Stabilization and activation of p53 induced by Cdk5 contributes to neuronal cell death

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Summary

The p53 tumor suppressor protein is a key regulator of cellular functions including responses to numerous stress signals, and triggers apoptosis in many cell types, including neurons. The major mechanisms known to regulate p53 stabilization and activation include phosphorylation and ubiquitin ligase-mediated proteasomal degradation. Cyclin-dependent kinase 5 (Cdk5), a proline-directed serine/threonine kinase, is most active in the central nervous system and plays a variety of roles in neuronal degeneration. Here, we demonstrate for the first time that Cdk5 interacts with p53 and increases its stability through posttranslational regulation, leading to accumulation of p53, particularly in the nucleus. We show that Cdk5 phosphorylates p53 on Ser15, Ser33 and Ser46 in vitro, and that increased Cdk5 activity in the nucleus mediates these phosphorylation events in response to genotoxic and oxidative stresses. Cdk5 mediates disruption of the interaction between p53 and Hdm2 (also known as Mdm2), and prevents Hdm2-induced p53 ubiquitylation and downregulation. Cdk5 additionally enhances phosphorylation-dependent binding of the p300 coactivator, inducing acetylation of p53. Cdk5-stabilized p53 protein is transcriptionally active, resulting in the induction of pro-apoptotic genes and subsequent mitochondria-mediated apoptosis in response to genotoxic or oxidative stress. Collectively, these novel findings help define the mechanisms underlying neuronal apoptosis occurring as a result of Cdk5-mediated p53 stabilization and transcriptional activation.

Supplementary material available online at
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Key words: p53, Cdk5, Phosphorylation, Acetylation, Ubiquitylation, Neuronal apoptosis

Introduction

Cyclin-dependent kinase 5 (Cdk5), a member of the cyclin-dependent kinase family, is primarily active in the central nervous system (CNS) because of the selective neuronal localization of its activator proteins, p35 and p39, or their truncated forms, p25 and p29 (Dhavan and Tsai, 2001). Cdk5 is a proline-directed serine/threonine (Ser/Thr) kinase that phosphorylates a diverse range of substrates, and plays crucial roles in neuronal differentiation, migration, axon outgrowth and synaptogenesis (Lee and Kim, 2004; Smith et al., 2001). However, increasing evidence has shown that abnormal activation of Cdk5 is toxic to neurons, leading to apoptosis under both physiological and pathological conditions (Shelton and Johnson, 2004). Deregulated Cdk5 activity has been reported in neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis, suggesting that this protein may play a pivotal role during pathological progression (Lau et al., 2002; Nguyen et al., 2001). Abnormal Cdk5 activity hyperphosphorylates tau and neurofilaments, leading to microtubule network destabilization, neuronal retraction and apoptosis (Bu et al., 2002). In addition, Cdk5 mediates neurotoxic effects through phosphorylation and inhibition of the transcription factor myocyte enhancer factor 2 (MEF2), a key regulator in neuronal survival (Tang et al., 2005). However, although these lines of evidence collectively suggest that Cdk5 plays an essential role in the regulation of neuronal apoptosis, the mechanisms by which active Cdk5 facilitates apoptosis remain unknown.

A previous report showed that Cdk5 activity is associated with increased expression and activation of the tumor suppressor protein p53 (Zhang et al., 2002). In addition, we recently showed that the extracellular-signal-related kinase (ERK) pathway induced neuronal death through regulation of Cdk5 and p53 in response to DNA damage (Lee and Kim, 2007). However, these findings do not fully describe the regulatory mechanisms of p53 and its physiological roles. In this study, we investigated the link between Cdk5 activity and p53 stabilization and activation. The p53 protein is situated at a crossroad of the pathways implicated in the regulation of cell survival and apoptotic death in response to genotoxic damage and other types of cellular stress (Culmsee and Mattson, 2005; Tan et al., 2005). Because of the short half-life of the p53 protein, p53 activity is maintained at low levels in the absence of stress. Upon exposure to a variety of stress conditions, the stability of p53 increases and the protein accumulates in the nucleus, where it is activated as a transcription factor (Brooks and Gu, 2003; Lee et al., 2006). The stabilization and activation of p53 is largely mediated through posttranslational modifications, such as phosphorylation and acetylation, as well as by protein-protein interactions (Appella and Anderson,

