Deregulated Cdk5 triggers aberrant activation of cell cycle kinases and phosphatases inducing neuronal death

Kuei-Hua Chang, Fabien Vincent and Kavita Shah*

Department of Chemistry and Purdue University Center for Cancer Research, Purdue University, West Lafayette, IN 47907, USA

*Author for correspondence (shah23@purdue.edu)

Accepted 5 July 2012
Journal of Cell Science 125, 5124–5137

doi: 10.1242/jcs.108183

Summary

Aberrant activation of cell cycle proteins is believed to play a critical role in Alzheimer's disease (AD) pathogenesis; although, the molecular mechanisms leading to their activation in diseased neurons remain elusive. The goal of this study was to investigate the mechanistic link between Cdk5 deregulation and cell cycle re-activation in β-amyloid$^{1-42}$ (Aβ^{1-42})-induced neurotoxicity. Using a chemical genetic approach, we identified Cdc25A, Cdc25B and Cdc25C as direct Cdk5 substrates in mouse brain lysates. We show that deregulated Cdk5 directly phosphorylates Cdc25A, Cdc25B and Cdc25C at multiple sites, which not only increases their phosphatase activities but also facilitates their release from 14-3-3 inhibitory binding. Cdc25A, Cdc25B and Cdc25C in turn activate Cdk1, Cdk2 and Cdk4 kinases causing neuronal death. Selective inhibition of Cdk5 abrogates Cdc25 and Cdk activations in Aβ^{1-42}-treated neurons. Similarly, phosphorylation-resistant mutants of Cdc25 isoforms at Cdk5 sites are defective in activating Cdk1, Cdk2 and Cdk4 in Aβ^{1-42}-treated primary cortical neurons, emphasizing a major role of Cdk5 in the activation of Cdc25 isoforms and Cdks in AD pathogenesis. These results were further confirmed in human AD clinical samples, which had higher Cdc25A, Cdc25B and Cdc25C activities that were coincident with increased Cdk5 activity, as compared to age-matched controls. Inhibition of Cdk5 confers the highest neuroprotection against Aβ^{1-42} toxicity, whereas inhibition of Cdc25 isoforms was partially neuroprotective, further emphasizing a decisive role of Cdk5 deregulation in cell-cycle-driven AD neuronal death.

Key words: Chemical genetic screen, Cdk5, Cdc25, Alzheimer's disease

Introduction

Alzheimer's disease (AD) is a fatal neurodegenerative disorder that has no known cure, nor is there a clear mechanistic understanding of the disease pathology. Accumulating evidence has highlighted the reactivation of cell cycle in AD neurons as a potential mechanism that drives cells towards neuronal atrophy. The presence of ectopic markers like phosphorylated histone H3, replicated DNA, active Cdc25 isoforms and active Cdk1 support alterations in cell cycle control in AD etiology (Ding et al., 2000; Vincent et al., 2001; Vincent et al., 1997; Ogawa et al., 2003). Active Cdk1 and cyclin B1 colocalize with AD-specific mitotic phospho-epitopes, suggesting that Cdk1 may phosphorylate neuronal tau protein causing neurofibrillary tangles (NFT) formation (Vincent et al., 1997). Notably, cell cycle disturbances emerge in the early stages of mild cognitive impairment (MCI) before the formation of amyloid plaques and neurofibrillary tangles (NFT), and persist as the patient's condition worsens (Vincent et al., 1996; Nagy et al., 1997a; McShea et al., 1997). Ectopic cell cycling occurs both in AD patients and mouse models, signifying that the loss of cell cycle control is an important pathological root of the disease. Similar to humans, in transgenic R1.40 AD mouse model, cell cycle markers appear months before the formation of amyloid plaques and NFT and persist throughout the disease process (Yang et al., 2003). Its activation pattern within the cortex also resembles the pattern in humans, with the upper layers activated first followed by the deeper layers, which further supports the hypothesis that cell cycle activation is an early and central event in AD pathogenesis, rather than being an ancillary symptom.

Moreover, ectopic expression of cell cycle markers spatially and regionally correlates well with neuronal cell death in AD patients (Busser et al., 1998; Nagy et al., 1997a; Yang et al., 2003). Early loss of neurons in the entorhinal cortex is preceded by the appearance of a large numbers of cell cycle markers (Yang et al., 2003). Only degenerating neurons are positive for different cell cycle proteins, suggesting that cell cycle antigens may be the best markers for dying neurons in all cell stages (Yang et al., 2003; Arendt et al., 2003). Additional evidence for the link between cell cycle and AD is the presence of higher levels of active cell cycle proteins in the hippocampus, the region most vulnerable in AD pathology. All these findings support the notion that cell cycle aberrations contribute significantly to AD pathogenesis. Thus, therapeutic interventions targeted toward ameliorating mitotic changes are predicted to have a profound and positive impact on AD progression. Small molecule inhibitors of Cdks are neuroprotective, supporting this hypothesis (Monaco and Vallano, 2003). However, despite the strong immunohistochemical correlation between cell cycle activation and neurodegeneration in AD patients and mouse models, the mechanism leading to their activation in AD brains and subsequent neuronal death remains incompletely understood, which has hindered the development of neuroprotective and target-based therapies in AD.

In this study, we unraveled a novel mechanism of cell cycle activation in post-mitotic neurons triggered by deregulated Cyclin

dependent kinase-5 (Cdk5). Cdk5 belongs to the Cyclin dependent kinase (Cdk) family of serine/threonine kinases, which play important regulatory roles in cell cycle progression. Unlike other members of Cdk family, Cdk5 is not activated by cyclins but by specific regulatory binding proteins, p35 or p39 (or their truncated forms, p25 and p29). While other Cdks are active in mitotic cells, Cdk5 is primarily active in post-mitotic neurons due to the restricted expression of its activators. Cdk5 is vital in regulating neuronal migration, neurite extension and synapse formation during embryogenesis (Ohshima et al., 1996). In adult brains, it regulates neuronal survival, synaptic plasticity, stress enhanced memory consolidation, associative learning and long-term behavioral changes (Hisanaga and Endo, 2010; Shea et al., 2003; Li et al., 2003; Zheng et al., 2010). Cdk5 is hyperactivated in several neurodegenerative diseases including AD and is highly neurotoxic.

Under pathological conditions such as β-amyloid (Aβ) exposure, oxidative stress, calcium dysregulation, mitochondrial dysfunction, excitotoxicity and inflammation, p35 and p39 are cleaved into p25 and p29 in AD (Patrick et al., 1999; Cruz and Tsai, 2004). These truncated proteins constitutively activate Cdk5 and change its subcellular localization from particulate to cytosolic and nuclear. Cdk5 can then access a variety of pathological substrates, triggering a cascade of pathways, contributing to all three hallmarks of AD: neurotoxic β amyloid (Aβ) and neurofibrillary tangles (NFT) formation and neuronal death (Patrick et al., 1999; Cruz et al., 2006; Wen et al., 2008; Kanungo et al., 2009). Interestingly, increased p25 levels in AD brains remain a contentious issue. While a few studies have reported an increase in the p25:p35 ratio and active calpain in AD brains (Grynspan et al., 1997; Patrick et al., 1999; Tseng et al., 2002), others have shown either reduced or equal levels of p25 (Yoo and Lubec, 2001; Taniguchi et al., 2001; Tandon et al., 2003). Nonetheless, significantly higher Cdk5 activity has been observed in AD brains compared to age-matched controls (Lee et al., 1999), suggesting additional Cdk5 regulators may exist and further contribute to Cdk5 deregulation observed in the diseased state. Recently, we identified glutathione *S*-transferase P1 (GSTP1) as a potent negative regulator of Cdk5 activity in human AD brains and various neuronal and non-neuronal cells. GSTP1 directly inhibits Cdk5 by dislodging p35/p25 and indirectly by eliminating oxidative stress, thereby uncovering an alternate mechanism for triggering Cdk5 deregulation in AD (Sun et al., 2011).

In AD, deregulated Cdk5-induced phosphorylation of signal transducer and activator of transcription 3 (STAT3) upregulates β secretase 1 (BACE1) levels, causing neurotoxic Aβ formation (Wen et al., 2008). Cdk5/p25-mediated tau and neurofilament hyperphosphorylation contributes significantly to NFT formation (Hashiguchi et al., 2002; Shea et al., 2004; Rudrabhatla et al., 2011). Our studies have demonstrated several novel neurotoxic roles of Cdk5 in primary neurons and AD mouse models. Cdk5 deregulation in neurotoxin-exposed primary neurons promotes robust Golgi fragmentation via GM130 phosphorylation (Sun et al., 2008a), and oxidative stress and mitochondrial dysfunction by inactivating peroxiredoxin-1 and peroxiredoxin-2 (Sun et al., 2008b). We identified c-Jun as a direct transcriptional target of Cdk5 in primary neurons and an AD mouse model, which causes neurotoxicity independent of JNK (Sun et al., 2009). We have determined the mechanism by which Cdk5 deregulation activates p38 MAPK in primary neurons and AD mouse model (Chang et al., 2010). We have also shown that deregulated Cdk5 triggers robust nuclear fragmentation by direct phosphorylation of lamin A and lamin B1 in neurons and AD animal model, which mislocalizes several proteins, including Cdk5 to the nucleus, which is highly neurotoxic (Chang et al., 2011).

Nuclear Cdk5 can trigger cell death by multiple mechanisms. Cdk5-mediated phosphorylation of p53 promotes apoptosis. Cdk5 also increases p53 expression via ATM phosphorylation causing cell death (Zhang et al., 2002; Lee and Kim, 2007; Tian et al., 2009). Cdk5 degrades pro-survival transcription factors MEF2A and MEF2D, promoting neuronal death (Tang et al., 2005). Nuclear Cdk5/p25 promotes cell death in ER-stressed neurons (Saito et al., 2007). Cdk5 activates DNA damage signaling by inhibiting histone deacetylase 1 (Kim et al., 2008) and apurinic/apyrimidinic endonuclease 1 (Huang et al., 2010), leading to cell death.

In this study, we unraveled a novel mechanism of AD pathogenesis following our discovery of mammalian cell division cycle 25 A (Cdc25A), Cdc25B and Cdc25C phosphatases as direct substrates of Cdk5/p25 in mouse brain lysates using a chemical genetic screen. Cdc25 phosphatases are key cell cycle regulators in proliferating cells, which initiate activation of Cdks by dual dephosphorylation of conserved Thr and Tyr residues. Interestingly, Cdc25A, Cdc25B and Cdc25C phosphatases are expressed in normal adult brain despite the absence of active cell cycle, and display basal enzymatic activities, although they are not known to play any roles. In contrast, in AD, their levels and activities are significantly increased, which is hypothesized to activate Cdk1/cyclin B and vice versa, leading to subsequent neurodegeneration. However, despite the strong immunohistochemical evidence demonstrating active Cdc25 phosphatases and Cdks in degenerating neurons, the mechanisms leading to their activation in AD remain elusive.

Results

Identification of Cdc25A, Cdc25B and Cdc25C as direct Cdk5 targets in mouse brain lysates

We have reported a chemical genetic approach for the identification of direct substrates of Cdk5 (Sun et al., 2008a; Sun et al., 2008b; Chang et al., 2011). A kinase of interest is engineered (analog-sensitive kinase), which in the presence of a radioactive orthogonal ATP analog (e.g. *N*6-phenethyl ATP) specifically transfers the radioactive tag to its substrates (Shah et al., 1997; Shah and Shokat, 2002; Shah and Shokat, 2003; Shah and Vincent, 2005; Kim and Shah, 2007; Sun et al., 2008a; Sun et al., 2008b; Chang et al., 2011; Johnson et al., 2011, Johnson et al., 2012). The engineered pocket is generated by replacing a conserved bulky residue (gatekeeper residue) with glycine in the active site of the kinase. The mutant kinase shows the same substrate specificity as the corresponding wild-type kinase. Since this ATP analog is biologically orthogonal to the endogenous kinases, this approach offers unbiased identification of direct substrates of the engineered kinase in a global environment. Using this chemical genetic approach, we have identified several direct targets of Cdk5/p25 in mouse brain lysates (Sun et al., 2008a; Sun et al., 2008b; Chang et al., 2011). In this study, we focused on novel Cdk5 substrates, Cdc25A, Cdc25B and Cdc25C phosphatases and their contributions in $A\beta^{1-42}$-mediated neurotoxicity.

Cdc25A, Cdc25B and Cdc25C are directly phosphorylated by Cdk5 *in vitro*

Since proteomic screens can often lead to false positives, Cdk5-mediated phosphorylation of Cdc25A, Cdc25B and Cdc25C were

examined using an *in vitro* kinase assay. Cdc25A, Cdc25B and Cdc25C were generated as 6xHis-tagged proteins and subjected to kinase assays with Cdk5/p25. Cdk5 efficiently phosphorylated all Cdc25 phosphatases confirming them as direct Cdk5 targets *in vitro* (Fig. 1A–C).

