antibodies

Antibodies against cyclin D1 (sc-753, 1 : 500), proliferating cell nuclear antigen (PCNA) (sc-56, 1 : 500), actin (Sc-47778, 1 : 2000), Cdk5 J-3 (Sc-6247; 1 : 500), Cdk5 C-8 (Sc-173; 1 : 500), p35 N20 (Sc-821; 1 : 500), p27 M-197 (Sc-776, 1 : 2000), and HA probe Y-11(Sc-805, 1 : 500) were from Santa Cruz Biotechnology, Santa Cruz, CA. Antibody against cleaved caspase 3 (D-175) (9661, 1 : 500), GFP (2555, 1 : 500), myc tag (9B11; 2276S, 1 : 2000) and phospho-S10 histone H3 (3377S; 1 : 1000) were from Cell Signaling technology. green fluorescent protein (GFP) (11814460001, 1 : 2000) from Roche Diagnostics and anti-BrdU (RPN202) from GE Healthcare Bio-Sciences, Piscataway, NJ, USA were used.

siRNA

siRNA against cyclin D1 and p27

Cyclin D1 and p27 siRNA duplex were custom synthesized from Dharmacon, USA.

Cyclin D1 siRNA: 5′-GCGAGGAGCAGAAGUGCGAUG-3′;

scrambled siRNA (ctrl_siRNA): 5′-GCGAGGAGAAGCA GUGCGAUG-3′.

p27 siRNA: 5′-AAGCACUGCCGAGAUAUGGAAUU-3′;

scrambled siRNA (ctrl_siRNA): 5′-AAGACCGAGCCAU UGAGGUAAUU-3′.

Plasmid DNA constructs

Cyclin D1-HA pCDNA 3.0 (Modi *et al.* 2012), p35 FLAG pCDNA 3.0 (Modi *et al.* 2012), myc p27 6xHis pCDNA and EGFP-C1 Cdk5 over-expression plasmids were used during this work. For Cdk5 over-expression, *Cdk5* was subcloned in EGFP-C1 vector between the *Hind III* and *Bam HI* restriction site. *Cdk5* cDNA was PCR amplified from the previously available construct of *Cdk5*-6xHis pCDNA using the following primers:

Cdk5_F: 5′-CCCaagcttTTATGCAGAAATACGAGAAACTGG-3′

Cdk5_R: 5′-CGCggatccCTAGGGCGGACAGAAGTCGG-3′

Letters in the lower case represent *Hind III* restriction site in F-primer and *Bam HI* restriction site in R-primer.

Preparation of lentivirus

To effectively knockdown p27 in primary cortical neurons, a specific shRNA was over-expressed using a lentivirus system, which is routinely used for this purpose. To prepare p27 lentiviral shRNA (short hairpin RNA), p27shRNA and a scrambled shRNA were designed as a stem loop structure, using the following oligonucleotides with *EcoRI* and *PacI* overhangs. The annealed oligos were then ligated between *EcoR1* and *PacI* site in pLKO.3G vector:

Lenti p27sh oligo1: 5′- aattcAAGCACTGCCGAGATAT GGAACTCGAGTTCCATATCTCGGCAGTGCTTTTTTTttaat -3′

Lenti p27sh oligo 2: 5′- taaAAAAAAAGCACTGCCGAG ATATGGAACTCGAGTTCCATATCTCGGCAGTGCTTg -3′

Lenti p27 CONsh oligo 1: 5′- aattcAAGACCGAGCCAT TGAGGTAACTCGAGTTACCTCAATGGCTCGGTCTTTTTTT ttaat-3′

Lenti p27 CONsh oligo 2: 5′- taaAAAAAAGACCGAGCC ATTGAGGTAACTCGAGTTACCTCAATGGCTCGGTCTTg -3′

The complementary oligonucleotides were annealed by first denaturing at 100°C for 2–3 min and then gradually cooled by incubation at 55°C for 10 min followed by 37°C for 15–20 min.

For lentivirus preparation, pLKO.3G containing p27 shRNA or control shRNA was co-transfected with packaging vector pCMV-dR8.2 and envelope vector pCMV- vsvg (4 : 3 : 1) in HEK293T cells. After 6 h of transfection, media was changed to serum-free medium containing N2 and B27 supplement, 0.48% Glucose, 1% antibiotic/antimycotic, and 2 mM L-glutamine. The supernatant was collected after 24 and 72 h and filtered, using 0.45 μM filter. The filtered supernatant was used in 1 : 1 ratio for transducing cortical

neurons and transduction efficiency was checked by counting the number of GFP positive cells.

Cell culture

Sprague–Dawley rats and amyloid precursor protein (APP)/presenillin 1 (PS1) transgenic mice were used as source of cortical neurons for *in vitro* culture. Sprague–Dawley rats were maintained in National Institute of Immunology, New Delhi, India. APP/PS1 transgenic (Tg) AD mice (strain name B6C3-Tg APPswe, PSEN1dE9 85Dbo/J; stock number 004462) were purchased by National Brain Research Centre (NBRC), Manesar, India from Jackson Laboratories. NBRC gifted these animals to National Institute of Immunology (NII), New Delhi, India and animals were subsequently maintained in NII animal facility. Cortical neurons from Embryonic day 18 (E18) Sprague–Dawley rats or Embryonic day 16 (E16) APP/PS1 transgenic AD mice of either sex were cultured as described previously (Modi *et al.* 2012). PC 12 (rat pheochromocytoma) cells were maintained in Dulbecco's Modified Eagles Medium (Gibco, Life technologies, Grand Island, NY, USA, 12800-017), + 10% heat inactivated Horse Serum (Gibco, Life technologies, Grand Island, NY, USA, 26050-088) + 5% heat inactivated Fetal Bovine Serum (Gibco, Life technologies, 10270-106) and 1 × Antibiotic/Antimycotic (Gibco, Life technologies, 15240-062). For differentiation, cells were cultured in Dulbecco's Modified Eagles Medium + 1% Fetal Bovine serum and treated with 50 ng/ml of 2.5S nerve growth factor (BD, 356005) for 5 days. NIH3T3 (fibroblast cell line), and HEK293T (Human Embryonic Kidney) cells were maintained in Dulbecco's Modified Eagles Medium (Gibco, Life technologies) supplemented with 10% fetal bovine serum (Gibco, Life technologies) and 1 × Antibiotic/Antimycotic (Gibco, Life technologies) at 37°C gassed with 5% CO_2. PC 12, NIH 3T3 and HEK 293T cells were purchased from ATCC (American Type Culture Collection).

Alzheimer's disease mouse model

APP/PS1 Tg mice were used as model for AD (hereafter referred to as Tg AD). The levels of $A\beta_{42}$ produced are significantly higher in these animals (Jankowsky *et al.* 2001, 2004). Wild-type and transgenic (Tg AD) mice were genotyped, using genomic DNA isolated from mouse tail. All experiments were performed in accordance with the guidelines for animal experiments of the Institutional Animal Ethical Committee of National Institute of Immunology.