2001). In this regard, the Hdm2 (also known as Mdm2) oncoprotein, an E3 ubiquitin ligase of p53, is a major negative regulator of p53 function. Hdm2 inhibits p53 function by blocking its N-terminal transcriptional activation domain and facilitating its nuclear export, ubiquitylation and proteasome-mediated degradation through E3 ubiquitin ligase activity (O'Brate and Giannakakou, 2003; Wsierska-Gadek and Horky, 2003; Yang et al., 2004). Phosphorylation is one of the mechanisms regulating p53 interactions with Hdm2 and transcriptional activity. The p53 protein is a substrate for diverse kinases, and may be phosphorylated at a minimum of 20 Ser/Thr residues within its N- and C-terminal regions (Culmsee and Mattson, 2005; Lee et al., 2006; Vega et al., 2004). Accumulating evidence suggests that the posttranslational modification of p53 at various sites is crucial for the regulation of cell death in response to different types of stress stimuli. For example, ATM (ataxia telangiectasia-mutated), ATR (ATM and Rad3-related) and DNA-PK (DNA-dependent protein kinase) induce p53 phosphorylation in response to DNA damage, resulting in impaired binding with Hdm2, and subsequent accumulation and functional activation of p53 (Shieh et al., 1997; Tibbetts et al., 1999). Checkpoint kinase 1 (Chk1), Chk2 and Jun amino-terminal kinase (JNK) additionally stabilize p53 through phosphorylation (Buschmann et al., 2001; Shieh et al., 2000). However the mechanisms responsible for regulating p53 activity in response to stress stimuli are yet to be clarified.

Here, we provide conclusive molecular evidence that Cdk5 associates with p53 and efficiently stabilizes it through phosphorylation at the Ser15, Ser33 and Ser46 residues. Furthermore, Cdk5 mediates nuclear accumulation of p53, affects the p53-Hdm2 interaction and decreases Hdm2-mediated ubiquitylation of p53. Our data additionally suggest that Cdk5 stimulates phosphorylation-driven p300 binding and acetylation at the C-terminal site of p53, leading to increases in transcriptional activity and subsequent mitochondria-mediated apoptosis. Thus, the present findings define an important molecular mechanism underlying Cdk5-mediated p53 stabilization and activation, which may form the basis for stress-induced neuronal apoptosis in the CNS.

Results

Cdk5 mediates regulation of p53 in response to mitomycin C-induced DNA damage and SNP-induced oxidative stress

Given the well-known function of p53 in regulating apoptosis (Culmsee and Mattson, 2005), we examined the contribution of p53 to genotoxic or cytotoxic stress-induced neuronal death by small interfering RNA (siRNA) knockdown of p53 in SH-SY5Y cells. Following mitomycin C-induced DNA damage or oxidative stress triggered by sodium nitroprusside (SNP), a nitric oxide donor, cell viability was found to be decreased in cells transfected with control scrambled siRNA, whereas cells transfected with siRNA against p53 were significantly protected against neuronal death (supplementary material Fig. S1A). Similar results were obtained in western blotting analysis, which showed that suppression of p53 expression abrogated mitomycin C- or SNP-induced caspase-3 cleavage (supplementary material Fig. S1B). In addition, suppression of endogenous p53 activity with the dominant-negative p53(R175H) markedly decreased TUNEL staining: from approximately 54% to 5% and from approximately 58% to 6% in mitomycin C- and SNP-treated SH-SY5Y cells, respectively (supplementary material Fig. S1C). Finally, western blotting showed that p53 levels were dramatically increased in SH-SY5Y cells following mitomycin C or SNP treatment (Fig. 1A). These findings collectively suggest that induction of p53 plays a crucial role in neuronal death induced by DNA damage and oxidative stress. To determine whether Cdk5 was responsible for the induction of p53 under these conditions, the effect of Cdk5 knockdown was examined. Blockage of Cdk5 expression markedly inhibited mitomycin C- and SNP-induced enhancement of p53 levels, compared with transfection of scrambled control siRNA (Fig. 1B). Consistent with this finding, inhibition of Cdk5 activity with the Cdk2/5 inhibitor N^4-(6-aminopyrimidin-4-yl)-sulfanilamide or roscovitine resulted in similar inhibitory effects on p53 expression (Fig. 1C). To examine whether Cdk5-mediated p53 regulation occurs posttranslationally, we determined p53 degradation rates in the presence and absence of a Cdk5 inhibitor when de novo protein synthesis was blocked with cycloheximide. Plotted p53 levels showed that rate of degradation increased in the presence of the Cdk5 inhibitor compared with untreated controls (Fig. 1D). Knockdown of Cdk5 expression with siRNA also markedly decreased the p53 degradation rate (supplementary material Fig. S2A), strongly suggesting that Cdk5 increases the stability of the p53 protein. To exclude any transcriptional effect of Cdk5 on the regulation of p53, the mRNA transcript levels of p53 were determined by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis. In contrast to the changes observed in protein expression, increased p53 transcript levels during DNA damage were not affected as a result of reduced Cdk5 activity. This result suggests that modulation of p53 expression by Cdk5 is likely to occur posttranslationally (Fig. 1E). Similarly, under conditions of SNP-induced oxidative stress, p53 mRNA levels remained unchanged regardless of Cdk5 activity. However, no increase in p53 mRNA levels was detected during SNP-induced oxidative stress, signifying that SNP-evoked p53 induction is because of a posttranslational event of p53 (supplementary material Fig. S2B).