Cdc25A, Cdc25B and Cdc25C associate with Cdk5 upon $A\beta^{1-42}$ stimulation

Since kinase substrate specificity *in vivo* is regulated by protein–protein interactions and subcellular localization, we investigated whether Cdk5 and Cdc25 associate in primary neurons under basal or neurotoxic conditions. Any insult that affects neurons leads to increased Aβ production. Thus, Cdk5 immune complexes were isolated from primary cortical neurons treated with oligomeric $A\beta^{1-42}$ (Fig. 1D), and probed for Cdc25A, Cdc25B and Cdc25C binding. Although little Cdc25–Cdk5 association was observed in untreated cells, their association increased significantly upon $A\beta^{1-42}$ stimulation (Fig. 1E–G). These results were verified by specifically isolating Cdc25A, Cdc25B and Cdc25C immune complexes from untreated and

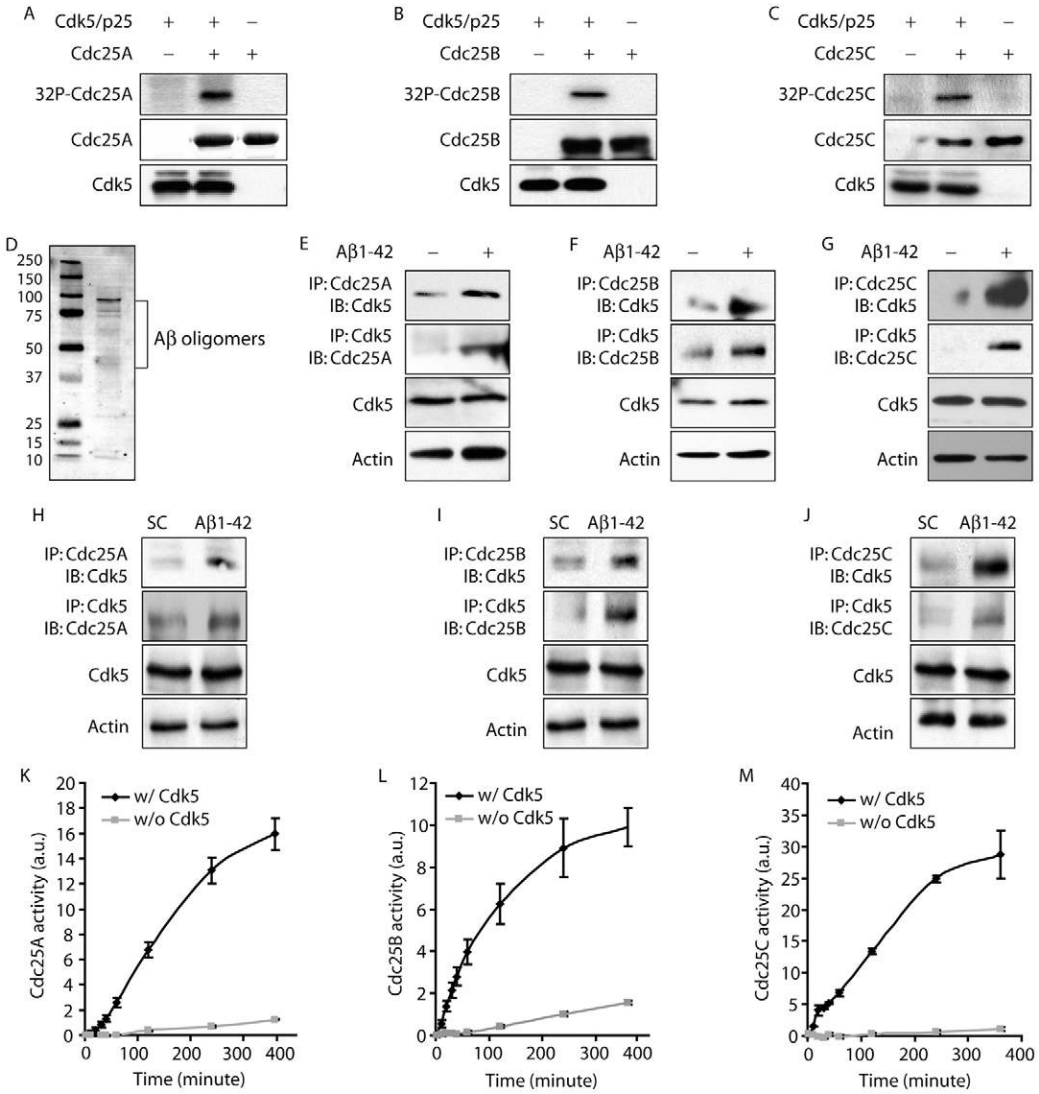

Fig. 1. Identification of Cdc25A, Cdc25B and Cdc25C as direct Cdk5 substrates. (**A–C**) Cdc25A, Cdc25B and Cdc25C are phosphorylated by Cdk5 *in vitro*. Recombinant 6xHis-Cdc25A, 6xHis-Cdc25B and 6xHis-Cdc25C were incubated with Cdk5/p25 complex-bound beads in the presence of [γ-^{32}P]ATP at 30℃ for 20 min. Phospho-Cdc25A, phospho-Cdc25B and phospho-Cdc25C were visualized using autoradiography. (**D**) Oligomeric $A\beta^{1-42}$ was prepared as described in Materials and Methods. (**E–G**) $A\beta^{1-42}$ increases the association of Cdc25A, Cdc25B or Cdc25C with Cdk5 in cells. Rat primary cortical neurons were treated with $A\beta^{1-42}$ (100 nM) for 0.5 h. Cdc25A, Cdc25B, Cdc25C or Cdk5 was immunoprecipitated from cell lysates and analyzed using the indicated antibodies. (**H–J**) $A\beta^{1-42}$ increases the association of Cdc25A, Cdc25B or Cdc25C with Cdk5 in primary neurons but scrambled $A\beta^{1-42}$ has no effect. Rat primary cortical neurons were treated with scrambled $A\beta^{1-42}$ (SC) or $A\beta^{1-42}$ (100 nM) for 0.5 h. Cdc25A, Cdc25B, Cdc25C or Cdk5 was immunoprecipitated from cell lysates and analyzed using the indicated antibodies. (**K**) Recombinant 6xHis-Cdc25A was preincubated with Cdk5/p25 complex bound on beads in the presence of ATP (10 μM) at 30℃ for 20 minutes. The supernatant containing Cdc25A was collected, followed by addition of OMFP (100 μM) at 30℃ at the indicated time points. The fluorescence intensity emitted from the product of the hydrolyzed OMFP was measured. (**L**) 6xHis-Cdc25B preincubated with Cdk5/p25 complex and ATP was incubated with OMFP for the indicated times and fluorescence intensity measured. (**M**) 6xHis-Cdc25C preincubated with Cdk5/p25 complex and ATP was incubated with OMFP for the indicated times and fluorescence intensity measured.

$A\beta^{1-42}$-treated neurons, which also revealed that Cdc25–Cdk5 association increases considerably in $A\beta^{1-42}$-treated neurons (Fig. 1E–G). We also compared Cdc25–Cdk5 association in scrambled $A\beta^{1-42}$-treated and $A\beta^{1-42}$-treated neurons by isolating Cdk5 and probing for Cdc25A, Cdc25B and Cdc25C association, or by isolating Cdc25 isoforms and analyzing Cdk5 binding. As shown in Fig. 1H–J, scrambled $A\beta^{1-42}$-treatment does not promote Cdk5–Cdc25 association, whereas $A\beta^{1-42}$ treatment specifically increases their association. These data thus support that Cdc25A, Cdc25B and Cdc25C are direct targets of deregulated Cdk5 in neurons.

Cdk5 increases phosphatase activities of Cdc25A, Cdc25B and Cdc25C *in vitro*

Since active Cdks directly phosphorylate Cdc25 isoforms causing their activation, we hypothesized that Cdk5 may similarly activate Cdc25 isoforms by phosphorylation. The phosphatase activities of recombinant Cdc25A, Cdc25B and Cdc25C were measured in the absence or presence of Cdk5/p25 in a time course experiment. Cdk5/p25 complex induced a rapid and profound increase in phosphatase activities of Cdc25A, Cdc25B and Cdc25C. Their basal activities in the absence of Cdk5/p25 remained unaltered during the course of the experiment, thereby suggesting that Cdk5/p25 positively regulates Cdc25A, Cdc25B and Cdc25C activities via direct phosphorylation (Fig. 1K–M).

$A\beta^{1-42}$ stimulates Cdc25A, Cdc25B and Cdc25C activation in a Cdk5-dependent manner

The *in vitro* activation of Cdc25 isoforms by Cdk5 prompted us to investigate this mechanism in neurotoxic $A\beta^{1-42}$-exposed primary neurons. Endogenous Cdk5 kinase activity and Cdc25 phosphatase activities were analyzed at different times in $A\beta^{1-42}$-treated primary cortical neurons (ranging from 0.5 h to 6 h), which revealed biphasic activation of Cdk5 (Fig. 2A). The first phase was initiated within 0.5 h of $A\beta^{1-42}$ stimulation, which gradually decreased, however remained higher than the basal levels. The appearance of secondary phase was triggered by prolonged $A\beta^{1-42}$ incubation (6 h). This result was similar to our previous report showing the biphasic pattern of Cdk5 activation in glutamate or $A\beta^{25-35}$-treated neuronal cells (Sun et al., 2009).

Cdc25A, Cdc25B and Cdc25C also showed biphasic activation upon $A\beta^{1-42}$ treatment in primary cortical neurons (Fig. 2B–D). The first wave was rapid (0.5 h), followed by a second wave of activation, both of which coincided with Cdk5 activation profile. However, the degree of activation varied between different isoforms. In contrast to Cdc25B and Cdc25C, where the levels of first and second phases of activation were similar, for Cdc25A, the second wave of activation was more profound. Notably, the endogenous levels of Cdc25A, Cdc25B and Cdc25C did not change significantly in response to $A\beta^{1-42}$ (Fig. 2E), suggesting that the observed Cdc25 activation arises due to Cdk5-mediated phosphorylation.

To test this hypothesis, we used TAT-fused Cdk5 inhibitory peptide (TAT–CIP) to specifically inhibit Cdk5 in primary neurons with high temporal control (Sun et al., 2008a; Sun et al., 2008b; Sun et al., 2009; Chang et al., 2010; Sun et al., 2011; Chang et al., 2011). CIP is a 126-mer artificial peptide containing $p35^{154–279}$, which inhibits Cdk5 specifically and potently by blocking Cdk5/p25 complex formation when expressed endogenously (Kesavapany et al., 2007). Transduction of TAT–CIP fully suppressed both early and late activations of Cdc25A, Cdc25B and Cdc25C induced by $A\beta^{1-42}$ in rat primary cortical neurons (Fig. 2F–H). These results confirm that Cdk5 is a key regulator of Cdc25A, Cdc25B and Cdc25C activities in neurons under neurotoxic conditions.

Cdk5 upregulates the kinase activities of Cdk1, Cdk2 and Cdk4

Multiple studies have documented active Cdks in AD neurons (Vincent et al., 1997; McShea et al., 1997; Busser et al., 1998). However, the mechanism underlying their activation has not been delineated. Since in proliferating cells Cdc25 phosphatases positively regulate the activities of Cdk by dual dephosphorylation at T14 and Y15 (for Cdk1), we postulated that activated Cdc25 proteins may be responsible for Cdk activation in AD neurons. To pursue this hypothesis, kinase activities of endogenous Cdk1, Cdk2 and Cdk4 were analyzed in $A\beta^{1-42}$-treated neurons, which gradually increased from 0.5 h to 6 h (Fig. 3A–C). For Cdk1, the maximal activation (2.5-fold) was observed at 1 h, Cdk2 (2.0-fold) at 4 h and for Cdk4 (1.5-fold) at 4 h; however, the levels of Cdk1, Cdk2 and Cdk4 remained unaltered in $A\beta^{1-42}$-treated neurons (Fig. 3D). In parallel, phosphotyrosine levels of Cdk1, Cdk2 and Cdk4 were also analyzed in primary cortical neurons, which decreased significantly in $A\beta^{1-42}$-exposed neurons suggesting possible dephosphosphorylation of the Y15 site by active Cdc25 phosphatases (Fig. 3E). Transduction of TAT–CIP completely prevented the Aβ-induced activation of Cdk1, Cdk2, and Cdk4 (Fig. 3F–H), suggesting that Cdk5-mediated activation of Cdc25 isoforms trigger Cdk activation in $A\beta^{1-42}$-treated post-mitotic neurons in the manner similar to proliferative cells. Most importantly, these results highlight Cdk5 as a critical upstream regulator that initiates re-activation of cell cycle proteins in neurons upon neurotoxic insults.

Cdk5 deregulation facilitates the release of active Cdc25 from 14-3-3ε

In proliferating cells, activities of Cdc25 phosphatases are regulated both by phosphorylation and protein–protein association. Scaffolding protein 14-3-3ε binds Cdc25 phosphatases, sequestering them in the cytoplasm, and rendering them inactive (Hermeking and Benzinger, 2006). This led us to postulate that Cdk5 may also regulate Cdc25 activities by inhibiting its binding to 14-3-3ε. As shown in Fig. 4A, $A\beta^{1-42}$ treatment induces the dissociation of Cdc25A, Cdc25B or Cdc25C from 14-3-3ε, which was prevented by transduction of TAT–CIP (Fig. 4B).