Transfections and cell treatment

Plasmid DNA transfections were performed, using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Cortical neurons, differentiated PC 12 cells, or NIH3T3 cells were transfected with 1–3 μg of plasmid DNA or 100 pmoles of siRNA (small interfering RNA) per well in a six well plate. 3–4 h after transfection, medium was changed to growth medium without antibiotic for 12 h. Subsequently, cultures were transferred to growth medium containing 1 × antibiotic and desired treatments were typically administered for 48 h. Adenovirus for GFP (control) or cyclin D1 (Modi *et al.* 2012) were used to over-express these proteins in neuronal PC 12 cells. 0.5 μM of soluble oligomers of $A\beta_{1\text{-}42}$ (R-peptide) were used as described previously (Dahlgren *et al.* 2002; Stine *et al.* 2003; Chang *et al.* 2012; Modi *et al.* 2016) for 48 h to induce CRNA.

Immunoblotting

Expression levels of different proteins were compared by immunoblot analysis. Cells were lysed using ice cold lysis buffer containing 500 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM $MgCl_2$, 5% NP-40 and 10% Glycerol. 1 mM Phenyl Methane Sulfonyl Fluoride, 1 mM Sodium Orthovanadate, 20 mM β-glycero phosphate and 1 × protease inhibitor cocktail were added before use. Lysis was performed by passing the lysate through 26 ½ gauze needle at least 25 times on ice. Lysate was cleared by centrifugation at 15 700 g for 25 min at 4°C. Protein estimation was done using bicinchoninic acid method (Pierce bicinchoninic acid protein assay kit, Pierce, Thermo Scientific, Rockford, USA). Proteins were electrophoresed using 10–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and transferred to nitrocellulose membrane (Merck Millipore, Tullagreen, Co. Cork, Ireland). The membranes were blocked with 5% non-fat dry milk and incubated with primary antibody prepared in 3% bovine serum albumin (BSA) at 4°C for ~12 h and with secondary antibody conjugated with horse radish peroxidase (1 : 5000 dilution, Bio-Rad) for 1.5 h at 25°C. The signal was detected on X-ray films, using chemiluminescence substrate West Pico or West Dura kit (Pierce).

Co-immunoprecipitation and Kinase assay

50–100 μg of protein was incubated with 1 μg of p35 (N20) or Cdk5 (J-3) antibody for 12 h at 4°C on shaker in 250 μL reaction volume. Subsequently, 50 μL of protein A+G Sepharose (GE Healthcare Ltd, Buckinghamshire, England) beads were washed and equilibrated in lysis buffer. Beads were then added to the antibody-protein complex and incubated on shaker for 5–7 h at 4°C. The beads were washed at 4°C to remove unbound proteins and resuspended in ice cold 1 × kinase assay buffer. The immunoprecipitate (IP) was used for immunoblotting as described above. IP-associated kinase activity was assessed, using 15 μL of IP in a reaction containing 50 mM Tris pH 7.4, 10 mM $MgCl_2$, 1 mM Dithiothreitol and 100 μM [γ-32P] ATP (6000 Ci/mmol) and phosphor acceptor substrate histone H1 (Sigma, St Louis, MO, USA) at 4°C. The kinase reaction was carried out at 30°C for 45 min and was stopped by boiling in laemmli's buffer followed by sodium dodecyl sulfate (SDS) PAGE. Incorporation of phosphate in histone H1 was analyzed by autoradiography or phosphorimaging of SDS-PAGE gels.

BrdU incorporation assay

5-bromo-2′-deoxyuridine (BrdU) labeling was performed to detect DNA replication as described previously (Modi *et al.* 2012, 2016). Briefly, cells were fixed using 4% paraformaldehyde for 20 min at 25°C, washed, and permeabilized, using 0.2% Triton- × 100 for 20 min. Blocking was done using 3% BSA in 1 × Phosphate Buffered Saline (PBS) for 1 h at 25°C. Cells were incubated with anti-BrdU antibody at 25°C, washed and incubated with alexa fluor conjugated secondary antibody for 1.5 h at 25°C. Nuclei were counter-stained with Hoechst 33342. Staining was visualized and imaged, using Zeiss AxioImager.Z1microscope and Axiovision release 4.8.2 software, Göttingen, Germany.

TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to detect cell death was performed, using the *In Situ* Cell Death Detection Kit, TMR red (12156792910; ROCHE, Roche diagnostics GmbH Mannheim, Germany) according to manufacturer's instructions. Cells were visualized using a Zeiss AxioImager.Z1 microscope and Axiovision release 4.8.2 software was used for image acquisition and manipulation.

Flow cytometry analysis

For flow cytometry, cells were initially trypsinized for 5 min. The activity of trypsin was inhibited by adding serum-containing media. Cells were pelleted at 400 g to remove media and trypsin and then washed with 1 × PBS by centrifugation for 5 min at 400 g. Subsequently, cells were fixed with 70% ethanol for ~12 h at 4°C, washed with 1 × PBS and permeabilized with 0.25% triton X-100 for 15 min on ice with intermittent tapping. For antibody staining, cells were incubated with the pS10-histone H3 primary antibody diluted in 1% BSA for 1 h at 25°C. Cells were subsequently washed and incubated with Alexafluor conjugated secondary antibody in 1% BSA for 30 min at 25°C. After washing, cells were resuspended in 1 × PBS constituted of 10 μg/mL of RNaseA, and 1 μg/mL of propidium iodide and incubated for 15–30 min at 25°C. Data were acquired using BD FACS calibur, San Jose, CA, USA and was analyzed, using the FlowJo software (Flow Jo LLC, Ashland, OR, USA).

Image and statistical analysis

Image J (NIH) software was used to perform densitometry of western blots. The band intensity of the loading control was used for normalization. Unless indicated otherwise, one-way Analysis of Variance (ANOVA) followed by Student–Newman–Keuls test was used for statistical analysis, using Prizm (Graph Pad software Inc., LaJolla, CA, USA). Data were represented as mean ± Standard error of mean (SEM) from three independent experiments unless indicated otherwise and $p < 0.05$ was taken as statistically significant in most cases.

Results

p27 stabilizes Cdk5/cyclin D1, but not Cdk5/p35, complex

Previous studies suggested that cyclin D1 induction promotes aberrant cell cycle reentry and apoptosis of neurons (Sakurai *et al.* 2000; Ino and Chiba 2001; Koeller *et al.* 2008; Modi *et al.* 2012), which it achieves by sequestering p35 from its partner Cdk5 resulting in attenuation of p35-associated Cdk5 activity (Modi *et al.* 2012). Since p27 is known to inhibit cyclin/Cdk complexes (Blain *et al.* 1997; Xu *et al.* 1999; Ray *et al.* 2009), we investigated if it contributes to Cdk5/cyclin D1 association. To this end, p27, Cdk5 and cyclin D1 were over-expressed in NIH3T3 fibroblast cell line in different combinations. Co-immunoprecipitation experiments were performed to analyze the effect of p27 on cyclin D1–Cdk5 interaction. While Cdk5 and cyclin D1 exhibited co-immunoprecipitation, their interaction was significantly increased upon p27 over-expression (Fig. 1a, panel c, lane 8 vs. lane 5). Furthermore, p27 also exhibited significantly enhanced binding to Cdk5 (Fig. 1a, panel a, lane 8 vs. lane 6) and cyclin D1 (Fig. 1a, panel b, lane 8 vs. lane 7) when cyclin D1 and Cdk5 were co-expressed with p27, respectively. These data suggested that the ternary complex formed between these three proteins is significantly more stable than the binary complex between any of the two proteins. The role of p27 in stabilizing Cdk5/cyclin D1 complex was further assessed in neuronal PC 12 cells, which were differentiated with 2.5 S nerve growth factor. Since expression of cyclin D1 is low in these cells as a result of neuronal differentiation, it was over-expressed, using adenoviruses (Modi *et al.* 2012, 2016) and p27 was knocked down, using specific siRNA. While Cdk5/cyclin D1 binding was observed, it was significantly reduced upon p27 knockdown (Fig. 1b, lane 3 vs. lane 1). Collectively, these observations suggested that p27 plays a key role in stabilizing Cdk5/cyclin D1 complex.