Cdk5 is activated in the nucleus and associates with p53

We recently reported that DNA damage increased Cdk5 activity through ERK-mediated p35 expression (Lee and Kim, 2007). In view of the finding that p53 is mainly localized and activated in the nucleus, we investigated whether Cdk5 is also activated in the nucleus in response to neurotoxic stress. Nuclear preparations from mitomycin C-treated SH-SY5Y cells showed increased expression of Cdk5 and its activator p35, as well as enhanced Cdk5 activity (Fig. 2A). Similarly, under oxidative stress we observed increased Cdk5 expression and elevated Cdk5 kinase activity (supplementary material Fig. S2C). It is interesting to note that oxidative stress increased proteolytic cleavage of p35 to p25, whereas mitomycin C treatment did not, suggesting that Cdk5 activity is differentially regulated in response to DNA damage and oxidative stress. We then examined whether Cdk5 interacted with p53. Co-immunoprecipitation analysis showed that Cdk5 was present in immunoprecipitates prepared with antibodies against p53, but not in those prepared with the control IgG antibody (Fig. 2B). To further verify that p53 binds to Cdk5, an in vitro glutathione

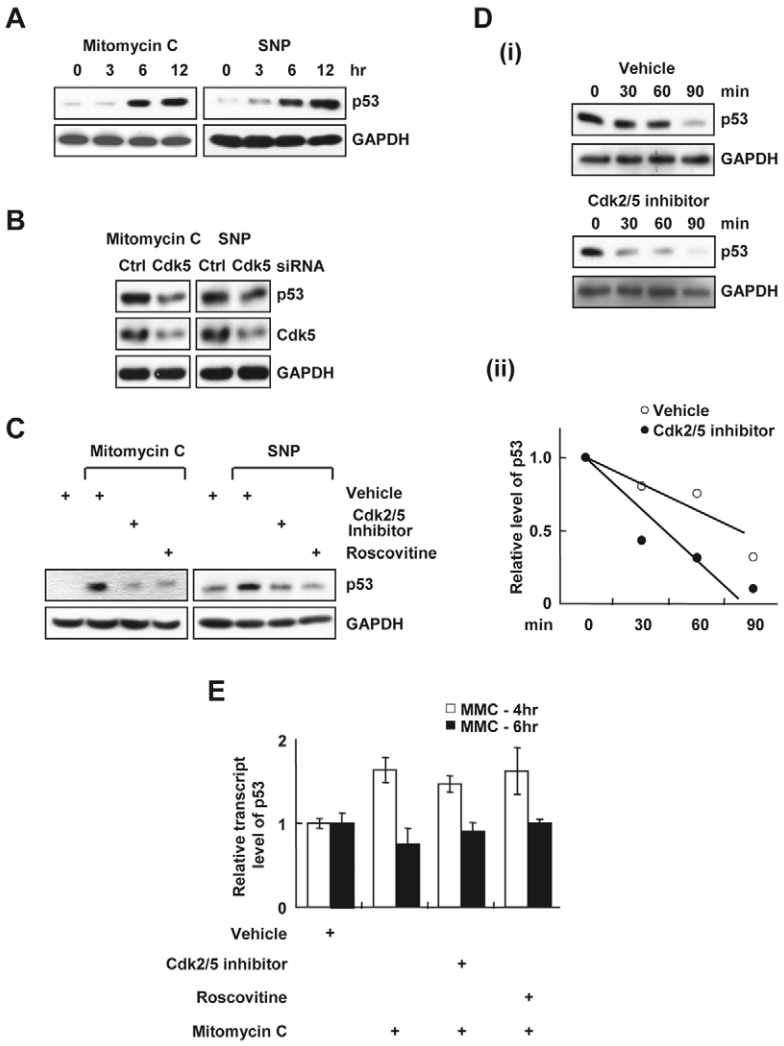

Fig. 1. Cdk5 regulates expression of the p53 tumor suppressor protein in response to genotoxic and oxidative stress. (A) SH-SY5Y cells were treated with 5 μM mitomycin C or 2 mM SNP for the indicated times, and cell lysates were subjected to immunoblotting with the indicated antibodies. (B) SH-SY5Y cells were transfected with Cdk5 targeting or scrambled control siRNA, treated with 5 μM mitomycin C or 2 mM SNP for 9 hours, and then subjected to immunoblotting with the indicated antibodies. (C) SH-SY5Y cells were pretreated with 10 μM roscovitine or Cdk2/5 inhibitor for 30 minutes and then incubated with 5 μM mitomycin C or 2 mM SNP for 9 hours. Cell lysates were subjected to immunoblot analysis with the indicated antibodies. (D) SH-SY5Y cells were treated with 5 μM mitomycin C for 6 hours and then incubated with 60 μg/ml cycloheximide for indicated times. Cdk2/5 inhibitor was added 30 minutes before the incubation with cycloheximide. (i) The amounts of p53 and GAPDH were determined by immunoblotting. (ii) Protein levels were quantified. (E) SH-SY5Y cells were treated as described in C for the indicated times, total RNA was isolated and quantitative real-time RT-PCR was performed. The results are from three independent experiments and are presented as the mean ± s.d. Each experiment was performed in triplicate.