Since 14-3-3 binding sequesters Cdc25 phosphatases in the cytoplasm in proliferating cells, we examined the sub-cellular distribution of Cdc25A, Cdc25B and Cdc25C in $A\beta^{1-42}$ neurons. We hypothesized that analogous to dividing cells, the release from 14-3-3ε will enable cytosolic Cdc25 phosphatases to migrate to the nucleus in primary neurons. However, subcellular fractionation analysis showed that in untreated primary cortical neurons, Cdc25A predominantly resides in the nucleus, Cdc25C primarily in the cytoplasm, and Cdc25B both in the nucleus and the cytoplasm. Upon $A\beta^{1-42}$ stimulation, increased amounts of Cdc25C were observed in the nuclear fraction of primary cortical neurons, whereas no obvious changes in the subcellular distribution of Cdc25A and Cdc25B could be detected (Fig. 4C). In agreement with these findings, immunofluorescence studies also revealed significant nuclear localization of Cdc25C in the nucleus in $A\beta^{1-42}$-treated neurons, but Cdc25A or Cdc25B sub-cellular

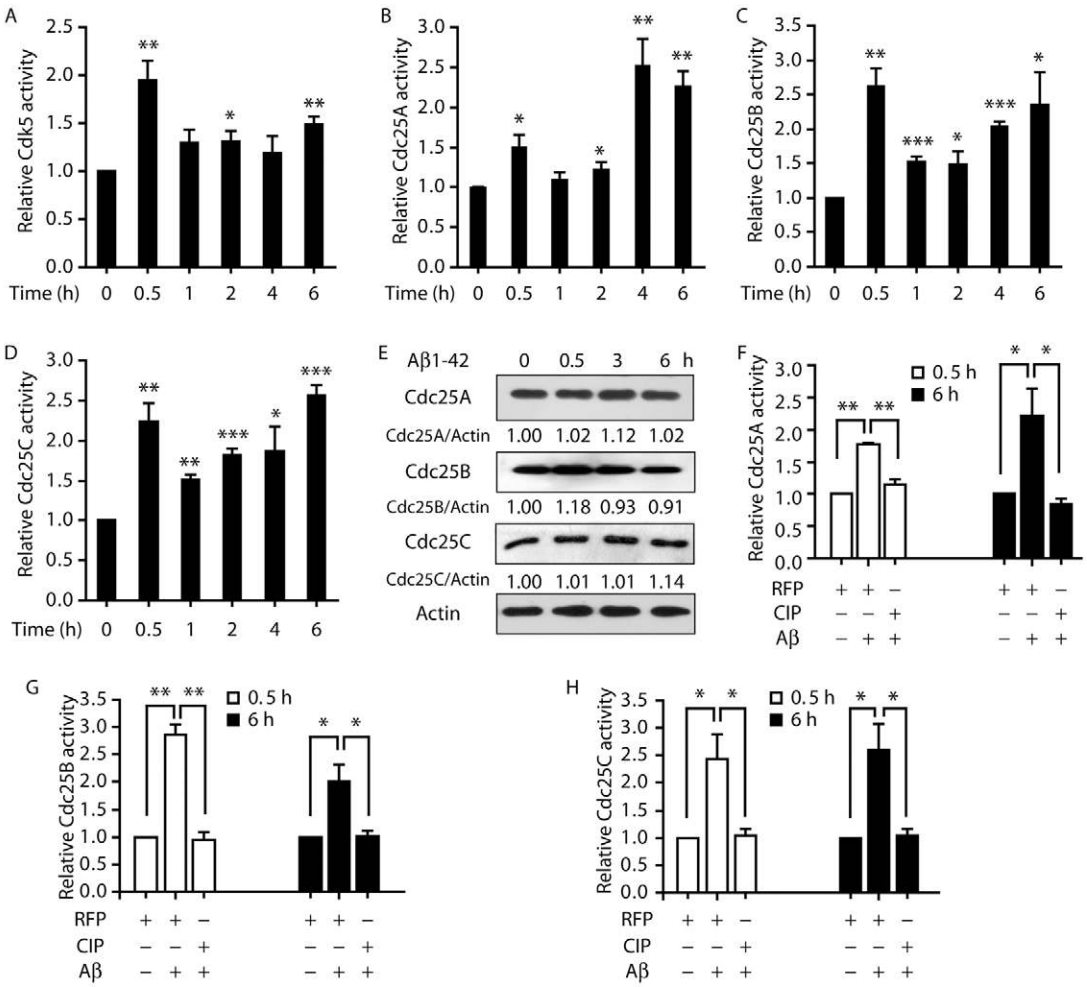

Fig. 2. Aβ$^{1-42}$ induces the activation of Cdc25A, Cdc25B and Cdc25C in a Cdk5-dependent manner. (**A**) Rat primary cortical neurons were treated with Aβ$^{1-42}$ (100 nM) for the indicated times. For the *in vitro* kinase assay, Cdk5 immunoprecipitated from lysed cells was incubated with the synthetic Cdk substrate peptide in the presence of [γ-^{32}P]ATP at 30℃ for 20 min. *P<0.05, **P<0.01 when comparing data obtained from Aβ$^{1-42}$-treated neurons at the indicated incubation times to the untreated samples (0 h). (**B–D**) Rat primary cortical neurons were treated with Aβ$^{1-42}$ (100 nM) for the indicated times. For the *in vitro* phosphatase assay, Cd25A, Cdc25B or Cdc25C immunoprecipitated from lysed cells was incubated with OMFP (100 μM) at 30℃ for 1 h. The fluorescence intensity emitted from the product of the hydrolyzed OMFP was measured. *P<0.05, **P<0.01, ***P<0.001 when comparing data obtained from Aβ$^{1-42}$-treated neurons to untreated ones (0 h). (**E**) Rat primary cortical neurons were treated with Aβ$^{1-42}$ (100 nM) for the indicated times. Levels of endogenous Cdc25A, Cdc25B and Cdc25C were analyzed using the indicated antibodies. (**F**) Rat primary cortical neurons were treated with TAT–CIP (200 nM) for 0.5 h, followed by incubation with Aβ$^{1-42}$ (100 nM) for 0.5 or 6 h. Cdc25A was immunoprecipitated for *in vitro* phosphatase assay. *P<0.05, **P<0.01 was determined for intergroup comparisons. (**G**) Rat primary cortical neurons were treated with TAT–CIP (200 nM) for 0.5 h, followed by incubation with Aβ$^{1-42}$ (100 nM) for 0.5 or 6 h. Cdc25B was immunoprecipitated for *in vitro* phosphatase assay. *P<0.05, **P<0.01 was determined for intergroup comparisons. (**H**) Rat primary cortical neurons were treated with TAT–CIP (200 nM) for 0.5 h, followed by incubation with Aβ$^{1-42}$ (100 nM) for 0.5 or 6 h. Cdc25C was immunoprecipitated for *in vitro* phosphatase assay. *P<0.05 was determined for intergroup comparisons. All experiments were performed at least five times independently; representative data are shown.

localization appeared almost unaltered (Fig. 4D). Since Cdc25A and Cdc25B already show significant nuclear residence in untreated neurons, dislodging from 14-3-3ε may not show an obvious increase in the nuclear pool. Taken together, these results suggest that increased catalytic activity and the release from the inhibitory binding of 14-3-3 are the two key mechanisms by which Cdk5 deregulation causes Cdc25 activation.

Identification of phosphorylation sites on Cdc25A, Cdc25B and Cdc25C

To gain further insight into Aβ-triggered activation of Cdc25, we investigated Cdk5-mediated phosphorylation sites on Cdc25A, Cdc25B and Cdc25C. Since Cdk5 is a proline-directed kinase with preference for basic residues close to the phosphorylation site, we generated multiple phosphorylation-resistant mutants of Cdc25 isoforms and subjected them to kinase assays using Cdk5/p25. As shown in Fig. 5A, Cdc25A was phosphorylated at S40, S116 and S261 residues by Cdk5/p25. S116 and S261 appeared to be the major phosphorylation sites, whereas S40 was a minor site. To confirm this finding, we generated (S116A, S261A) Cdc25A double mutant and (S40A, S116A, S261A) triple mutant and conducted the kinase assays with Cdk5/p25. While the double mutant showed severely reduced phosphorylation, the triple mutant completely

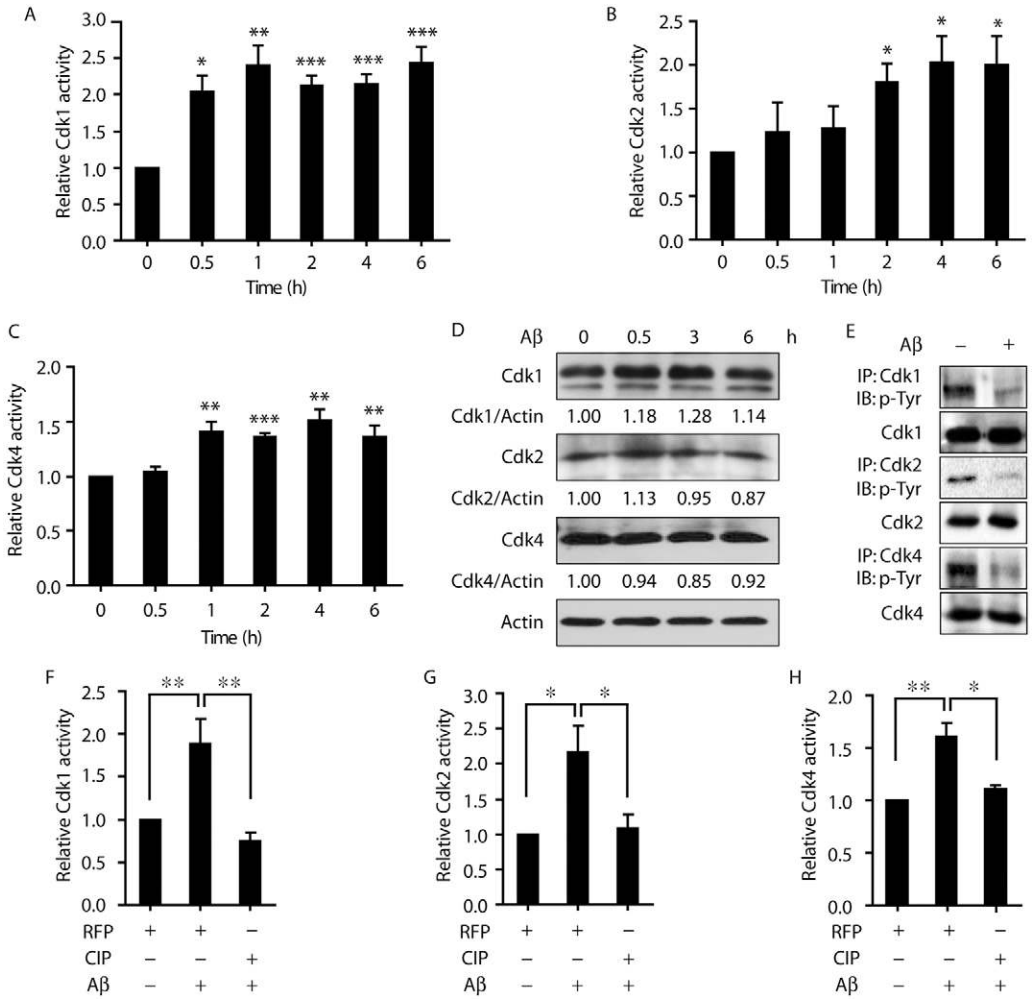

Fig. 3. Aβ$^{1-42}$ induces the activation of Cdk1, Cdk2 and Cdk4 in a Cdk5-dependent manner. (**A–C**) Rat primary cortical neurons were treated with Aβ$^{1-42}$ (100 nM) for the indicated times. For the *in vitro* kinase assay, Cdk1, Cdk2 or Cdk4 immunoprecipitated from lysed cells was incubated with the synthetic Cdk substrate peptide in the presence of [γ-^{32}P]ATP at 30℃ for 20 min. *P<0.05, **P<0.01, ***P<0.001 when comparing data obtained from Aβ$^{1-42}$-treated neurons at the indicated incubation times to the untreated samples (0 h). (**D**) Levels of endogenous Cdk1, Cdk2 and Cdk4 in Aβ$^{1-42}$-treated cortical neurons were analyzed using indicated antibodies. (**E**) Rat primary cortical neurons were treated with Aβ$^{1-42}$ (100 nM) and Cdk1, Cdk2 or Cdk4 immunoprecipitated from lysed cells, and their tyrosine phosphorylation analyzed using phosphotyrosine antibody. (**F–H**) Cells were incubated with Aβ$^{1-42}$ (100 nM) and TAT–CIP (200 nM) for 6 h. 100 nM TAT–CIP was re-added in the middle of the experiment. Total cell lysates were prepared and endogenous Cdk1, Cdk2, or Cdk4 immunoprecipitates were subjected to *in vitro* kinase assay. TAT–RFP was chosen as the control. *P<0.05, **P<0.01 was determined for intergroup comparisons. All experiments were performed at least five times independently; representative data are shown.

obliterated it, suggesting that all three sites are phosphorylated by Cdk5/p25 *in vitro* (Fig. 5B). This result also confirms that S40, S116 and S261 are the only sites on Cdc25A that are phosphorylated by Cdk5/p25.