Given that p35 is a physiological activator of Cdk5 with which it associates in neuronal cells, it was pertinent to test the effect of p27 on association between Cdk5 and p35. p27 did not exhibit any detectable binding with p35 even upon Cdk5 over-expression (Fig. 1c, panel b, lane 5, 7, 8). In addition, p35 over-expression did not result in Cdk5–p27 interaction (Fig. 1c, panel a, lane 7 vs. lane 2), which is in contrast to enhanced Cdk5-p27 binding upon cyclin D1 over-expression (Fig. 1c, panel a, lane 6 vs. lane 2). These data suggested that p27 does not interact directly with Cdk5/p35 complex. Since Cyclin D1 is involved in dissociation of Cdk5/p35 complex (Modi *et al.* 2012), we explored the possibility of the role of p27 in preventing the association between Cdk5 and p35. To this end, Cdk5 was immunoprecipitated and its association with p35 was ascertained upon p27 over-expression. Expectedly, p35 co-immunoprecipitated with Cdk5 (Fig. 1c, panel c, lane 4), however, over-expression of p27 significantly abrogated this interaction (Fig. 1c, panel c, lane 7 vs. lane 4). Moreover, over-expression of cyclin D1 further reduced the association of Cdk5 with p35 (Fig. 1c, panel c, lane 8 vs. lane 7). Collectively, these data indicated that p27 forms a stable complex with Cdk5 in the presence of cyclin D1 resulting in destabilization of Cdk5-p35.

$A\beta_{42}$ promotes association of Cdk5/cyclin D1 *via* p27, which results in dissociation of Cdk5 from p35

It has been previously demonstrated that during $A\beta_{42}$ induced neurotoxic stress, cyclin D1 causes dissociation of Cdk5/p35 complex and forms inactive complex with Cdk5 (Modi *et al.* 2012). Cyclin D1 sequesters Cdk5 from p35 (but not p25) resulting in a loss of p35-associated Cdk5 activity (Modi *et al.* 2012), which is important for various neuronal processes (Nikolic *et al.* 1996; Ko *et al.* 2001; Tanaka *et al.* 2001; Lagace *et al.* 2008). As p27 stabilizes Cdk5/cyclin D1 complex, we tested if p27- mediated stability of this complex has any consequence on association of Cdk5