S-transferase (GST) pulldown assay was performed. As shown in Fig. 2C, an association between GST-p53 and Cdk5 was evident. For further confirmation, lysates from H1299 ($p53^{-/-}$) cells transfected with expression vectors for Flag-tagged Cdk5, His-tagged p35 and p53 were immunoprecipitated with anti-Cdk5 antibody, and detected with antibodies against either His or p53. As shown in Fig. 2D, p53 and p35 co-precipitated with Cdk5 but not the control IgG. These results collectively indicate that Cdk5-p35 interacts with p53.

Cdk5 interferes with binding of p53 to Hdm2 and decreases subsequent ubiquitylation of p53

Considerable evidence has demonstrated that Hdm2 protein negatively regulates p53 through ubiquitylation and proteasome-mediated degradation (Michael and Oren, 2003; Paajarvi et al., 2005). Consistent with previous results, we observed significant reduction of p53 levels following transient co-transfection of H1299 ($p53^{-/-}$) cells with increasing amounts of an *HDM2*-expressing vector (data not shown). However, expression of a ligase-deficient RING domain mutant of Hdm2, Hdm2(C464A), could not destabilize p53 under the same conditions, indicating that Hdm2 negatively regulates p53 through its E3-ligase activity (data not shown). To investigate whether Cdk5 could affect this regulatory process, increasing amounts of an expression vector encoding the Cdk5 activator p25 were co-transfected with expression vectors encoding p53 and Hdm2. Because transient expression of p35 or p25 did not significantly alter the activation of Cdk5 or the subsequent regulation of p53 (Fig. 3D, Fig. 5D) and p25 functioned as an activator of Cdk5 during oxidative stress (supplementary material Fig. S2C), we used p25 to activate Cdk5. Increased Cdk5 activity prevented the Hdm2-induced reduction of p53, instead leading to accumulation of p53 (Fig. 3A). By contrast, dominant-negative Cdk5(D144N) did not protect p53 from Hdm2, suggesting that this effect is Cdk5 activity-dependent (Fig. 3B). The observed Cdk5-mediated p53 stabilization could be explained by two different mechanisms, i.e. counteraction of the Hdm2-induced degradation of p53 or an effect that is independent of Hdm2 function. To further elucidate the involvement of Cdk5 in Hdm2-mediated p53 regulation, the above experiments were repeated using a mouse embryonic fibroblast (MEF) cell line derived from a *p53/Mdm2* double-knockout mouse. Overexpression of Cdk5-p25 in *p53/Mdm2* double-null ($p53^{-/-}/Mdm2^{-/-}$) MEFs did not result in the accumulation of transfected p53 (Fig. 3C), but did prevent the reduction in p53 levels caused by co-transfection of Hdm2, in a manner similar to that shown in Fig. 3A,B (data not shown). These results

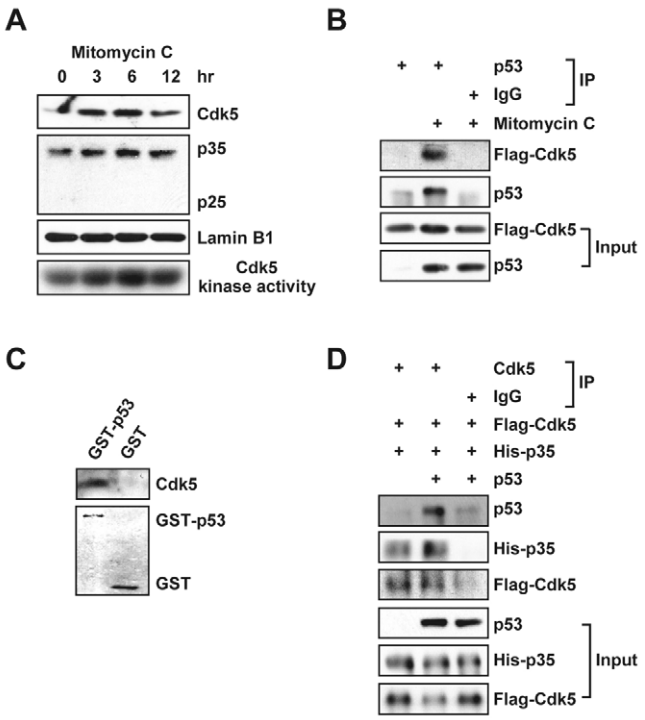

Fig. 2. Cdk5 activity increases in the nucleus, and Cdk5 interacts with p53. (A) SH-SY5Y cells were treated with 5 μM mitomycin C for the indicated times, and nuclear fractions were subjected to immunoblotting. Cdk5 kinase activity was determined by autoradiography using histone H1 as a substrate. (B) SH-SY5Y cells were transfected with a vector encoding Flag-Cdk5 and then treated with 5 μM mitomycin C or vehicle for 6 hours. Cell lysates were immunoprecipitated with antibodies against p53 or control IgG. Membranes were immunoblotted with anti-Flag and/or anti-p53 antibodies, with 5% of the cell lysates used as input controls. (C) Lysates from SH-SY5Y cells treated with 5 μM mitomycin C for 3 hours were used for pulldown with GST-p53 or GST, and probed with antibodies against Cdk5 or GST. (D) H1299 cells were transfected with vectors encoding Flag-Cdk5, His-p35 or p53 as indicated. Cell lysates were immunoprecipitated with antibodies against Cdk5 or control IgG, and then immunoblotted with anti-p53, anti-His and/or anti-Flag, with 5% of the cell lysates used as input controls.