Using similar approaches to those described for Cdc25A, we identified S50, T69, S160, S321 and S470 as Cdk5 phosphorylation sites on Cdc25B (Fig. 5C). Among these, S50A, T69A, S321A and S470A substitutions were more resistant to Cdk5-mediated phosphorylation, while S160A mutant showed relatively enhanced phosphorylation, indicating that Ser160 is the minor phosphorylation site compared to other sites. These results were verified by generating the corresponding double (S50, T69A), triple (S50, T69, S321A), quadruple (S50, T69, S321A, S470A) and quintuple mutants of Cdc25B (S50, T69, S160, S321, S470A) and subjecting them to Cdk5-mediated phosphorylation (Fig. 5D). While all five sites were phosphorylated by Cdk5, phosphorylation-resistant quintuple mutant did not show any phosphorylation, thereby confirming that S50, T69, S160, S321, S470 are the only Cdk5 sites on Cdc25B.

Similarly, we identified T48, T67, S122, T130, S168 and S214 as direct Cdk5 sites on Cdc25C (Fig. 5E). Among these T48, S122, T130, S168 and S214 were identified as the major Cdk5 sites, while T67 appeared to be relatively weak site. Phosphorylation-resistant double, triple, quadruple, quintuple and sextuple Cdc25C mutants further confirmed these findings (Fig. 5F). Taken together, our results demonstrate that Cdc25A is phosphorylated at S40, S116, S261 residues, Cdc25B at S50, T69, S160, S321, S470 sites, and Cdc25C at T48, T67, S122, T130, S168, S214 sites by Cdk5/p25 (Fig. 5B–F). Sequence alignment of Cdc25A, Cdc25B and Cdc25C revealed that the three Cdk5 phosphorylation sites (S40, S116 and S261 on

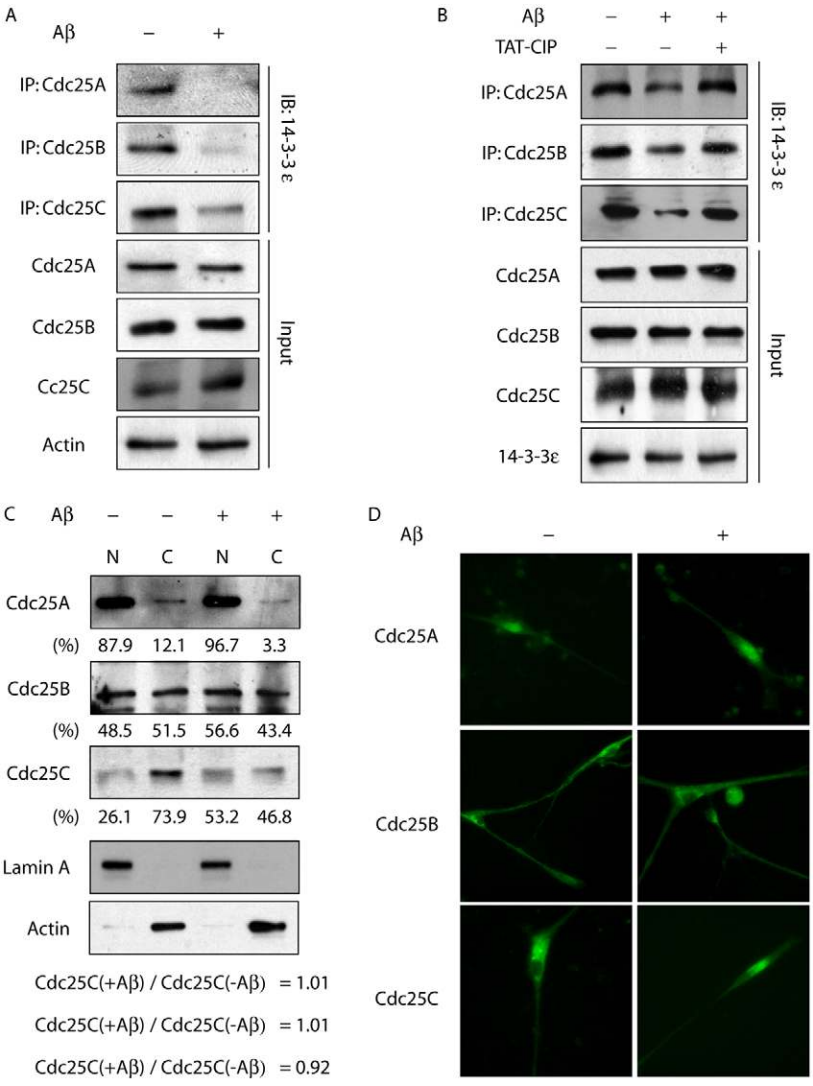

Fig. 4. $A\beta^{1-42}$ facilitates the release of Cdc25 phosphatases from 14-3-3 inhibitory binding. (**A**) Rat primary cortical neurons were treated with 100 nM $A\beta^{1-42}$ for 6 h. Total cell lysates were immunoprecipitated using antibodies against Cdc25A, Cdc25B or Cdc25C, and the immunoprecipitates (IPs) were analyzed using antibodies against 14-3-3ε. (**B**) Rat primary cortical neurons were treated with $A\beta^{1-42}$ (100 nM) for 6 h in the presence of TAT–CIP (200 nM). Cdc25 immunoprecipitates were analyzed using antibodies against 14-3-3ε. (**C**) Rat primary cortical neurons were treated with $A\beta^{1-42}$ (100 nM) for 6 h. Total cell lysates were separated into nuclear (N) and cytoplasmic (C) fractions upon centrifugation. Each fraction was analyzed using antibodies against Cdc25A, Cdc25B and Cdc25C. (**D**) Rat primary cortical neurons were treated with $A\beta^{1-42}$ (100 nM) for 6 h. Cells were fixed and immunostained using antibodies against Cdc25A, Cdc25B or Cdc25C (green).

Cdc25A) were conserved among the three Cdc25 isoforms, while T69 was conserved between Cdc25B and Cdc25C (Fig. 5G). Other Cdk5-mediated phosphorylation sites were unique in Cdc25B and Cdc25C (Fig. 5G).

We also monitored the phosphatase activities of phosphorylation-resistant $Cdc25A^{S40,\ S116,\ S261A}$ (Cdc25A3A), $Cdc25B^{S50,\ T69,\ S160,\ S321,\ S470A}$ (Cdc25B5A), and $Cdc25C^{T48,\ T67,\ S122,\ T130,\ S168,\ S214}$ (Cdc25C6A) mutants in the absence or the presence of Cdk5/p25 using OMFP as the substrate. In contrast to their wild-type counterparts, the presence of Cdk5/p25 did not induce the activation of these Cdc25 mutants (Fig. 5H–J), which further support that Cdc25 phosphatases are activated via direct phosphorylation by Cdk5.

Phosphorylation-resistant Cdc25 isoforms prevents $A\beta^{1-42}$-induced Cdk1, Cdk2 and Cdk4 activations in cortical neurons

To determine the consequences of Cdk5-mediated phosphorylation of Cdc25 isoforms in primary neurons, we generated 6xHis-tagged TAT-fused wild-type and phosphorylation-resistant mutants of Cdc25 isoforms. Fusion of TAT sequence allows rapid and efficient transduction of the recombinant proteins into the cells (Becker-Hapak et al., 2001). TAT-fused wild-type Cdc25 proteins ($Cdc25^{WT}$) and the corresponding mutants $Cdc25A^{S40,\ S116,\ S261A}$ (Cdc25A3A), $Cdc25B^{S50,\ T69,\ S160,\ S321,\ S470A}$ (Cdc25B5A) and $Cdc25C^{T48,\ T67,\ S122,\ T130,\ S168,\ S214}$ (Cdc25C6A) were transduced into primary neurons, and the kinase activities of endogenous Cdk1, Cdk2 and Cdk4 were analyzed. Transduction of TAT–$Cdc25A^{WT}$ markedly promoted the activation of Cdk1, Cdk2 and Cdk4, whereas the phosphorylation-resistant TAT–Cdc25A3A mutant was unable to activate Cdk1, Cdk2 and Cdk4 in cortical neurons (Fig. 6A), although similar levels of transduced WT and mutant Cdc25A were detected (Fig. 6B). In a similar manner, TAT–$Cdc25B^{WT}$ induced significant activation of Cdk1, Cdk2 and Cdk4, but the neurons transduced with TAT–Cdc25B5A mutant merely displayed basal kinase activities for Cdk1, Cdk2 and Cdk4 (Fig. 6C,D). Endogenous Cdk1 was specifically activated by transduction of TAT–$Cdc25C^{WT}$ in primary cortical neurons but none of other Cdks were activated by transduction of TAT–Cdc25C mutants (Fig. 6E,F). These results in primary neurons are similar to previous reports in proliferating cells showing Cdc25A and Cdc25B-mediated activation of Cdk1, Cdk2 and Cdk4, while