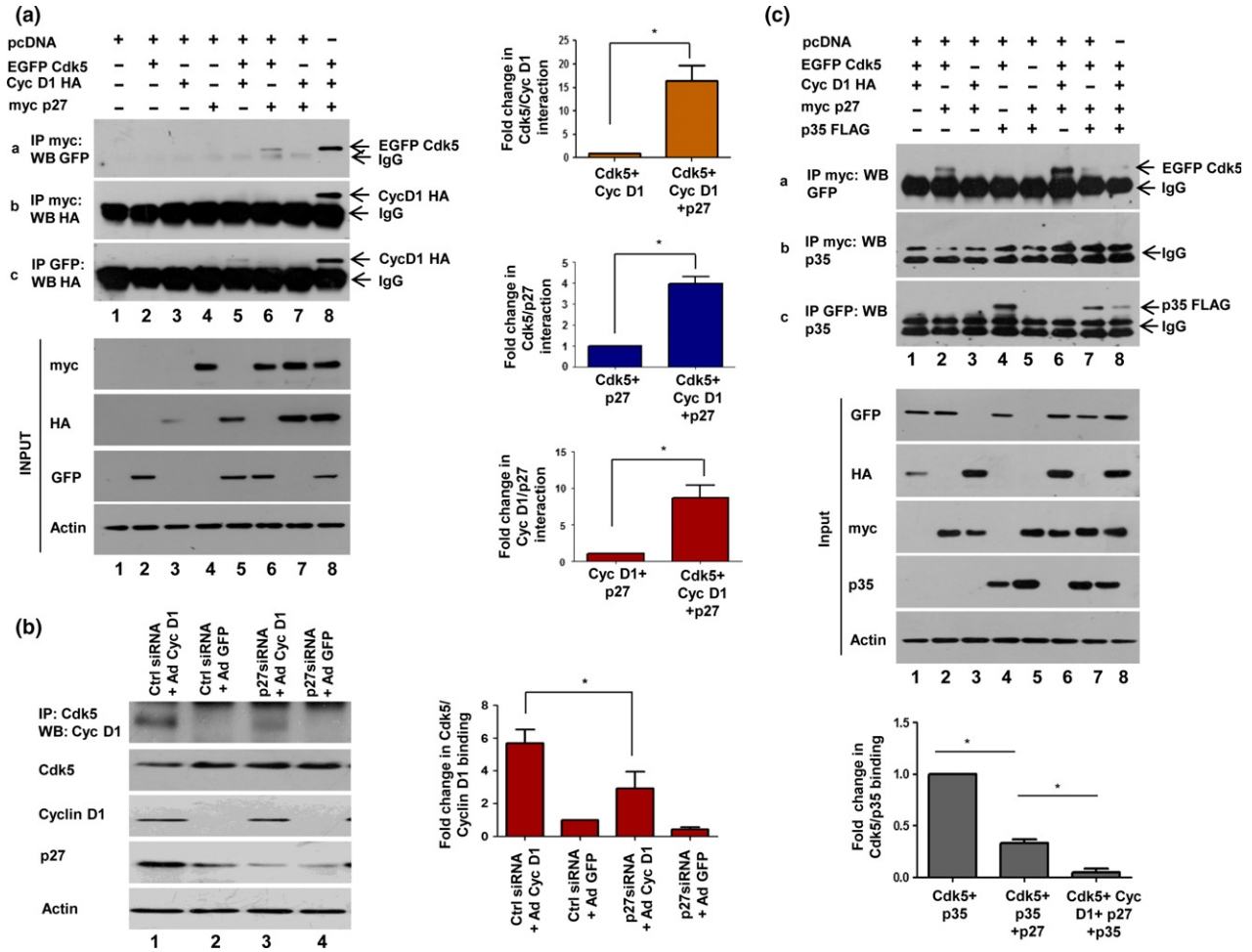

Fig. 1 p27 stabilizes Cdk5/cyclinD1 complex. (a) NIH3T3 cells were transfected with plasmid constructs for EGFP-Cdk5, cyclinD1-HA and myc-p27 as indicated. Cells lysates were prepared 48 h post-transfection and immunoprecipitation was performed using anti-myc and anti-GFP antibody followed by western blotting with indicated antibodies. Western blotting was also performed on total cell lysates. *Right Panel,* Cdk5/Cyclin D1, Cdk5/p27 and Cyclin D1/p27 binding were quantified by densitometry. Data represent mean ± SEM, *t*-test, $*p < 0.05$, $n = 3$. (b) Neuronal PC 12 cells were transfected with p27 siRNA or control siRNA followed by treatment with recombinant adenovirus for Cyclin D1 (Ad-CycD1) or control virus for GFP (Ad-GFP). After 48 h, cells lysates were prepared and immunoprecipitation was done using anti-Cdk5 antibody followed by western blotting with anti-Cyclin D1 antibody. Western blotting on total cell lysates was performed with indicated antibodies. *Right Panel,* association between Cdk5 and Cyclin D1 was quantified by densitometry. Data represent mean ± SEM, ANOVA, $*p < 0.05$, $n = 3$. (c) NIH3T3 cells were transfected with plasmid constructs for EGFP-Cdk5, Cyclin D1-HA, myc - p27 and p35-FLAG as indicated. Cell lysates were prepared 48 h post-transfection and immunoprecipitation was performed using anti-myc and anti-GFP antibody followed by western blotting with indicated antibodies. Western blotting was also performed on total cell lysates. *Lower Panel,* Cdk5/p35 binding was quantified by densitometry. Data represent mean ± SEM, ANOVA, $*p < 0.05$, $n = 2$.

with p35. To test this, p27 was knocked down in cortical neurons in the presence of $A\beta_{42}$. Subsequently, p35 was immunoprecipitated using an antibody against its N-terminus which does not recognize its cleaved product p25. Cyclin D1–Cdk5 interaction was severely disrupted upon p27 knock down (Fig. 2a, panel a, lane 3 vs. lane 2). In contrast, p35–Cdk5 interaction, which was lost upon $A\beta_{42}$ treatment as reported earlier (Modi *et al.* 2012), was significantly restored upon p27 knock down (Fig. 2a, panel b, lane 3 vs. lane 2). Next, we tested if p27 influenced p35-associated Cdk5 activity in the presence of $A\beta_{42}$. $A\beta_{42}$ caused a significant loss in p35-associated Cdk5 activity as adjudged by kinase activity associated with p35 IP (Fig. 2b, lane 2 vs. lane 1). When p27 was knocked down, a significant restoration of its activity was observed (Fig. 2b, lane 3 vs. lane 2), which corroborated well with reduced p35–Cdk5 association (Fig. 2a, panel b). Deregulated p35-associated Cdk5 activity has been previously demonstrated to result in aberrant activation of MEK-ERK pathway as p35-Cdk5 negatively regulates the pathway (Sharma *et al.* 2002). The knockdown of p27 could revert the aberrant activation of MEK-ERK pathway induced by $A\beta_{42}$ to normal levels (Fig. 2c lane 3 vs.

lane 2). Collectively, these data suggested that p27 contributes to the loss of p35–Cdk5 association in response to $A\beta_{42}$ by promoting cyclin D1–Cdk5 interaction.

p27 promotes $A\beta_{42}$ induced neuronal apoptosis

Previous studies suggested that $A\beta_{42}$ promotes the reentry of neurons into the cell cycle which causes their apoptosis (Copani *et al.* 1999; Yang *et al.* 2001; Herrup and Yang 2007; Modi *et al.* 2012). The mechanism of cell-cycle-mediated neuronal apoptosis involves aberrant activation of the MEK-ERK pathway, which leads to overproduction of cyclin D1. Cyclin D1 sequesters Cdk5 from p35 thereby preventing negative regulation of this pathway via p35/Cdk5 (Modi *et al.* 2012). Since present findings indicated that p27