indicate that Cdk5 stabilizes p53 at least in part by counteracting Hdm2 function, but do not strictly rule out the possible involvement of Cdk5 in Hdm2-independent mechanisms. To investigate whether Cdk5 could also affect Hdm2-mediated ubiquitylation of p53, H1299 cells were co-transfected with expression vectors encoding p53, Hdm2, Cdk5-p35 and/or Cdk5-p25, and then treated with the proteasome inhibitor MG132, in order to prevent proteasome-mediated degradation of p53. Immunoprecipitation experiments with an antibody against p53 revealed a marked increase in p53 ubiquitylation following coexpression of Hdm2. However, coexpression of Cdk5-p35 or Cdk5-p25 decreased Hdm2-specific p53 ubiquitylation and also decreased the amount of Hdm2 bound to p53 (Fig. 3D). Inhibition of Cdk5 activity by expression of Cdk5(D144N) reversed the Cdk5-mediated reduction of p53 ubiquitylation and the associated disruption of p53-Hdm2 interactions (Fig. 3E). These results demonstrate that the effect of Cdk5 on p53 is at least partly mediated by disruption of the p53-Hdm2 interaction and inhibition of Hdm2-mediated ubiquitylation of p53. However, it is important to note that the ubiquitylation of p53 was not completely blocked by Cdk5-p35 or Cdk5-p25, and Cdk5(D144N) did not completely reverse the effects of Cdk5. Because other ubiquitin ligases, such as COP1 and Pirh2, are known to target p53 for proteasome-mediated degradation (Dornan et al., 2004; Leng et al., 2003), it is possible that Cdk5 also mediates p53 downregulation through these or other negative regulators of p53.

Cdk5-mediated nuclear localization of p53

Hdm2 negatively regulates p53 transactivation by stimulating its nuclear export through ubiquitylation (Goldberg et al., 2002; Oh et al., 2006). Because Cdk5 inhibited the binding of Hdm2 to p53 and the subsequent ubiquitylation of p53, we investigated whether nuclear export of p53 was also affected by Cdk5. Immunofluorescence analysis showed that p53 resided almost entirely in the nucleus of mitomycin C-treated SH-SY5Y cells. However, p53 was observed in both the nuclei and cytoplasm of cells in which Cdk5 activity had been inhibited. Cell counts demonstrated that up to 75% of the Cdk5-inhibited cells showed cytoplasmic distribution of p53 (Fig. 4A). Moreover, mitomycin C treatment of cells transfected with Cdk5-targeting siRNA was associated with increased levels of cytoplasmic p53 and decreased nuclear levels of p53, compared with mitomycin C-treated cells transfected with scrambled control siRNA (Fig. 4B). However, it is important to note that nuclear p53 was not totally abolished by Cdk5 inhibition, as shown in immunocytochemical and immunoblotting analysis (Fig. 4A,B). Considerable amounts of p53 were still detected in the nucleus of Cdk5-suppressed cells, suggesting that Cdk5 is not the only factor capable of inducing nuclear localization of p53. In a complementary experiment to determine the subcellular localization of p53 in response to expression of Hdm2 and Cdk5, H1299 ($p53^{-/-}$) cells were transfected with DsRed-p53 plasmids. Cells expressing p53 only displayed nuclear localization, whereas those expressing both p53 and Hdm2 presented cytoplasmic distribution of p53. Notably, p53 accumulated in the nucleus when cells were additionally transfected with enhanced green fluorescent protein (EGFP)-Cdk5-p35, even in the presence of Hdm2, whereas expression of EGFP-Cdk5(D144N)-p35 did not affect Hdm2-mediated cytoplasmic localization of p53 (supplementary material Fig. S3A). Consistent with these findings, overexpression of Cdk5-p25 reduced nuclear export of p53 in HEK293T cells transiently transfected with Hdm2 (supplementary material Fig. S3B). Taken together, our observations support the theory that Cdk5 mediates the accumulation of p53 within the nucleus by preventing Hdm2-mediated nuclear export.