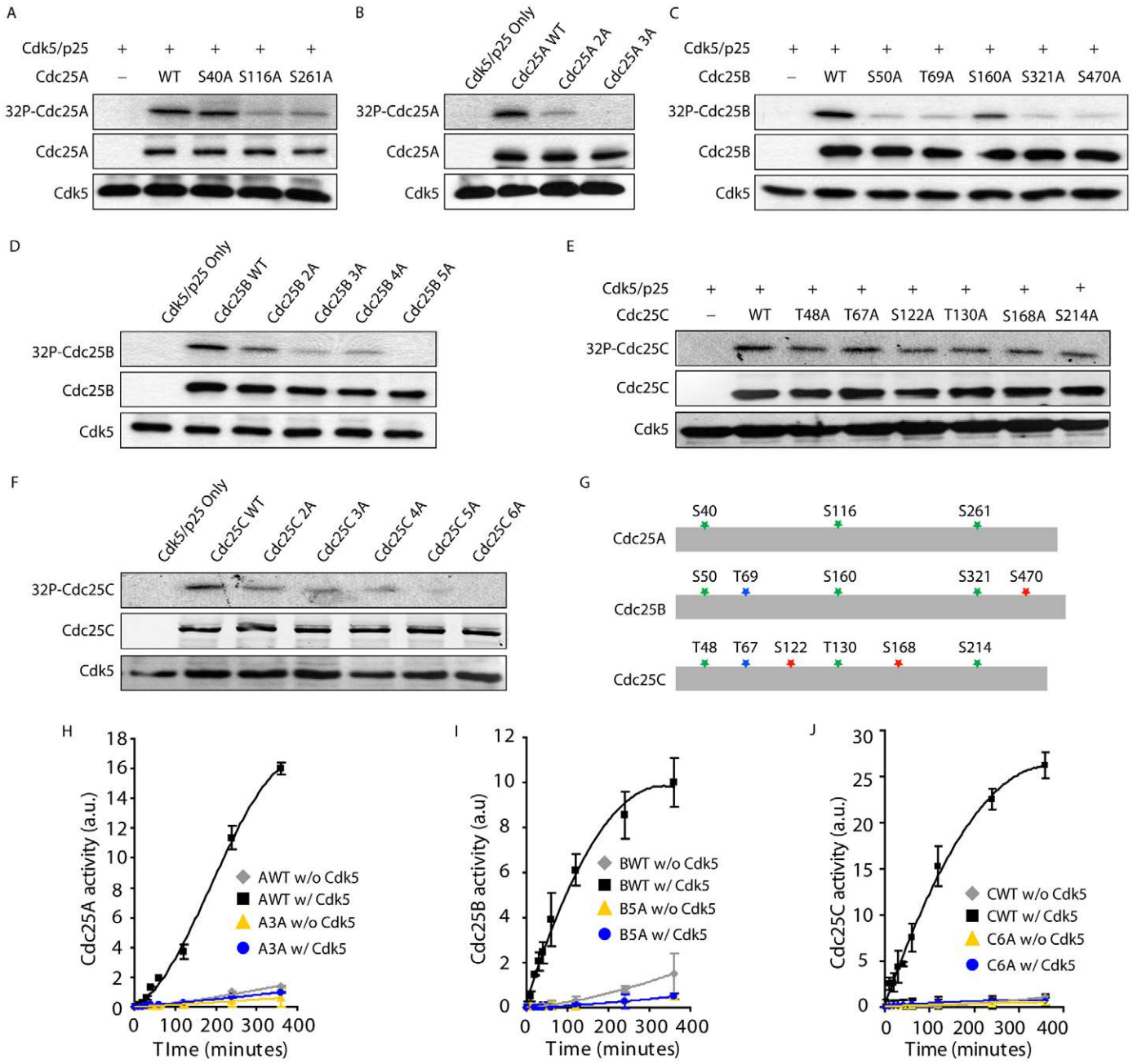

Fig. 5. Identification of phosphorylation sites on Cdc25 phosphatases. (**A**) *In vitro* kinase assay of 6xHis-Cdc25A proteins with different single mutation sites using 6xHis-Cdk5/p25 complex and [γ-^{32}P]ATP. The radioactive intensity of ^{32}P was visualized on X-ray film. (**B**) Kinase assay of 6xHis-Cdc25A proteins with multiple mutation sites using 6xHis-Cdk5/p25 complex and [γ-^{32}P]ATP. Cdc25A2A=Cdc25A (S116, S261A); Cdc25A3A=Cdc25A (S40, S116, S261A). (**C**) Kinase assay of 6xHis-Cdc25B proteins with different single mutation sites using 6xHis-Cdk5/p25 complex and [γ-^{32}P]ATP. (**D**) Kinase assay of 6xHis-Cdc25B proteins with multiple mutation sites using 6xHis-Cdk5/p25 complex and [γ-^{32}P]ATP. Cdc25B2A=Cdc25B (S50, T69A); Cdc25B3A=Cdc25B (S50, T69, S321A); Cdc25B4A=Cdc25B (S50, T69A, S321, S470A); Cdc25B5A=Cdc25B (S50, T69A, S160, S321, S470A). (**E**) Kinase assay of 6xHis-Cdc25C proteins with different single mutation sites using 6xHis-Cdk5/p25 complex and [γ-^{32}P]ATP. (**F**) Kinase assay of 6xHis-Cdc25C proteins with multiple mutation sites using 6xHis-Cdk5/p25 complex and [γ-^{32}P]ATP. Cdc25C2A=Cdc25C (T48, T67A); Cdc25C3A=Cdc25C (T48, T67, S122A); Cdc25C4A=Cdc25C (T48, T67, S122, T130A); Cdc25C5A=Cdc25C (T48, T67, S122, T130, S168A); Cdc25C6A=Cdc25C (T48, T67, S122, T130, S168, S214A). (**G**) Cdk5-mediated phosphorylation sites on Cdc25A, Cdc25B and Cdc25C. Green stars indicate phosphorylation sites are conserved in all three Cdc25 isoforms. Blue stars indicate phosphorylation sites are conserved in Cdc25B and Cdc25C. Red stars indicate unique phosphorylation sites on Cdc25 isoforms. (**H**) Recombinant 6xHis-Cdc25AWT or 6xHis-Cdc25A3A mutant was preincubated with Cdk5/p25 complex bound on beads in the presence of ATP (10 μM) at 30℃ for 20 minutes. The supernatant containing Cdc25A was collected, followed by addition of OMFP (100 μM) at 30℃ for the indicated times. The fluorescence intensity emitted from the product of the hydrolyzed OMFP was measured. (**I**) 6xHis-Cdc25BWT or 6xHis-Cdc25B5A mutant preincubated with Cdk5/p25 complex and ATP was incubated with OMFP for the indicated times and fluorescence intensity measured. (**J**). 6xHis-Cdc25CWT or 6xHis-Cdc25C4A mutant preincubated with Cdk5/p25 complex and ATP was incubated with OMFP for the indicated times and fluorescence intensity measured.

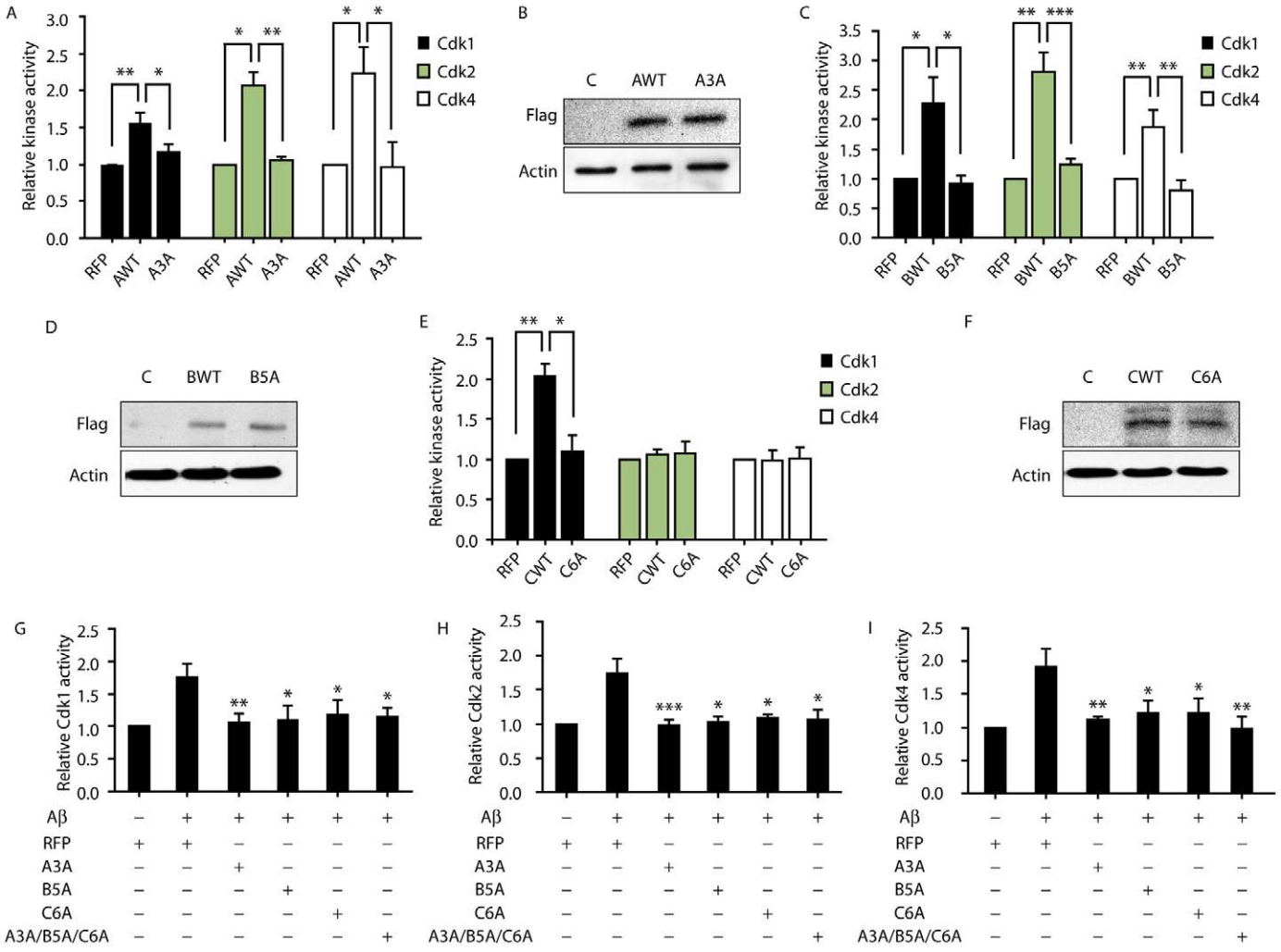

Fig. 6. Cdk5-phosphorylation-resistant mutants of Cdc25 prevents Aβ$^{1-42}$-induced activation of Cdk. (**A**) Rat primary cortical neurons were transduced with TAT–RFP (200 nM), TAT–Cdc25AWT (200 nM) or TAT–Cdc25A3A (200 nM) for 1 h. Total cell lysates were immunoprecipitated with antibodies against Cdk1, Cdk2 and Cdk4. The immunoprecipitates were further subjected to *in vitro* kinase assay. *P<0.05, **P<0.01 was determined for intergroup comparisons. (**B**) Rat primary cortical neurons were transduced with TAT–RFP (200 nM), TAT–Cdc25AWT (200 nM) or TAT–Cdc25A3A (200 nM) for 1 h. Total cell lysates were immunoblotted using FLAG antibody, which shows transduced levels of wt and mutant Cdc25A in neurons. TAT–RFP lacks Flag tag. (**C**) Cells transduced with TAT–RFP, TAT–Cdc25BWT, or TAT–Cdc25B5A were lysed and immunoprecipitated using antibodies against Cdk1, Cdk2 and Cdk4 for *in vitro* kinase assay. *P<0.05, **P<0.01, ***P<0.001 was determined for intergroup comparisons. (**D**) Rat primary cortical neurons were transduced with TAT–RFP (200 nM), TAT–Cdc25BWT (200 nM), or TAT–Cdc25B5A (200 nM) for 1 h. Total cell lysates were immunoblotted using FLAG antibody, which shows transduced levels of wt and mutant Cdc25B in neurons. (**E**) Cells transduced with TAT–RFP, TAT–Cdc25CWT or TAT–Cdc25C6A were lysed and immunoprecipitated using antibodies against Cdk1, Cdk2 and Cdk4 for *in vitro* kinase assay. *P<0.05, **P<0.01 was determined for intergroup comparisons. (**F**) Rat primary cortical neurons were transduced with TAT–RFP (200 nM), TAT–Cdc25CWT (200 nM) or TAT–Cdc25C6A (200 nM) for 1 h. Total cell lysates were immunoblotted using FLAG antibody, which shows transduced levels of wt and mutant Cdc25C in neurons. (**G–I**) Primary cortical neurons were treated with Aβ$^{1-42}$ upon transduction of TAT–RFP, TAT–Cdc25A3A, TAT–Cdc25B5A, TAT–Cdc25C6A for 6 h. Total cell lysates were immunoprecipitated against the indicated antibodies for *in vitro* kinase assay. Cdc25A3A=Cdc25A (S40, S116, S261A); Cdc25B5A=Cdc25B (S50, T69A, S160, S321, S470A); Cdc25C6A=Cdc25C (T48, T67, S122, T130, S168, S214). *P<0.05, **P<0.01, ***P<0.001 when comparing data to the Aβ$^{1-42}$- and TAT–RFP-treated neurons. All experiments were performed at least five independent times. Representative data are shown.

Cdc25C specifically activating Cdk1 (Lyon et al., 2002; Donzelli and Draetta, 2003). These findings also confirm that activation of Cdc25 via phosphorylation is essential for activating Cdks in neurons.

We next investigated whether dominant-negative phosphorylation-resistant Cdc25 mutants could inhibit endogenous Cdk activation in Aβ-treated neurons. The primary cortical neurons were treated with Aβ$^{1-42}$ in the presence of Cdc25-phosphorylation-resistant mutants and Cdk activities analyzed. Aβ$^{1-42}$ induced significant activation of Cdk1, which was largely inhibited by TAT–Cdc25A, TAT–Cdc25B or TAT–Cdc25C phosphorylation-resistant mutants (Fig. 6G). Similarly, complete suppression of Cdk2, and Cdk4 activation by Aβ$^{1-42}$ was observed in cortical neurons by transducing TAT–Cdc25A, TAT–Cdc25B or TAT–Cdc25C phosphorylation-resistant mutants (Fig. 6H,I). Interestingly, transduction of three phosphorylation-resistant mutants together exhibited similar inhibition of Cdks as transduction of

either one of them, indicating that transducing one species of Cdc25 phosphorylation mutants is sufficient to prevent the activation of Cdks induced by $A\beta^{1-42}$ stimulation (Fig. 6G–I). It is likely that the TAT–Cdc25 phosphorylation-resistant mutants can either interact with Cdk5 or Cdks, thereby hindering the access of Cdk5 to endogenous Cdc25 or the access of endogenous Cdc25 to Cdks.

Inhibition of Cdk5 provides the highest neuroprotection against $A\beta^{1-42}$ toxicity

Accumulating evidence suggests that cell cycle reactivation is a critical element in AD pathogenesis, which prompted us to investigate Cdk5′s role in promoting synaptic toxicity and neuronal death. The distribution of PSD95, a postsynaptic protein, was analyzed to examine the integrity of cortical neurons upon cellular treatments. The immunofluorescence studies showed that the cortical neuron immunostained with antibodies against PSD95 (green) displayed the intact neurites, but $A\beta^{1-42}$ treatment caused severe neuritic shrinkage (Fig. 7A). Inhibition of Cdk5 using roscovitine or TAT–CIP significantly prevented the shrinkage of neurites (Fig. 7A,B). In agreement with this, $A\beta^{1-42}$ treatment showed significant decrease in cell viability (40%). Pre-treatment of pan Cdc25 inhibitor NSC95397 in $A\beta^{1-42}$-exposed neurons was considerably neuroprotective, suggesting that the activation of Cdc25 phosphatases is neurotoxic in $A\beta^{1-42}$-induced signaling. More importantly, inhibition of Cdk5 using roscovitine or TAT–CIP treatment conferred higher neuroprotection than NSC95397 (Fig. 7C), revealing an upstream role of deregulated Cdk5 in Cdc25 signaling cascade.