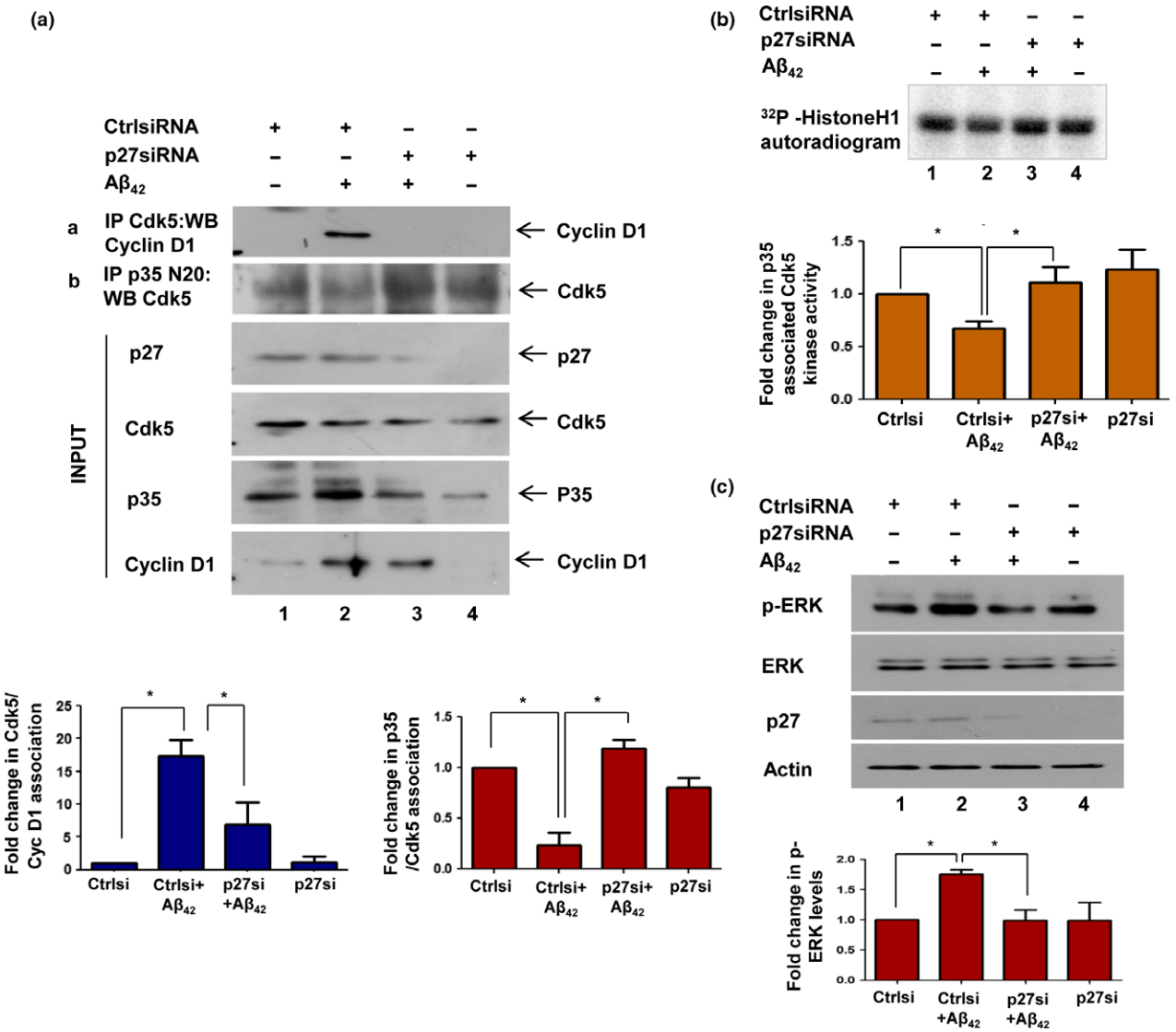

Fig. 2 $A\beta_{42}$ promotes association of Cdk5/Cyclin D1 via p27. Rat cortical neurons were transfected with p27 siRNA or control siRNA followed by $A\beta_{42}$ treatment and cell lysates were prepared after 48 h. Immunoprecipitation was performed using anti-Cdk5 or anti-p35 N20 antibody. Co-immunoprecipitation of indicated proteins was assessed by western blotting (a) or p35-associated Cdk5 activity was determined by kinase assay, using histone H1 as substrate (b). Phosphorylation of histone H1 was assessed by phosphorimaging of the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel on which kinase assay mix was separated. Kinase activity (histone H1 phosphorylation) was normalized with respect to total p35 in the lysate. Western blotting on total cell lysates was performed with indicated antibodies. Quantitation of Cdk5/cyclinD1 binding, Cdk5/p35 binding and p35-associated Cdk5 activity was done by densitometry. Data represent mean ± SEM, ANOVA, $*p < 0.05$, $n = 3$. (c) Rat cortical neurons were transfected with p27 siRNA or its scrambled control followed by $A\beta_{42}$ treatment. After 48 h, lysates were prepared and western blotting was done to monitor p-ERK levels. *Lower panel,* Densitometry for p-ERK levels normalized to ERK. Data represent mean ± SEM, ANOVA, $*p < 0.05$, $n = 3$.

stabilizes Cdk5/cyclin D1 binding, it was worth pursuing the contribution of p27 to neuronal apoptosis. To this end, p27 was knocked down in the presence of $A\beta_{42}$. Cleaved caspase3 levels, which indicated active apoptosis, were elevated upon $A\beta_{42}$ treatment and p27 knockdown significantly reverted this increase (Fig. 3a lane 3 vs. lane 2). Furthermore, TUNEL assay was performed in rat cortical neurons subsequent to lentivirus-mediated knockdown of p27 and $A\beta_{42}$ treatment. Since lentiviral vector has GFP over-expression cassette, apoptosis could be measured in lentivirus transduced cells, which expressed GFP. The assay revealed that the $A\beta_{42}$ induced increase in apoptotic cells was significantly reduced upon p27 knockdown (Fig. 3b). Collectively, these data suggest that p27 plays a critical role in promoting $A\beta_{42}$ induced neuronal apoptosis.

p27 regulates cell cycle progression of the neuronal cell cycle

In order to establish whether p27 regulates neuronal apoptosis by altering neuronal cell cycle, the status of cell cycle markers in $A\beta_{42}$-treated cells was assessed. It was interesting to observe that the levels of cyclin D1 and PCNA, which are indicative of G1/S phase, remained largely unchanged upon p27 knockdown (Fig. 4a, lane 3 vs. lane 2). In addition, no significant change in the number of BrdU positive cells was observed (Fig. 4b). These data suggested that p27 depletion may not prevent G1/S entry. Therefore, the involvement of p27 in the later stages of cell cycle was investigated. To this end, the presence of $A\beta_{42}$-treated cells in the M-phase cells was determined by flow cytometry (Fig. 5a) and western blotting (Fig. 5b) using an antibody against S10

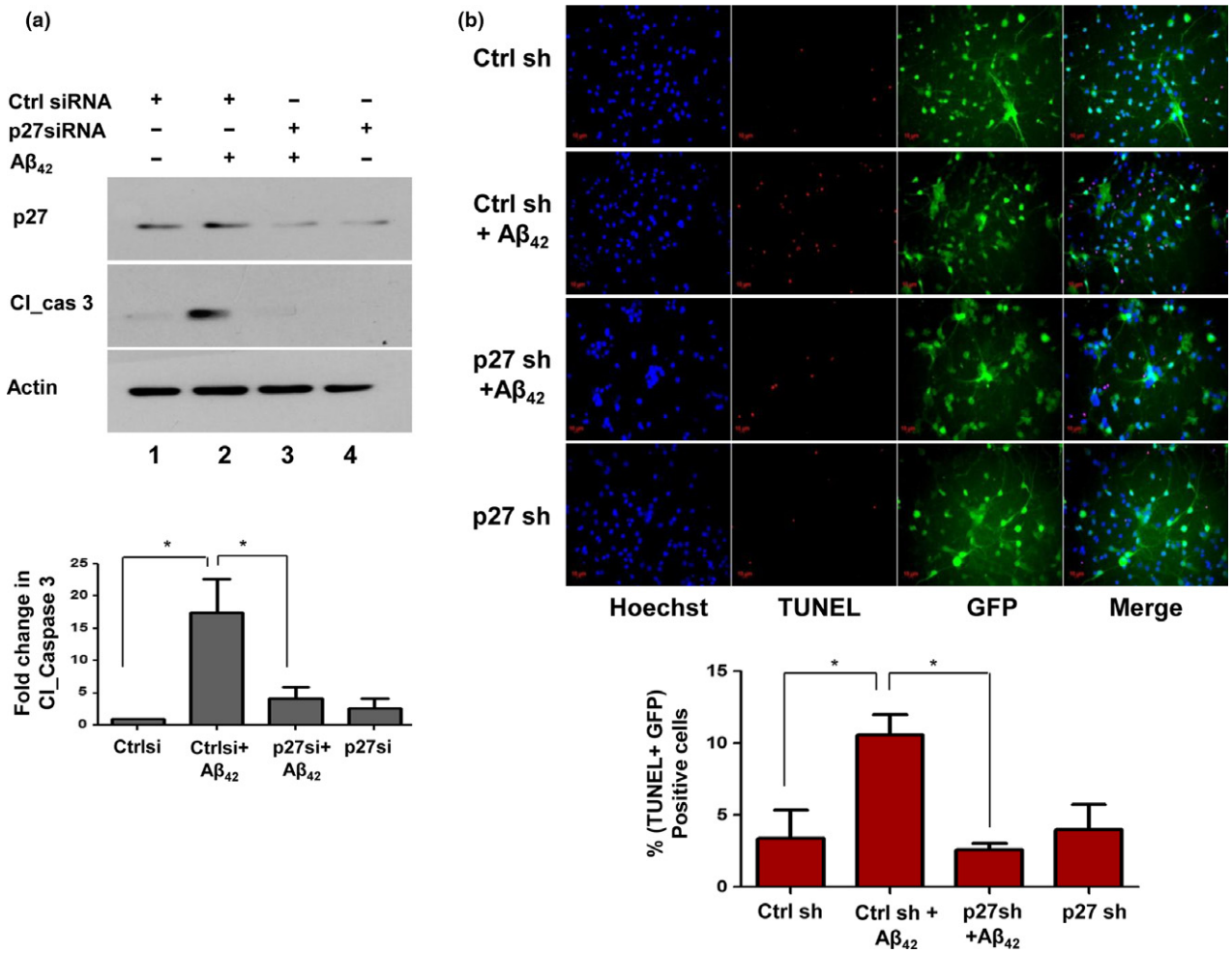

Fig. 3 p27 promotes $A\beta_{42}$ induced neuronal apoptosis. (a) Rat cortical neurons were transfected with p27 siRNA or control siRNA followed by $A\beta_{42}$ treatment. Cell lysates were prepared after 48 h and Western blotting was performed for indicated proteins. Actin was used as loading control. Levels of cleaved caspase 3 were quantified by densitometry. Data represent mean ± SEM, ANOVA, $*p < 0.05$, $n = 4$. (b) Rat cortical neurons were transduced with lentivirus for p27 shRNA or control shRNA. After 24 h virus was removed and fresh medium was added followed by $A\beta_{42}$ treatment for 48 h. Subsequently, TUNEL assay was performed on these cells to determine apoptosis. GFP positive neurons were scored as transduction positive and both GFP^+ and $TUNEL^+$ cells were counted and normalized with respect to total GFP^+ cells. Data represent mean ± SEM, ANOVA, $*p < 0.05$, $n = 3$.