Cdk5 mediates p53 phosphorylation at multiple sites including Ser15, Ser33 and Ser46

Given that phosphorylation is a well-known posttranslational mechanism capable of regulating p53, we examined the contribution of Cdk5 to the phosphorylation and subsequent regulation of p53. In vitro kinase assay showed that p53 was phosphorylated to a greater extent by the recombinant active Cdk5-p35 protein. However, inhibition of Cdk5 activity with roscovitine dramatically blocked p53 phosphorylation,

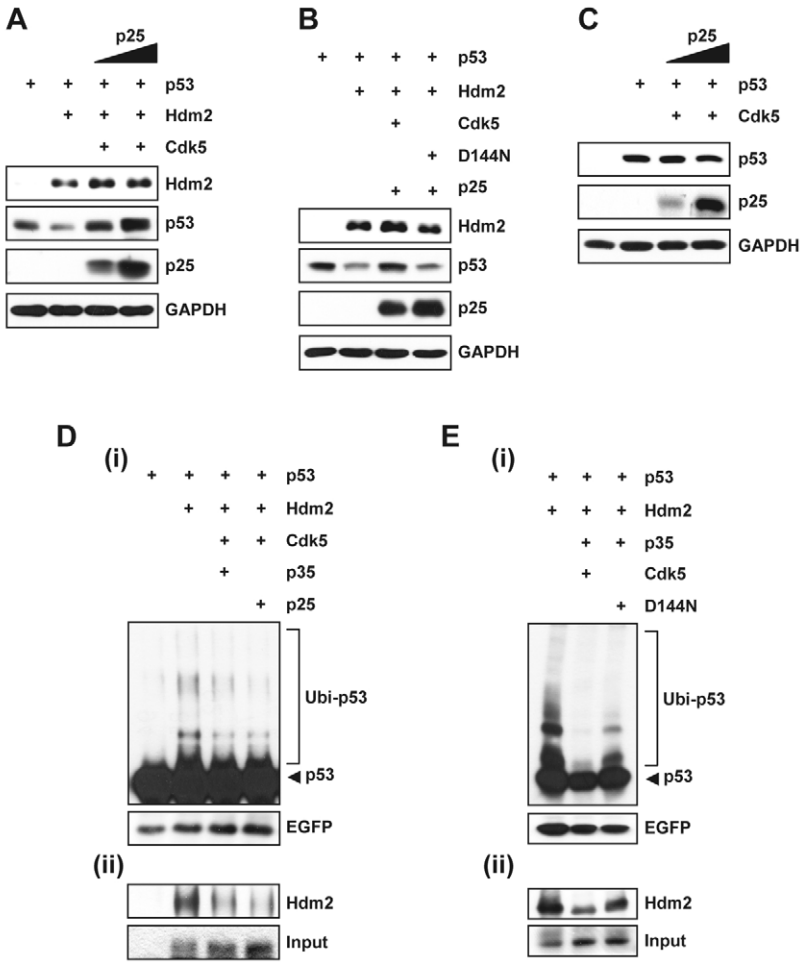

Fig. 3. Cdk5 stabilizes p53 in the presence of Hdm2. (A) H1299 cells were transiently co-transfected with a fixed amount of vectors encoding p53/Hdm2/Cdk5 (0.5 μg/1.5 μg/2 μg) and the various amounts of vector encoding p25 (0, 2 and 4 μg). Cell lysates were subjected to immunoblotting with the indicated antibodies. (B) In similar experiments as described in A, cells were transfected with a fixed amount of p53/Hdm2/p25 (0.5 μg/1.5 μg/2 μg) as indicated. For the inhibition of Cdk5, cells were transfected with Cdk5(D144N) instead of Cdk5 as indicated. (C) Mouse embryonic fibroblasts derived from double-knockout mice ($p53^{-/-}/Mdm2^{-/-}$) were transiently co-transfected with a fixed amount of vectors encoding p53/Cdk5 (0.5 μg/2 μg) and the various amounts of vector encoding p25 (0, 2 and 4 μg). Cell lysates were subjected to immunoblotting with the indicated antibodies. (D,E) H1299 cells were transfected with the indicated combinations of vectors encoding p53 (0.5 μg), Hdm2 (1.5 μg), Cdk5-D144N (2 μg) or p35/p25 (2 μg). 20 μM MG132 was added 36 hours after transfection for 8 hours. (i) Total p53 was immunoprecipitated with a polyclonal antibody and detected with a monoclonal antibody. The ubiquitylated form of p53 is indicated. EGFP was used as the transfection control. (ii) For detecting the association of p53 with Hdm2, cell lysates were immunoprecipitated with anti-p53 and then immunoblotted with anti-Hdm2, with 5% of the cell lysates used as input controls.