Upregulation of Cdk5, Cdc25A, Cdc25B and Cdc25C in AD tissues

To investigate the clinical significance of our findings, we examined Cdk5 and Cdc25 enzymatic activities in human AD and age-matched control brain tissues. We focused on prefrontal cortex area 9 (Brodmann's area 9), a prefrontal association region located in the superior frontal gyrus of the brain, which is severely affected in AD (Braak and Braak, 1991). These neurons are especially vulnerable in AD and their loss strongly correlates with the severity of the disease, with >90% loss occurring in the end stages of AD (Bussière et al., 2003). Cdk5 activity and Cdc25A, Cdc25B and Cdc25C phosphatase activities were analyzed in tissues obtained from AD patients at moderate stage ($n=2$), severe stage ($n=2$), and end stage ($n=2$) along with age-matched controls ($n=4$). The upregulation of Cdk5, Cdc25A, Cdc25B and Cdc25C activities began at the moderate stage of AD (Fig. 8). The highest activities of Cdk5 and Cdc25C were observed in AD patients at moderate stage, while the activity of Cdc25A peaked at the severe stage. The intensity of Cdc25B activation remained the same at all stages and persisted till the end stage of AD. This suggests that Cdk5 deregulation is one of

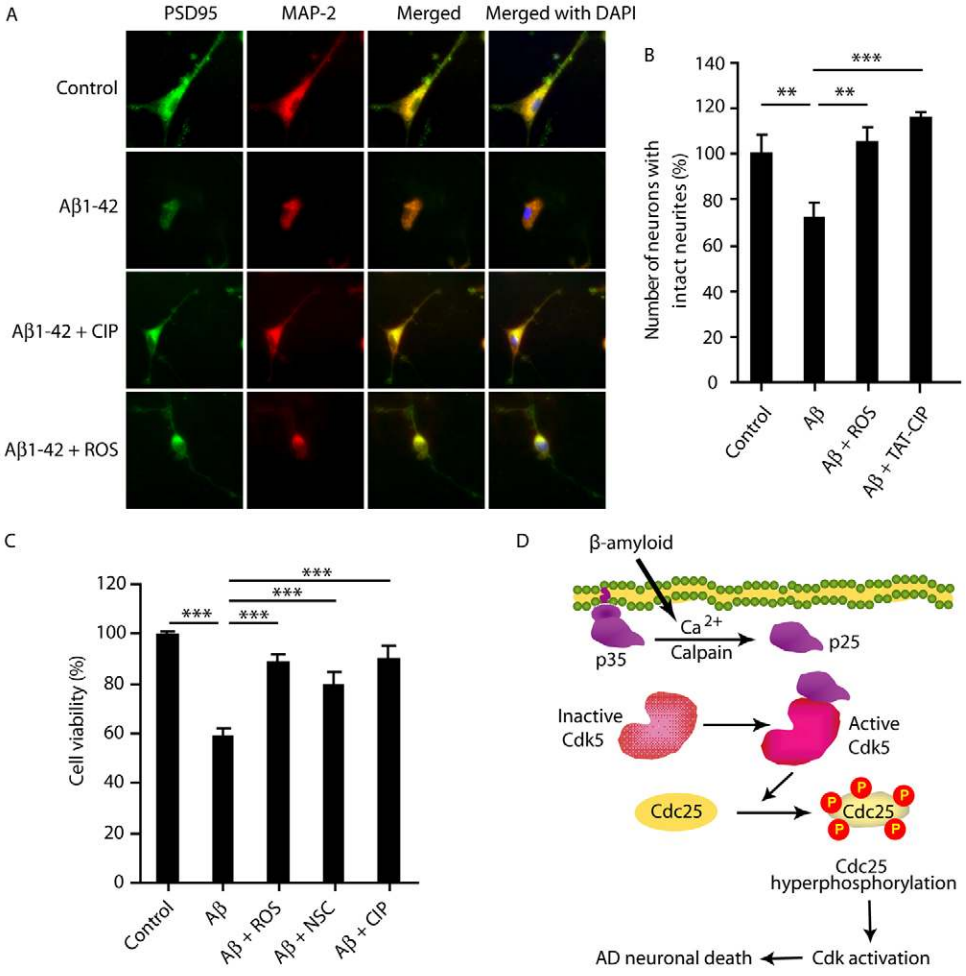

Fig. 7. Cdk5 inhibition effectively suppresses $A\beta^{1-42}$-mediated neuronal death. (**A**) Immunofluorescence studies of PSD95, MAP-2 and DAPI staining in rat primary cortical neurons in response to $A\beta^{1-42}$ toxicity. Cells were treated with roscovitine (ROS) (10 μM) or TAT–CIP (200 nM) 0.5 h prior to $A\beta^{1-42}$ (100 nM). After 24 h, cells were fixed and immunostained with antibodies against PSD95 and MAP-2. (**B**) Quantification of the number of $A\beta^{1-42}$-treated rat primary cortical neurons with intact neurites with or without roscovitine (ROS) or TAT–CIP treatment. $^{**}P<0.01$, $^{***}P<0.001$ for intergroup comparisons. (**C**) Cortical neurons were treated with $A\beta^{1-42}$ in the absence or presence of roscovitine (10 μM), NSC95397 (NSC; 2.5 μM) or TAT–CIP (200 nM). After 72 hours, MTT was added and the absorbance at 590 nm was measured. All experiments were performed at least five times independently. Representative data are shown. $^{***}P<0.001$ for intergroup comparisons. (**D**) Our model showing an upstream role of Cdk5 in activating Cdc25 phosphatases and Cdks in Aβ-mediated neurotoxicity.

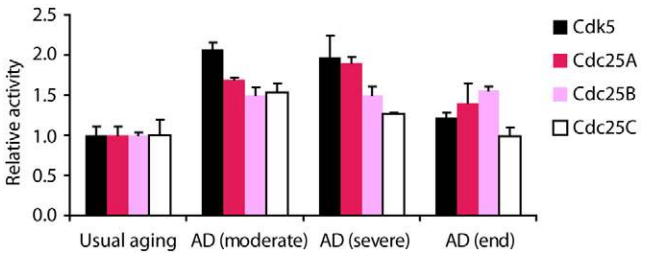

Fig. 8. Upregulation of Cdk5, Cdc25A, Cdc25B and Cdc25C activities in AD patients. Cdk5, Cdc25A, Cdc25B and Cdc25C activities were analyzed in tissues obtained from AD patients at moderate stage ($n=2$), severe stage ($n=2$), and end stage ($n=2$) along with age-matched controls ($n=4$). The immune complexes were subjected to *in vitro* kinase assay and phosphatase assay. All experiments were performed at least five times independently. Representative data are shown.

the earliest pathological events in AD, which triggers the reactivation of Cdc25 proteins upon neurotoxic insults.

Discussion

The re-activation of cell cycle proteins in AD neurons is supported by multiple lines of investigation. In addition to active Cdc25 and Cdks, ectopic upregulation of Cdk1, Cdk4, phospho-pRb, Cyclin B, Cyclin D, proliferating cell nuclear antigen (PCNA), Histone H3 (pSer10) and Plk have been reported in AD neurons (Nagy et al., 1997a; Nagy et al., 1997b; Busser et al., 1998; McShea et al., 1997; Thakur et al., 2008; Ding et al., 2000; Vincent et al., 2001; Ogawa et al., 2003; Harris et al., 2000). Studies from cultured cortical neurons and AD mouse models further demonstrate that Aβ induces the activation or the levels of DNA polymerase-β, Cdk4, pRb, PCNA (Copani et al., 2006; Lopes et al., 2009; Varvel et al., 2008); however, the mechanism remains unclear.

In this study, we unraveled the mechanistic link between Cdk5 deregulation and activation of Cdc25 phosphatases and Cdk kinases in Aβ-induced neurotoxicity. Cdk5 directly phosphorylates Cdc25A, Cdc25B and Cdc25C at multiple serine and threonine sites, which not only stimulate their catalytic activities, but also facilitate their release from inhibitory sequestration of 14-3-3. Interestingly, unlike proliferating cells, where phosphorylation of Cdc25 proteins by Cdks enhances their protein levels, our data revealed that Cdc25 proteins levels are not altered in $A\beta^{1-42}$-treated neurons, suggesting that increase in Cdc25 proteins levels is not the key mechanism by which deregulated Cdk5 initiates the activation of Cdc25 isoforms in Aβ-induced neurotoxicity.

More importantly, Aβ-treated primary cortical neuron model not only captures the deregulation of Cdk5, Cdc25A, Cc25B and Cdc25C observed in human AD brain samples, but also elucidates the molecular mechanism leading to re-activation of Cdks in AD neurons (Lee et al., 1999; McShea et al., 1997; Giovanni et al., 1999). Deregulated Cdk5 triggers the activation of neuronal Cdk1, Cdk2 and Cdk4 in $A\beta^{1-42}$-exposed neurons by activating Cdc25 isoforms. Accordingly, phosphorylation-resistant mutants of Cdc25 proteins at Cdk5 sites are defective in activating Cdks both *in vitro* and in $A\beta^{1-42}$-treated neurons. This finding is in contrast with the mechanisms operative in proliferating cells, where Cdks are known to activate Cdc25 proteins. Thus, despite the presence of active Cdks in AD neurons, our results show that Cdk activation occurs after Cdc25 activation, and inhibition of Cdc25 prevents Cdk activation in $A\beta^{1-42}$-treated cortical neurons. These findings support the notion that Cdk5 is responsible for the deregulation of Cdc25 isoforms and Cdk kinases observed in AD. These results were further confirmed in human AD clinical samples which displayed higher Cdc25A, Cdc25B and Cdc25C activities that coincided with increased Cdk5 activity as compared to age-matched controls.

Importantly, we show that inhibition of Cdc25 activity is neuroprotective in Aβ-induced neurotoxicity, thereby suggesting that cell cycle re-activation is an important pathway causing AD neurodegeneration (Copani et al., 2006; Giovanni et al., 1999). Although previous studies posited that aberrant activations of Cdc2 and Cdc25 is fatal in AD pathogenesis (Shi et al., 1994; Niida et al., 2005; Castedo et al., 2002; Yang et al., 2003; Webber et al., 2005), the mechanism by which they cause AD neuronal death is not fully understood. Active Cdk1 and cyclin B1 are present in AD neurons and colocalize with AD-specific mitotic phospho-epitopes, suggesting that they may contribute to tau pathology (Vincent et al., 1996; Vincent et al., 1997).

In addition, the loss of cell cycle control might be another mechanism underlying cell cycle-driven AD neurodegeneration. Despite the presence of several cell cycle markers in AD patients and mouse models, these neurons do not complete mitosis, as there is no evidence of mitotic spindle formation or chromosome condensation in any study. Ectopic expression of cell cycle proteins can make it through S or G2/M phases, but not any further. Thus, their death could be due to the genetic imbalance caused by aneuploidy (Yang et al., 2001). While one study showed that 4% of hippocampal pyramidal and basal forebrain neurons undergo partial or full DNA replication (Yang et al., 2001); another study found ~20% of vulnerable AD neurons possessing 2n–4n DNA, which were also cyclin B1 positive (Mosch et al., 2007). No such anomalies were discovered in unaffected regions of the AD brain or in non-demented age-matched controls. Active Cdk1 promotes neuronal death in post-mitotic granule neurons by phosphorylating FOXO1 upon depolarization (Yuan et al., 2008); however, whether this pathway exists in β-amyloid-mediated neurotoxicity in AD models remains to be seen. Thus, aberrant activation of cell cycle proteins can trigger neuronal death by multiple mechanisms. Future studies are needed to uncover these pathways.

In conclusion, we identified Cdc25 phosphatases as novel Cdk5 substrates. Cdk5-mediated hyperphosphorylation of Cdc25 isoforms increases their enzymatic activities, and trigger their dissociation from 14-3-3ε inhibitory binding. Active Cdc25 isoforms, in turn activate Cdk1, Cdk2 and Cdk4 leading to neuronal death in response to Ab1-42, underscoring a crucial role of deregulated Cdk5 in AD pathogenesis. Inhibition of Cdk5 is highly neuroprotective, strongly supporting the idea that Cdk5 is an important therapeutic target in AD.

Materials and Methods

Materials

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), NSC95397, 3-O-methylfuorescein phosphate cyclohexylammonium salt (OMFP), and poly-L-lysine were obtained from Sigma (St Louis, MO). $A\beta^{1-42}$ was purchased from AnaSpec (Freemont, CA). Antibodies against actin (C-2), cdc2 p34 (C-19), Cdk2 (H-298), Cdk4 (DCS-35), Cdk5 (C-8), Cdk5 (J-3), Cdc25A (144), Cdc25B (H-85), Cdc25C (C-20), 14-3-3ε (T-16), PSD95 (7E3) and MAP-2 (H-300) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit and Texas-Red-labeled goat anti-mouse antibodies were bought from Invitrogen (Carlsbad, CA).

Expression plasmids and constructs

To generate 6xHis-tagged TAT fusion proteins, Cdc25A (human) in pGEX-4T-1 vector was subcloned to pET-28b-TAT (V2.1) at *Bam*HI and *Eco*RI sites, and Cdc25B (human) and Cdc25C (human) in pGEX-4T-1 vector were subcloned to pET-28b-TAT (V2.1) at *Bam*HI and *Sal*I sites. Serine to alanine substitution mutation on Cdc25 proteins were generated using overlapping PCR.

Isolation of primary cortical neurons

Time pregnant Sprague Dawley rats were purchased from Charles River (Wilmington, MA). Cortices isolated from E17 rat embryos were treated with trypsin (0.2 mg/ml) at 37℃ for 30 minutes and triturated in minimum essential medium (MEM) supplemented with DNase (130 U/ml) and 10% FBS, and passed through the cell strainer (40 μM mesh). After centrifugation at 1000 rpm for 4 minutes, the isolated cortical neurons were further seeded on poly-L-lysine-coated coverslips in a 24-well plate at the density of 5×10^4 cells or in a 6-well plate at the density of 1×10^6 cells in MEM supplemented with 5% FBS, 5% horse serum, 0.5 mM glutamine, 2.6 g/l glucose, 2.2 g/l $NaHCO_3$, and 1 mM pyruvate. The medium was changed after overnight incubation and half amount of medium was replaced every 5 days. All experiments were conducted after 10 DIV (days *in vitro*).