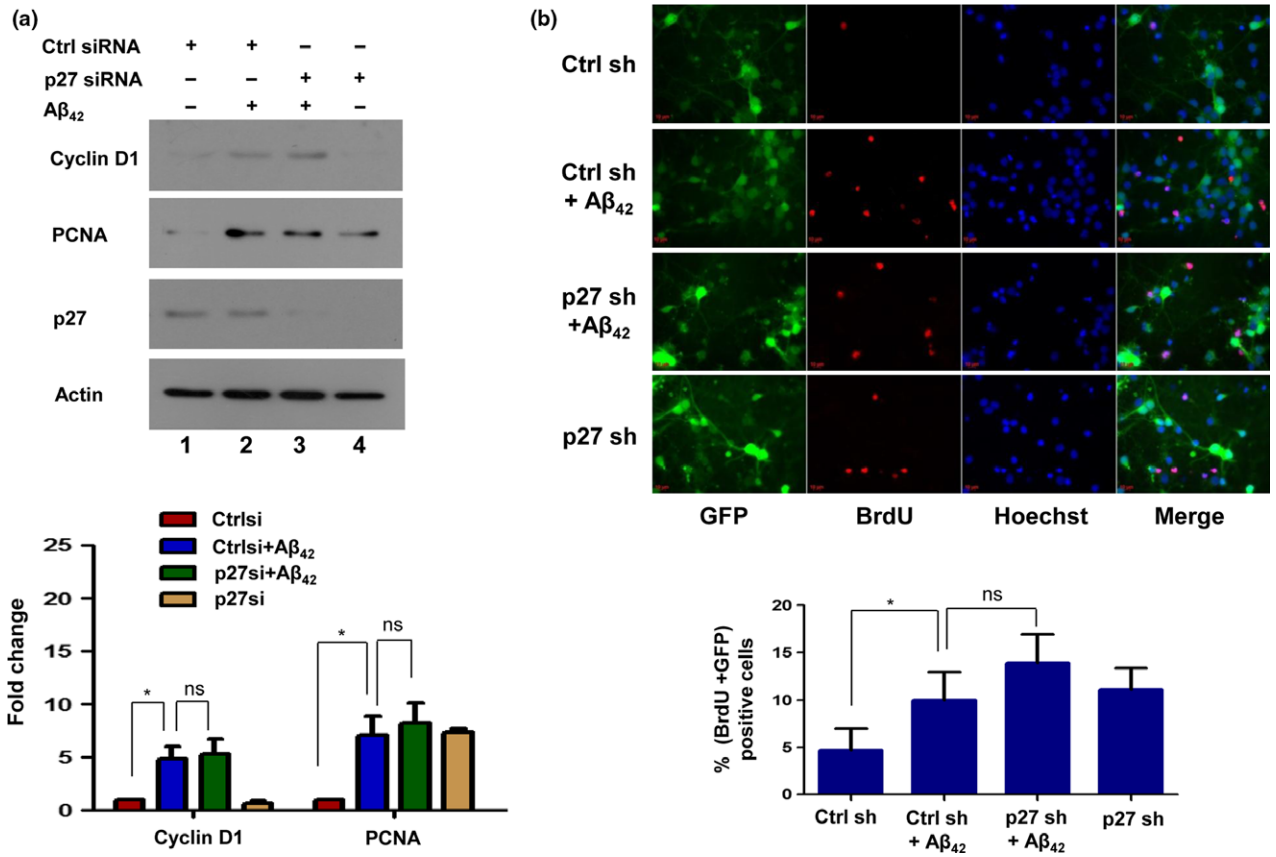

Fig. 4 p27 does not alter $A\beta_{42}$ induced G1 and S-phase of the cell cycle. (a) Rat cortical neurons were transfected with p27 siRNA or its scrambled control followed by $A\beta_{42}$ treatment. Cell lysates were prepared after 48 h for western blotting for indicated proteins. Expression level of cyclin D1 and proliferating cell nuclear antigen (PCNA) was quantified by densitometry. Data represent mean ± SEM, ANOVA, $*p < 0.05$, $n = 4$. (b) Rat cortical neurons were transduced with lentivirus for p27 shRNA or its scrambled control. After 24 h, virus was removed and fresh medium was added along with 5′ Bromo- 2′-deoxy Uridine (BrdU) followed by $A\beta_{42}$ treatment for 48 h. Subsequently, BrdU incorporation assay was performed on these cells to monitor DNA replication. GFP positive neurons were scored as transduction positive. Cells that were both GFP^+ and $BrdU^+$ were counted and normalized with respect to total GFP positive cells. Data represent mean ± SEM, *t*-test, $*p < 0.05$, $n = 3$.

phosphorylated histone H3 (pS10- histone H3). Since phosphorylation of histone H3 at S10 is necessary for mitosis, cells exhibiting enhanced phosphorylation of S10 will be indicative of M-Phase (Hendzel *et al.* 1997; Cheung *et al.* 2000). A significant fraction of cells in the M-phase was significantly reduced upon p27 knockdown in the presence of $A\beta_{42}$ (Fig. 5a and b). These observations suggested that p27 may contribute to neuronal apoptosis by promoting M-phase progression.

p27 promotes apoptosis in cortical neurons of APP/PS1 transgenic AD mice

Next, the role of p27 in neuronal apoptosis was tested in a transgenic mouse model of AD (Tg AD). APP and PS1 are over expressed in these animals, which results in high levels of $A\beta_{42}$ production (Jankowsky *et al.* 2001, 2004) and has been used to study cell cycle and cell death (Li *et al.* 2011; Modi *et al.* 2016). The neurons from Tg AD exhibited elevated expression of cleaved caspase 3 as reported previously (Modi *et al.* 2016), which was significantly prevented by p27 depletion (Fig. 6a, panel d, lane 3 vs. lane 2). However, PCNA expression remained unaltered (Fig. 6a, panel c, lane 3 vs. lane 2) as observed in earlier experiments with $A\beta_{42}$ treatment (Fig. 4). The role of p27 in Cdk5/p35 association was also studied in Tg AD mouse neurons. To this end, amount of Cdk5 associated with p35-IP from neurons of Tg AD and wild-type (WT) neurons was compared. p35-associated Cdk5 was significantly reduced in Tg AD neurons (Fig. 6b, panel b, lane 2 vs. lane 1), which was restored upon p27 knockdown (Fig. 6b, panel b, lane 3 vs. lane 2). As reported earlier, Tg AD neurons exhibit aberrant expression of cyclin D1 (Modi *et al.* 2016), which results in dissociation of Cdk5 from p35 (Fig. 6b, panel b, lane 2 vs. lane 1) and reduced p35-Cdk5 activity (Fig. 6B,