suggesting that p53 is a direct substrate for Cdk5 (Fig. 6A). Because Cdk5 is a proline-directed protein kinase and p53 has multiple putative sites for phosphorylation by Cdk5, including Ser33, Ser46 and Ser315, we performed western blot analysis with phosphorylation site-specific antibodies after an in vitro kinase assay. When p53 was incubated with recombinant Cdk5-p35 protein, phosphorylation was detected at Ser33 and Ser46, but not Ser315 (Fig. 5A). Surprisingly, Ser15, which is not part of the consensus sequence for Cdk5, was additionally phosphorylated by Cdk5. Phosphorylation of Ser15, Ser33 and Ser46 was completely inhibited by roscovitine, indicating that these sites are targeted by Cdk5. Additional immunoblotting analysis with other phosphorylation site-specific antibodies (Ser6, Ser9, Ser20 and Ser37) failed to detect phosphorylation, indicating that Cdk5 specifically phosphorylated Ser15, Ser33 and Ser46 (data not shown). We then investigated whether this phosphorylation was under conditions of genotoxic or oxidative stress. Consistent with the results of the in vitro kinase assay, phosphorylation of p53 at Ser15, Ser33 and Ser46 increased after treatment with mitomycin C or SNP (Fig. 5B). To further examine Cdk5-mediated phosphorylation under stress conditions, we suppressed Cdk5 expression with siRNA. Given that downregulation of Cdk5 markedly inhibited mitomycin C-induced p53 expression (Fig. 1B,C), we blocked proteasome-mediated p53 degradation with MG132. In this system, siRNA-mediated knockdown of Cdk5 suppressed mitomycin C-induced phosphorylation of p53 at Ser15, Ser33 and Ser46 (Fig. 5C). Similar results were obtained under conditions of SNP-induced oxidative stress. However, in the SNP-treated system phosphorylation at Ser15 was only marginally suppressed by transfection with Cdk5 siRNA, suggesting that other factors are likely to be involved in this process during SNP-induced oxidative stress. To examine whether overexpression of Cdk5-p35 or Cdk5-p25 is sufficient to induce p53 phosphorylation and its subsequent stabilization, SH-SY5Y cells were transiently transfected with Cdk5-p35 or Cdk5-p25. Immunoblotting analysis revealed that ectopic expression of Cdk5-p35 or Cdk5-p25 could induce phosphorylation of p53 and increase its levels regardless of neurotoxic stress. Downregulation of Cdk5 activity with Cdk5(D144N) failed to induce phosphorylation or upregulation of p53, suggesting that Cdk5 activity plays an important role in the phosphorylation and stabilization of p53 (Fig. 5D). Taken together, these results present clear evidence that Ser15, Ser33 and Ser46 of p53 are the phosphorylation sites for Cdk5, and form potentially important regions for posttranslational modification and stabilization of p53. However, these findings do not completely rule out the possibility that Cdk5 could activate other kinases that subsequently phosphorylate one or more of these residues. Moreover, it is also possible that Cdk5 regulates p53 through collaboration with additional signaling pathways. Because PI3-