Preparation of soluble $A\beta^{1-42}$ oligomers

Soluble oligomers of $A\beta^{1-42}$ were prepared by modifying the protocol as described previously (Stine et al., 2003). 1 mM of $A\beta^{1-42}$ solution was prepared in hexafluoroisopropanol (HFIP), which was immediately separated into aliquots (10 μl each). HFIP was removed using a Speed Vac for 10 minutes and the formed peptide film was stored in desiccators at −20℃. The peptide film was resuspended in DMSO (DMSO; 2 μl), vortexed for 30 seconds, sonicated for 5 minutes, and centrifuged. 100 μM of $A\beta^{1-42}$ were prepared by addition of medium (98 μl), which was incubated at 37℃, 225 rpm for 24 hours. Upon centrifugation at 13.2 rpm for 10 minutes, the supernatant containing soluble oligomeric $A\beta^{1-42}$ was collected for further cellular experiments.

In vitro phosphorylation of Cdc25 by Cdk5/p25

Purified recombinant 6xHis-Cdc25 (20 μg) was incubated with Cdk5/p25 complex-bound beads (10 μl) in the presence of 10 μCi of [γ-^{32}P]ATP in kinase assay buffer (50 mM Tris pH 8.0, 20 mM $MgCl_2$) at 30℃ for 30 minutes. Proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore). The intensity of radioactivity was visualized following exposure on X-ray film.

In vitro phosphatase assay for Cdc25A, Cdc25B, and Cdc25C

Purified recombinant 6xHis-Cdc25 proteins (20 μg) were incubated with Cdk5/p25 complex-bound beads (10 μl) in the presence of 10 μM ATP in kinase assay buffer (50 mM Tris pH 8.0, 20 mM $MgCl_2$) at 30℃ for 20 minutes. At the end of kinase reaction, the supernatant was collected and incubated with 3-*O*-methylfuorescein phosphate cyclohexylammonium salt (OMFP; 100 μM) in phosphatase assay buffer (50 mM Tris pH 8,0, 50 mM NaCl, 5 mM EDTA, 1 mM DTT, 20% glycerol). The reactions were conducted at 30℃, 225 rpm ranging from 0 to 360 minutes. The fluorescence intensity was measured by excitation at 485 nm and emission at 535 nm using a microplate reader Tecan Spectrafluor Plus (Männedorf, Switzerland).

Co-immunoprecipitation

Primary cortical neurons were treated with $A\beta^{1-42}$ (100 nM) for 0.5, 1, 3 or 6 hours. Cells were lysed in 1% NP40 lysis buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP40, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) and placed on ice for 30 minutes. Upon centrifugation at 10,000 rpm, 4℃ for 20 minutes, the homogenous cell lysates were incubated with protein-A–Sepharose (Sigma) and indicated antibodies at 20 rpm, 4℃ for 6 hours. Proteins were separated by 12% SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membrane, and blocked with 5% non-fat milk in TBST. The membrane was incubated with the indicated antibodies (1:1000 dilution in 2% milk/TBST) at 4℃ for overnight. After washed with TBST, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit (1:5000 dilution in 5% milk/TBST). The X-ray film was exposed using a Pierce ECL western blot substrate kit (Thermo Scientific, Rockford, IL).

In vitro phosphatase assay for Cdc25A, Cdc25B and Cdc25C immunocomplex

Cultured cortical neurons (DIV10–15) were treated with $A\beta^{1-42}$ for 0.5, 1, 2, 4 or 6 hours in the absence or presence of 200 nM TAT–CIP. Cells were lysed in 1% NP40 lysis buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP40, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin), and put on ice for 30 minutes. Upon centrifugation at 10,000 rpm, 4℃ for 20 minutes, the homogenous cell lysates were immunoprecipitated using antibodies against Cdc25A, Cdc25B or Cdc25C at 4℃ for 4 hours. The immunocomplex-bound beads were incubated with 100 μM 3-*O*-methylfluorescein phosphate cyclohexylammonium salt (OMFP) in phosphatase assay buffer. The reaction was conducted at 30℃, 225 rpm for 1 hour. The fluorescence intensity was measured by excitation at 485 nm and emission at 535 nm using a microplate reader Tecan Spectrafluor Plus (Männedorf, Switzerland).

Transduction of TAT-fusing proteins

TAT–Cdc25A, TAT–Cdc25B, TAT–Cdc25C or TAT–CIP (200 nM) proteins were directly added to primary cortical neurons 0.5 hour prior to $A\beta^{1-42}$ treatment. For 6-hour incubation, the half amount of TAT-fusing proteins (100 nM) was added 3 hours later. For more than 24-hour incubation, 100 nM TAT–CIP was added every 6 hours until the end of the $A\beta^{1-42}$ treatment. TAT–GFP or TAT–RFP was used as the control in untreated cells. The proteins concentration was determined by Bradford assay.

In vitro kinase assay for endogenous Cdk1, Cdk2, Cdk4 or Cdk5 activities

After cellular treatments, cells were lysed in 1% NP40 lysis buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP40, 1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml aprotinin). Upon centrifugation at 10,000 rpm, 4℃ for 20 minutes, the homogenous cell lysate was incubated with protein A Sepharose (Sigma) and antibodies against Cdk1, Cdk2, Cdk4, or Cdk5 at 20 rpm, 4℃ for 2 hours. The beads were washed with kinase assay buffer (50 mM Tris pH 8.0, 20 mM $MgCl_2$) three times, and incubated with 5 μg of Cdk substrate peptide (KHHKSPKHR) in kinase assay buffer at 30℃, 225 rpm for 20 minutes. The solution was evenly spotted onto P81 phosphocellulose disc (Whatman, Clifton, NJ), placed in 10% acetic acid at room temperature for 30 minutes, washed with 0.5% phosphoric acid, and rinsed with acetone. Finally, the disc was placed in a scintillation bottle containing complete counting cocktail 'Budget Solve' (Research Products International, Mount prospect, IL). The reading was recorded by Packard Tri-Carb liquid scintillation counter (GMI, Ramsey, MN).

Immunofluorescence studies

Rat primary cortical cells were treated with $A\beta^{1-42}$ (100 nM) for the indicated times in the absence or presence of TAT–CIP (200 nM). At the end of the treatment, the medium was aspirated and the cells were fixed with 4% formaldehyde for 15 minutes, rinsed with phosphate-buffered saline (PBS), and incubated for 20 minutes with 5% BSA and 0.1% Triton X-100. Cells were then immunostained using antibodies against Cdc25A, Cdc25B, Cdc25C, PSD95 or MAP2 at a 1:50 dilution in PBS for 3 hours at room temperature. FITC-labeled goat anti-rabbit and Texas-Red-labeled goat anti-mouse antibodies were used at a 1:250 dilution in PBS, together with DAPI (1 μg/ml). The coverslips were incubated at room temperature for 1 hour in the dark. After three washes with PBS and one wash with water, coverslips were mounted on microscope slides using Mowiol 4-88 reagent (Calbiochem). Images were taken by a Nikon Eclipse E600 microscope (Nikon Instruments, Melville, NY).

MTT assay

Roscovitine (10 μM), NSC95397 (2.5 μM) and TAT–CIP (200 nM) were added to cultured primary cortical neurons in a 96-well plate. After 0.5 hours, $A\beta^{1-42}$ (100 nM) was added and incubated at 37℃ for 72 hours. During the period of treatment, TAT–CIP (100 nM) was added every 6 hours. At the end of treatment, MTT (0.5 mg/ml) was added and incubated for additional 30 minutes. The media were aspirated and the cells on wells were dissolved in DMSO (100 μl). The absorbance value at 590 nm was measured using a microplate reader Tecan Spectrafluor Plus (Männedorf, Switzerland).

Statistical analysis

Bar graphs show the means ± s.e.m. Significance was determined using one-way ANOVA followed by *post hoc* analysis.

Funding

This work was funded by the Indiana Alzheimer Disease Center [grant number(s) IUPUI-4685888-PU to K.S.].

References

Arendt, T. (2003). Synaptic plasticity and cell cycle activation in neurons are alternative effector pathways: the 'Dr. Jekyll and Mr. Hyde concept' of Alzheimer's disease or the yin and yang of neuroplasticity. *Prog. Neurobiol.* **71**, 83-248.

Becker-Hapak, M., McAllister, S. S. and Dowdy, S. F. (2001). TAT-mediated protein transduction into mammalian cells. *Methods* **24**, 247-256.

Braak, H. and Braak, E. (1991). Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathol.* **82**, 239-259.

Busser, J., Geldmacher, D. S. and Herrup, K. (1998). Ectopic cell cycle proteins predict the sites of neuronal cell death in Alzheimer's disease brain. *J. Neurosci.* **18**, 2801-2807.

Bussière, T., Giannakopoulos, P., Bouras, C., Perl, D. P., Morrison, J. H. and Hof, P. R. (2003). Progressive degeneration of nonphosphorylated neurofilament protein-

enriched pyramidal neurons predicts cognitive impairment in Alzheimer's disease: stereologic analysis of prefrontal cortex area 9. *J. Comp. Neurol.* **463**, 281-302.

Castedo, M., Perfettini, J. L., Roumier, T. and Kroemer, G. (2002). Cyclin-dependent kinase-1: linking apoptosis to cell cycle and mitotic catastrophe. *Cell Death Differ.* **9**, 1287-1293.

Chang, K. H., de Pablo, Y., Lee, H. P., Lee, H. G., Smith, M. A. and Shah, K. (2010). Cdk5 is a major regulator of p38 cascade: relevance to neurotoxicity in Alzheimer's disease. *J. Neurochem.* **113**, 1221-1229.

Chang, K. H., Multani, P. S., Sun, K. H., Vincent, F., de Pablo, Y., Ghosh, S., Gupta, R., Lee, H. P., Lee, H. G., Smith, M. A. et al. (2011). Nuclear envelope dispersion triggered by deregulated Cdk5 precedes neuronal death. *Mol. Biol. Cell* **22**, 1452-1462.

Copani, A., Hoozemans, J. J., Caraci, F., Calafiore, M., Van Haastert, E. S., Veerhuis, R., Rozemuller, A. J., Aronica, E., Sortino, M. A. and Nicoletti, F. (2006). DNA polymerase-beta is expressed early in neurons of Alzheimer's disease brain and is loaded into DNA replication forks in neurons challenged with beta-amyloid. *J. Neurosci.* **26**, 10949-10957.

Cruz, J. C. and Tsai, L. H. (2004). Cdk5 deregulation in the pathogenesis of Alzheimer's disease. *Trends Mol. Med.* **10**, 452-458.

Cruz, J. C., Kim, D., Moy, L. Y., Dobbin, M. M., Sun, X., Bronson, R. T. and Tsai, L. H. (2006). p25/cyclin-dependent kinase 5 induces production and intraneuronal accumulation of amyloid beta in vivo. *J. Neurosci.* **26**, 10536-10541.

Ding, X. L., Husseman, J., Tomashevski, A., Nochlin, D., Jin, L. W. and Vincent, I. (2000). The cell cycle Cdc25A tyrosine phosphatase is activated in degenerating postmitotic neurons in Alzheimer's disease. *Am. J. Pathol.* **157**, 1983-1990.

Donzelli, M. and Draetta, G. F. (2003). Regulating mammalian checkpoints through Cdc25 inactivation. *EMBO Rep.* **4**, 671-677.

Giovanni, A., Wirtz-Brugger, F., Keramaris, E., Slack, R. and Park, D. S. (1999). Involvement of cell cycle elements, cyclin-dependent kinases, pRb, and E2F x DP, in B-amyloid-induced neuronal death. *J. Biol. Chem.* **274**, 19011-19016.

Grynspan, F., Griffin, W. R., Cataldo, A., Katayama, S. and Nixon, R. A. (1997). Active site-directed antibodies identify calpain II as an early-appearing and pervasive component of neurofibrillary pathology in Alzheimer's disease. *Brain Res.* **763**, 145-158.

Harris, P. L., Zhu, X., Pamies, C., Rottkamp, C. A., Ghanbari, H. A., McShea, A., Feng, Y., Ferris, D. K. and Smith, M. A. (2000). Neuronal polo-like kinase in Alzheimer disease indicates cell cycle changes. *Neurobiol. Aging* **21**, 837-841.

Hashiguchi, M., Saito, T., Hisanaga, S. and Hashiguchi, T. (2002). Truncation of CDK5 activator p35 induces intensive phosphorylation of Ser202/Thr205 of human tau. *J. Biol. Chem.* **277**, 44525-44530.

Hermeking, H. and Benzinger, A. (2006). 14-3-3 proteins in cell cycle regulation. *Semin. Cancer Biol.* **16**, 183-192.

Hisanaga, S. and Endo, R. (2010). Regulation and role of cyclin-dependent kinase activity in neuronal survival and death. *J. Neurochem.* **115**, 1309-1321.

Huang, E., Qu, D., Zhang, Y., Venderova, K., Haque, M. E., Rousseaux, M. W., Slack, R. S., Woulfe, J. M. and Park, D. S. (2010). The role of Cdk5-mediated apurinic/apyrimidinic endonuclease 1 phosphorylation in neuronal death. *Nat. Cell Biol.* **12**, 563-571.

Johnson, E. O., Chang, K. H., de Pablo, Y., Ghosh, S., Mehta, R., Badve, S. and Shah, K. (2011). PHLDA1 is a crucial negative regulator and effector of Aurora A kinase in breast cancer. *J. Cell Sci.* **124**, 2711-2722.

Johnson, E. O., Chang, K. H., Ghosh, S., Venkatesh, C., Giger, K., Low, P. S. and Shah, K. (2012). LIMK2 is a crucial regulator and effector of Aurora-A-kinase-mediated malignancy. *J. Cell Sci.* **125**, 1204-1216.