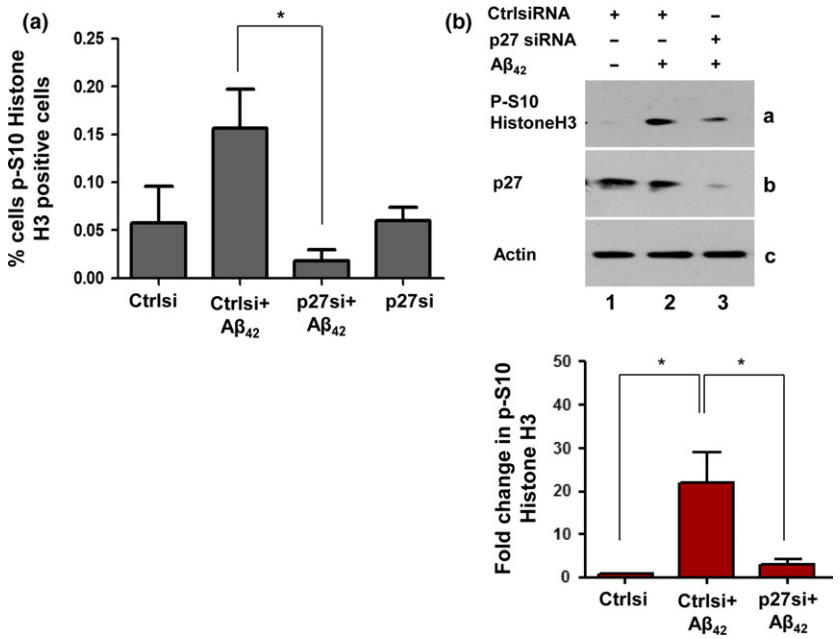

Fig. 5 p27 regulates M-phase of the neuronal cell cycle. (a) Rat cortical neurons were transfected with p27 siRNA or its scrambled control followed by $A\beta_{42}$ treatment. Cells were harvested after 48 h and staining was performed with pS10-histone H3 and propidium iodide for FACS analysis. Data represent mean ± SEM, ANOVA, $*p < 0.05$, $n = 4$. (b) Rat cortical neurons were transfected with p27 siRNA or its scrambled control followed by $A\beta_{42}$ treatment. Cells lysates were prepared after 48 h and western blotting was done with anti-pS10-histone H3 antibody. Data represent mean ± SEM, ANOVA, $*p < 0.05$, $n = 4$.

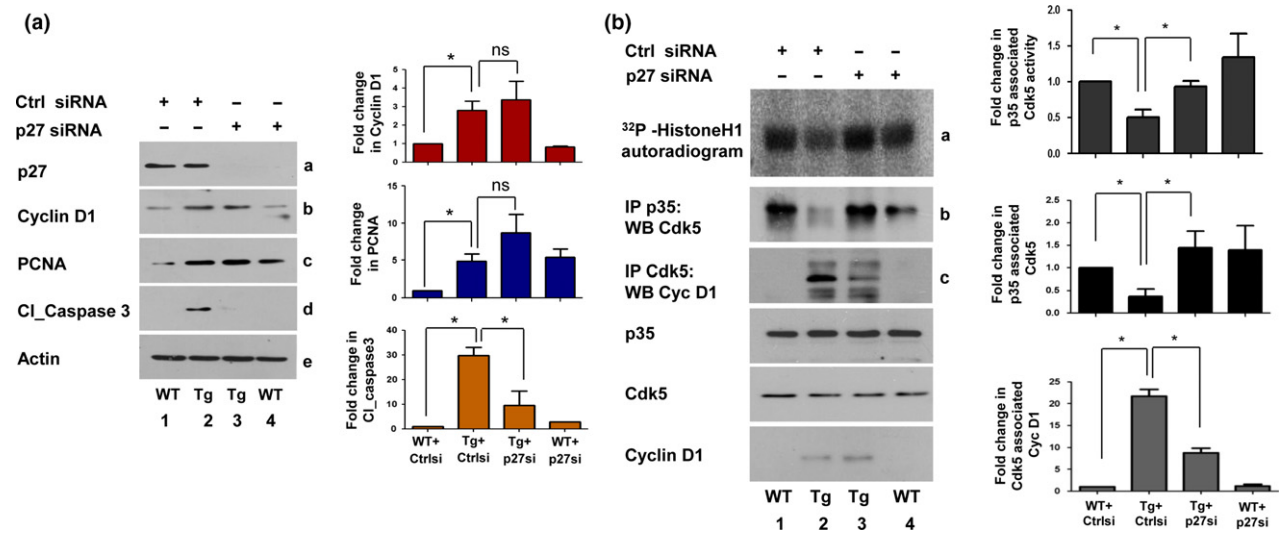

Fig. 6 p27 may promote apoptosis of cortical neurons from transgenic Alzheimer's disease (AD) mouse model. (a) Cortical neurons from amyloid precursor protein (APP)/presenillin 1 (PS1) transgenic mice (Tg) or their wild-type (WT) littermates were transfected with p27 siRNA or control siRNA. Cell lysates were prepared after 48 h and western blotting was performed for indicated proteins. The expression of cyclin D1, proliferating cell nuclear antigen (PCNA) and cleaved caspase 3 was quantified by densitometry. Data represent mean ± SEM, ANOVA, $*p < 0.05$, $n = 3$. (b) Cortical neurons from APP/PS1 transgenic mice (Tg) or their wild-type (WT) littermates were transfected with p27 siRNA or control siRNA. Immunoprecipitation was done, using anti-Cdk5 or anti-p35 N20 antibody. Co-immunoprecipitation of indicated proteins was checked by western blotting or p35-associated Cdk5 activity was determined by kinase assay, using histone H1 as substrate. Western blotting on total cell lysates was performed with indicated antibodies. Quantitation of Cdk5/cyclinD1 binding, Cdk5/p35 binding and p35-associated Cdk5 activity was done by densitometry. Data represent mean ± SEM, ANOVA; $*p < 0.05$. For Cdk5/cyclinD1 binding, $n = 3$; Cdk5/p35 binding and p35-associated Cdk5 activity, $n = 4$.

panel a, lane 2 vs. lane 1). Consistent with these findings, the amount of cyclin D1 bound to Cdk5 was significantly higher in Tg neurons in comparison to WT animals (Fig. 6b, panel c, lane 2 vs. lane1). However, p27 siRNA significantly reduced the cyclin D1–Cdk5 interaction (Fig. 6b, panel c, lane 3 vs. lane 2) without altering cyclin D1 levels (Fig. 6b)

and restored Cdk5–p35 association (Fig. 6b, panel b, lane 3 vs. lane 2) as well as p35-associated Cdk5 activity (Fig. 6B, panel a, lane 3 vs. lane 2). Given the above described observations, it suffices to conclude that cyclin D1 may use p27 to interact strongly with Cdk5 which results in its dissociation from p35. The p27 knockdown restored the p35-associated Cdk5 activity. Collectively, these data suggested that p27 abrogates the p35/Cdk5 association by sequestering Cdk5 from p35 in Tg AD neurons. Given the importance of p35 activation of Cdk5 in essential neuronal functions (Nikolic *et al.* 1996; Tanaka *et al.* 2001; Hisanaga and Endo 2010), it may explain the observed neuronal apoptosis.