Kanungo, J., Zheng, Y. L., Amin, N. D. and Pant, H. C. (2009). Targeting Cdk5 activity in neuronal degeneration and regeneration. *Cell. Mol. Neurobiol.* **29**, 1073-1080.

Kesavapany, S., Zheng, Y. L., Amin, N. and Pant, H. C. (2007). Peptides derived from Cdk5 activator p35, specifically inhibit deregulated activity of Cdk5. *Biotechnol. J.* **2**, 978-987.

Kim, S. and Shah, K. (2007). Dissecting yeast Hog1 MAP kinase pathway using a chemical genetic approach. *FEBS Lett.* **581**, 1209-1216.

Kim, D., Frank, C. L., Dobbin, M. M., Tsunemoto, R. K., Tu, W., Peng, P. L., Guan, J. S., Lee, B. H., Moy, L. Y., Giusti, P. et al. (2008). Deregulation of HDAC1 by p25/Cdk5 in neurotoxicity. *Neuron* **60**, 803-817.

Lee, J. H. and Kim, K. T. (2007). Regulation of cyclin-dependent kinase 5 and p53 by ERK1/2 pathway in the DNA damage-induced neuronal death. *J. Cell. Physiol.* **210**, 784-797.

Lee, K. Y., Clark, A. W., Rosales, J. L., Chapman, K., Fung, T. and Johnston, R. N. (1999). Elevated neuronal Cdc2-like kinase activity in the Alzheimer disease brain. *Neurosci. Res.* **34**, 21-29.

Li, B. S., Ma, W., Jaffe, H., Zheng, Y., Takahashi, S., Zhang, L., Kulkarni, A. B. and Pant, H. C. (2003). Cyclin-dependent kinase-5 is involved in neuregulin-dependent activation of phosphatidylinositol 3-kinase and Akt activity mediating neuronal survival. *J. Biol. Chem.* **278**, 35702-35709.

Lopes, J. P., Oliveira, C. R. and Agostinho, P. (2009). Cdk5 acts as a mediator of neuronal cell cycle re-entry triggered by amyloid-beta and prion peptides. *Cell Cycle* **8**, 97-104.

Lyon, M. A., Ducruet, A. P., Wipf, P. and Lazo, J. S. (2002). Dual-specificity phosphatases as targets for antineoplastic agents. *Nat. Rev. Drug Discov.* **1**, 961-976.

McShea, A., Harris, P. L., Webster, K. R., Wahl, A. F. and Smith, M. A. (1997). Abnormal expression of the cell cycle regulators P16 and CDK4 in Alzheimer's disease. *Am. J. Pathol.* **150**, 1933-1939.

Monaco, E. A., 3rd and Vallano, M. L. (2003). Cyclin-dependent kinase inhibitors: cancer killers to neuronal guardians. *Curr. Med. Chem.* **10**, 367-379.

Mosch, B., Morawski, M., Mittag, A., Lenz, D., Tarnok, A. and Arendt, T. (2007). Aneuploidy and DNA replication in the normal human brain and Alzheimer's disease. *J. Neurosci.* **27**(26), 6859-6867.

Nagy, Z., Esiri, M. M., Cato, A. M. and Smith, A. D. (1997a). Cell cycle markers in the hippocampus in Alzheimer's disease. *Acta Neuropathol.* **94**, 6-15.

Nagy, Z., Esiri, M. M. and Smith, A. D. (1997b). Expression of cell division markers in the hippocampus in Alzheimer's disease and other neurodegenerative conditions. *Acta Neuropathol.* **93**, 294-300.

Niida, H., Tsuge, S., Katsuno, Y., Konishi, A., Takeda, N. and Nakanishi, M. (2005). Depletion of Chk1 leads to premature activation of Cdc2-cyclin B and mitotic catastrophe. *J. Biol. Chem.* **280**, 39246-39252.

Ogawa, O., Zhu, X., Lee, H. G., Raina, A., Obrenovich, M. E., Bowser, R., Ghanbari, H. A., Castellani, R. J., Perry, G. and Smith, M. A. (2003). Ectopic localization of phosphorylated histone H3 in Alzheimer's disease: a mitotic catastrophe? *Acta Neuropathol.* **105**, 524-528.

Ohshima, T., Ward, J. M., Huh, C. G., Longenecker, G., Veeranna, Pant, H. C., Bran, R. O., Martin, L. J. and Kulkarni, A. B. (1996). Targeted disruption of the cyclin-dependent kinase 5 gene results in abnormal corticogenesis, neuronal pathology and perinatal death. *Proc. Natl. Acad. Sci. USA* **93**, 11173-11178.

Patrick, G. N., Zukerberg, L., Nikolic, M., de la Monte, S., Dikkes, P. and Tsai, L. H. (1999). Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. *Nature* **402**, 615-622.

Rudrabhatla, P., Jaffe, H. and Pant, H. C. (2011). Direct evidence of phosphorylated neuronal intermediate filament proteins in neurofibrillary tangles (NFTs): phosphoproteomics of Alzheimer's NFTs. *FASEB J.* **25**, 3896-3905.

Saito, T., Konno, T., Hosokawa, T., Asada, A., Ishiguro, K. and Hisanaga, S. (2007). p25/cyclin-dependent kinase 5 promotes the progression of cell death in nucleus of endoplasmic reticulum-stressed neurons. *J. Neurochem.* **102**, 133-140.

Shah, K. and Shokat, K. M. (2002). A chemical genetic screen for direct v-Src substrates reveals ordered assembly of a retrograde signaling pathway. *Chem. Biol.* **9**, 35-47.

Shah, K. and Shokat, K. M. (2003). A chemical genetic approach for the identification of direct substrates of protein kinases. *Methods Mol. Biol.* **233**, 253-271.

Shah, K. and Vincent, F. (2005). Divergent roles of c-Src in controlling platelet-derived growth factor-dependent signaling in fibroblasts. *Mol. Biol. Cell* **16**, 5418-5432.

Shah, K., Liu, Y., Deirmengian, C. and Shokat, K. M. (1997). Engineering unnatural nucleotide specificity for Rous sarcoma virus tyrosine kinase to uniquely label its direct substrates. *Proc. Natl. Acad. Sci. USA* **94**, 3565-3570.

Shea, T. B., Jung, C. and Pant, H. C. (2003). Does neurofilament phosphorylation regulate axonal transport? *Trends Neurosci.* **26**, 397-400.

Shea, T. B., Yabe, J. T., Ortiz, D., Pimenta, A., Loomis, P., Goldman, R. D., Amin, N. and Pant, H. C. (2004). Cdk5 regulates axonal transport and phosphorylation of neurofilaments in cultured neurons. *J. Cell Sci.* **117**, 933-941.

Shi, L., Nishioka, W. K., Th'ng, J., Bradbury, E. M., Litchfield, D. W. and Greenberg, A. H. (1994). Premature p34cdc2 activation required for apoptosis. *Science* **263**, 1143-1145.

Stine, W. B., Jr, Dahlgren, K. N., Krafft, G. A. and LaDu, M. J. (2003). In vitro characterization of conditions for amyloid-beta peptide oligomerization and fibrillogenesis. *J. Biol. Chem.* **278**, 11612-11622.

Sun, K. H., de Pablo, Y., Vincent, F., Johnson, E. O., Chavers, A. K. and Shah, K. (2008a). Novel genetic tools reveal Cdk5's major role in Golgi fragmentation in Alzheimer's disease. *Mol. Biol. Cell* **19**, 3052-3069.

Sun, K. H., de Pablo, Y., Vincent, F. and Shah, K. (2008b). Deregulated Cdk5 promotes oxidative stress and mitochondrial dysfunction. *J. Neurochem.* **107**, 265-278.

Sun, K. H., Lee, H. G., Smith, M. A. and Shah, K. (2009). Direct and indirect roles of cyclin-dependent kinase 5 as an upstream regulator in the c-Jun NH2-terminal kinase cascade: relevance to neurotoxic insults in Alzheimer's disease. *Mol. Biol. Cell* **20**, 4611-4619.

Sun, K. H., Chang, K. H., Clawson, S., Ghosh, S., Mirzaei, H., Regnier, F. and Shah, K. (2011). Glutathione-S-transferase P1 is a critical regulator of Cdk5 kinase activity. *J. Neurochem.* **118**, 902-914.

Tandon, A., Yu, H., Wang, L., Rogaeva, E., Sato, C., Chishti, M. A., Kawarai, T., Hasegawa, H., Chen, F., Davies, P. et al. (2003). Brain levels of CDK5 activator p25 are not increased in Alzheimer's or other neurodegenerative diseases with neurofibrillary tangles. *J. Neurochem.* **86**, 572-581.

Tang, X., Wang, X., Gong, X., Tong, M., Park, D., Xia, Z. and Mao, Z. (2005). Cyclin-dependent kinase 5 mediates neurotoxin-induced degradation of the transcription factor myocyte enhancer factor 2. *J. Neurosci.* **25**, 4823-4834.

Taniguchi, S., Fujita, Y., Hayashi, S., Kakita, A., Takahashi, H., Murayama, S., Saido, T. C., Hisanaga, S., Iwatsubo, T. and Hasegawa, M. (2001). Calpain-mediated degradation of p35 to p25 in postmortem human and rat brains. *FEBS Lett.* **489**, 46-50.

Thakur, A., Siedlak, S. L., James, S. L., Bonda, D. J., Rao, A., Webber, K. M., Camins, A., Pallàs, M., Casadesus, G., Lee, H. G. et al. (2008). Retinoblastoma protein phosphorylation at multiple sites is associated with neurofibrillary pathology in Alzheimer disease. *Int. J. Clin. Exp. Pathol* **1**, 134-146.

Tian, B., Yang, Q. and Mao, Z. (2009). Phosphorylation of ATM by Cdk5 mediates DNA damage signalling and regulates neuronal death. *Nat. Cell Biol.* **11**, 211-218.

Tseng, H. C., Zhou, Y., Shen, Y. and Tsai, L. H. (2002). A survey of Cdk5 activator p35 and p25 levels in Alzheimer s disease brains. *FEBS Lett.* **523**, 58-62.

Varvel, N. H., Bhaskar, K., Patil, A. R., Pimplikar, S. W., Herrup, K. and Lamb, B. T. (2008). Abeta oligomers induce neuronal cell cycle events in Alzheimer's disease. *J. Neurosci.* **28**, 10786-10793.

Vincent, I., Rosado, M. and Davies, P. (1996). Mitotic mechanisms in Alzheimer's disease? *J. Cell Biol.* **132**, 413-425.

Vincent, I., Jicha, G., Rosado, M. and Dickson, D. W. (1997). Aberrant expression of mitotic cdc2/cyclin B1 kinase in degenerating neurons of Alzheimer's disease brain. *J. Neurosci.* **17**, 3588-3598.

Vincent, I., Bu, B., Hudson, K., Husseman, J., Nochlin, D. and Jin, L. (2001). Constitutive Cdc25B tyrosine phosphatase activity in adult brain neurons with M phase-type alterations in Alzheimer's disease. *Neuroscience* **105**, 639-650.

Webber, K. M., Raina, A. K., Marlatt, M. W., Zhu, X., Prat, M. I., Morelli, L., Casadesus, G., Perry, G. and Smith, M. A. (2005). The cell cycle in Alzheimer disease: a unique target for neuropharmacology. *Mech. Ageing Dev.* **126**, 1019-1025.

Wen, Y., Yu, W. H., Maloney, B., Bailey, J., Ma, J., Marié, I., Maurin, T., Wang, L., Figueroa, H., Herman, M. et al. (2008). Transcriptional regulation of beta-secretase by p25/cdk5 leads to enhanced amyloidogenic processing. *Neuron* **57**, 680-690.

Yang, Y., Geldmacher, D. S. and Herrup, K. (2001). DNA replication precedes neuronal cell death in Alzheimer's disease. *J. Neurosci.* **21**, 2661-2668.

Yang, Y., Mufson, E. J. and Herrup, K. (2003). Neuronal cell death is preceded by cell cycle events at all stages of Alzheimer's disease. *J. Neurosci.* **23**, 2557-2563.

Yoo, B. C. and Lubec, G. (2001). p25 protein in neurodegeneration. *Nature* **411**, 763-764.

Yuan, Z., Becker, E. B., Merlo, P., Yamada, T., DiBacco, S., Konishi, Y., Schaefer, E. M. and Bonni, A. (2008). Activation of FOXO1 by Cdk1 in cycling cells and postmitotic neurons. *Science* **319**, 1665-1668.

Zhang, Y., McLaughlin, R., Goodyer, C. and LeBlanc, A. (2002). Selective cytotoxicity of intracellular amyloid beta peptide1-42 through p53 and Bax in cultured primary human neurons. *J. Cell Biol.* **156**, 519-529.

Zheng, Y. L., Li, B. S., Rudrabhatla, P., Shukla, V., Amin, N. D., Maric, D., Kesavapany, S., Kanungo, J., Pareek, T. K., Takahashi, S. et al. (2010). Phosphorylation of p27Kip1 at Thr187 by cyclin-dependent kinase 5 modulates neural stem cell differentiation. *Mol. Biol. Cell* **21**, 3601-3614.