Discussion

Cell cycle activity has been observed in apoptotic neurons from patients of various neurodegenerative disorders (Busser *et al.* 1998; Yang *et al.* 2003). Previous studies demonstrated that $A\beta_{42}$ induced stress in cortical neurons leads to aberrant activation of MEK-ERK pathway thus causing CRNA (Modi *et al.* 2012). Cdk5, which suppresses MEK-ERK pathway and cyclin D1 expression, prevents CRNA (Modi *et al.* 2012). Under physiological conditions, Cdk5 is activated by its cognate partner p35 (Tsai *et al.* 1994). It has been shown that $A\beta_{42}$ treatment of cultured neurons or other neurotoxic insults cause calpain-mediated cleavage of p35 to form a smaller form p25 (Kusakawa *et al.* 2000; Lee *et al.* 2000). Since p25 is more stable, it causes aberrant and sustained activation of Cdk5 (Patrick *et al.* 1999), which promotes neuronal apoptosis by aberrantly phosphorylating its substrates like microtubule-associated protein tau (Patrick *et al.* 1999). $A\beta_{42}$ promotes cyclin D1 expression in neurons, which leads to dissociation of p35–Cdk5 complex, as cyclin D1 sequesters Cdk5 into an inactive complex resulting neuronal cell-cycle reentry and apoptosis (Modi *et al.* 2012). It had remained unknown how despite the presence of its cognate partner p35, Cdk5 is associated with cyclin D1 in a stable complex. Since CKIs have affinity for cdk/cyclin complexes, we investigated the possibility of the involvement of p27 in this process. P27 inhibits Cdk2/cyclin E complex to prevent G1/S transition of the cell cycle (Xu *et al.* 1999). In neuronal cells, besides cell cycle regulation, p27 also plays an important role in neuronal differentiation, neuronal migration (Zindy *et al.* 1999; Zheng *et al.* 2010) etc. p27 was previously demonstrated to be a substrate of Cdk5, which phosphorylates it at S10 (Kawauchi *et al.* 2006). While p27 interacts with Cdk5 (Lee *et al.* 1996; Kawauchi *et al.* 2006; Zhang *et al.* 2010; Zhang and Herrup 2011), it does not inhibit active Cdk5/p35 (Lee *et al.* 1996). Our findings provide insights into p27 association with Cdk5 under neurotoxic conditions; the presence of p27 results in a stable complex with cyclin D1 and Cdk5, which comes into play when cyclin D1 levels elevate upon exposure of neurons to $A\beta_{42}$. As a result, Cdk5 is sequestered from p35 and is rendered inactive, which results in neuronal apoptosis. $A\beta_{42}$ induced neuronal apoptosis could be prevented by p27 knockdown (Fig. 3a). Investigation of the link between apoptosis and cell cycle suggested that PCNA expression and BrdU incorporation remained almost unaltered upon p27 knockdown. As reported previously, the depletion of cyclin D1 prevented cell-cycle reentry and apoptosis by blocking progression into the S-phase (Modi *et al.* 2012, 2016). However, p27 seems to play a major role in M-phase progression of $A\beta_{42}$-treated neurons as its depletion prevented the S10-phosphorylation of histone H3 (Fig. 5a and b). p27 inhibits G1/S phase cyclin–Cdk complexes like cyclin E-Cdk2 (Hengst and Reed 1998; Sherr and Roberts 1999; Xu *et al.* 1999) and cyclin D-Cdk4 (Blain *et al.* 1997;

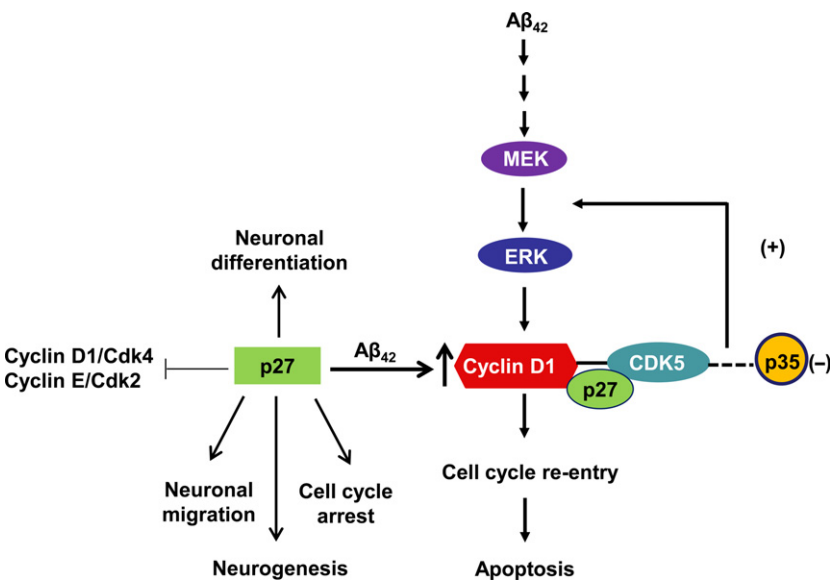

Fig. 7 Molecular mechanisms via which p27 may contribute to the process of $A\beta_{42}$ induced neuronal apoptosis. Previous studies have highlighted the importance of p27 for several important neuronal functions (see text for details). This study demonstrates that $A\beta_{42}$ results in p27-mediated Cdk5/cyclin D1 complex formation, which is inactive (Modi *et al.* 2012). As a result, Cdk5 is sequestered from p35 resulting in the down-regulation of p35-Cdk5. Since Cdk5 keeps check on MEK1 in neurons (Modi *et al.* 2012; Sharma *et al.* 2002), its inactivation results in aberrant activation of MEK-ERK pathway (Modi *et al.* 2012) leading to cell cycle reentry and neuronal apoptosis.

Ray *et al.* 2009), which drives G1-S transition and S-phase entry. Therefore, it is possible that p27 knockdown in $A\beta_{42}$-treated neurons caused activation of these kinases, which are activated by cognate cyclins like cyclin D1, contributing to S-phase entry. As a result, the effect of p27 depletion on the suppression of S-phase was masked by possible aberrant activation of these G1–S cyclin/Cdk complexes. Typically, in conventional cell-cycle progression of dividing cells, p27 undergoes degradation during G1–S and is present in low amounts subsequently (Nourse *et al.* 1994; Auld *et al.* 2007). In contrast, it is evident from this study that it continues to be expressed in neurons after $A\beta_{42}$ triggered cell cycle reentry. It is clear that its continued presence is deleterious to neurons as its depletion prevented the M-phase progression and apoptosis.

Present findings suggest that p27 may perform dual function in neuronal cell cycle. While it may promote neuronal differentiation on one hand (Lee *et al.* 1996; Hengst and Reed 1998; Baldassarre *et al.* 2000; Zheng *et al.* 2010; Andreu *et al.* 2015), it seems to contribute to neuronal cell death in response to stress like $A\beta_{42}$. Its ability to interact and stabilize Cdk5/cyclin D1 complex plays a critical role in this process as neuronal survival and cell-cycle suppression-mediated by Cdk5 is attenuated upon its dissociation from its regulatory partner p35 (Fig. 7).

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Author contributions

PS and SJ designed experiments and analyzed the data. SJ performed experiments. PS and SJ wrote the manuscript and conceived the idea. All authors discussed the results and edited the manuscript.

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