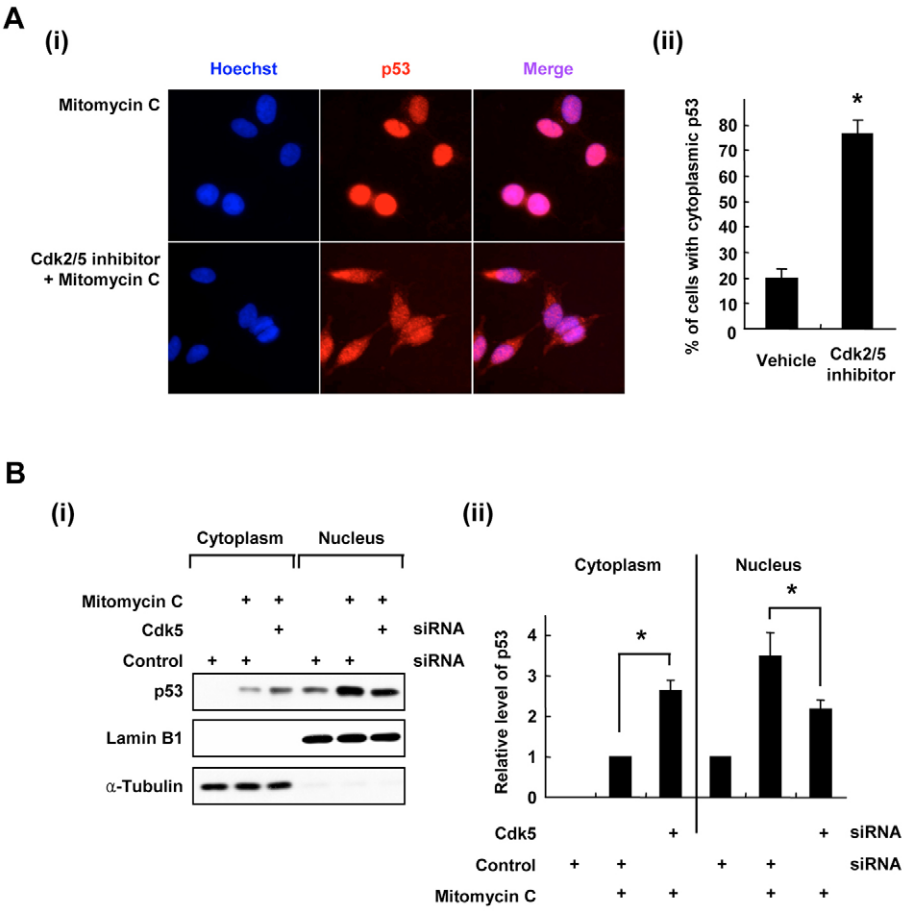

Fig. 4. Cdk5 activity mediates efficient nuclear accumulation of p53. (A) SH-SY5Y cells were pretreated with 10 μM Cdk2/5 inhibitor or an equal volume of vehicle for 30 minutes and then incubated with 5 μM mitomycin C for 6 hours. (i) Cells were double-labeled with Hoechst (blue) and anti-p53 (red) and then analyzed by fluorescence microscopy. (ii) Percentage of cells showing cytoplasmic distribution of p53. Data are the mean ± s.d. from three independent experiments. **P*<0.0001. (B) SH-SY5Y cells were transfected with Cdk5-targeting or scrambled control siRNA, and then incubated with 5 μM mitomycin C for 6 hours. (i) Nuclear and cytoplasmic fractions were prepared as described in the Materials and Methods, and subjected to immunoblotting with the indicated antibodies. Lamin B1 and α-tubulin were used as nucleus- and cytoplasm-specific controls, respectively. (ii) The results displayed in the bar graphs represent quantitatively analyzed data from three independent experiments and are given as the mean ± s.d. For quantitative analysis, each blot was normalized with respect to the internal controls, lamin B1 and α-tubulin. **P*<0.05.

kinase family members (e.g. ATM, ATR and DNA-PK) are known to induce p53 stabilization through phosphorylation in response to DNA damage (Appella and Anderson, 2001), we examined whether these enzymes could be involved in the phosphorylation of p53 in our system. Treatment of Cdk5-knockdown cells with wortmannin, a potent inhibitor of PI3-kinase family members, significantly suppressed mitomycin C-induced phosphorylation of p53 on residues Ser15 and Ser46, whereas phosphorylation of Ser33 was not affected, indicating that the PI3-kinase family is likely to mediate p53 phosphorylation on Ser15 and Ser46 residues in cooperation with Cdk5 (Fig. 5E). Consistent with these findings with regard to phosphorylation of p53, the levels of p53 protein were decreased following wortmannin treatment of Cdk5-suppressed cells (Fig. 5F). These results collectively suggest that p53 is likely to be maximally stabilized through collaboration of Cdk5 with PI3-kinase family members in response to mitomycin C-induced DNA damage.

Cdk5-dependent phosphorylation mediates stabilization of p53

To establish whether these phosphorylation events are associated with Cdk5-mediated stabilization and accumulation of p53, we generated mutant p53-SDM, in which residues Ser15, Ser33 and Ser46 were substituted with alanine residues. An in vitro kinase assay showed that phosphorylation by Cdk5 was completely blocked in p53-SDM, clearly indicating that the mutated sites are targeted by Cdk5 (Fig. 6A). Having established that replacement of these three serine residues with alanine efficiently blocks Cdk5-mediated p53 phosphorylation, we explored whether these phosphorylation events were related to the Cdk5-mediated stabilization of p53. The degradation rate of p53-SDM declined rapidly relative to that of wild-type p53 (p53-WT), suggesting that phosphorylation of residues Ser15, Ser33 and Ser46 plays an important role in Cdk5-mediated posttranslational regulation (supplementary material Fig. S4A). Because Cdk5 promotes p53 stabilization by counteracting Hdm2 activity, we hypothesized that the unphosphorylated form of p53 would not be protected from Hdm2-mediated ubiquitylation by Cdk5. As expected, expression of Cdk5-p35 did not affect the ubiquitylation of p53-SDM by Hdm2, but inhibited that of p53-WT (Fig. 6B). In addition, expression of Cdk5-p35 did not affect the amount of Hdm2 bound to p53-SDM, suggesting that disruption of p53-Hdm2 interactions is at least partly mediated by Cdk5-mediated phosphorylation. To further verify the requirement of Cdk5 in regulating p53 stabilization, we examined p53 ubiquitylation in response to mitomycin C treatment in Cdk5 knockdown cells. Immunoprecipitation experiments with anti-p53 showed that p53 ubiquitylation was markedly decreased following mitomycin C treatment but not in Cdk5 knockdown cells (Fig. 6C). Furthermore, inhibition of Cdk5 expression reversed mitomycin C-mediated reduction of p53-Hdm2 interactions. Taken together, these observations suggest that the Cdk5-mediated stabilization of p53 is because of posttranslational

modification of p53, particularly phosphorylation at Ser15, Ser33 and Ser46.

Cdk5 mediates phosphorylation-dependent p53 acetylation and cooperation of p53 with p300

Although recent studies have demonstrated that Cdk5 regulates the transcriptional activity of p53 (Zhang et al., 2002), the regulatory mechanism underlying this process has not yet been established. In view of the crucial role of p53 acetylation in both transcriptional activity and stabilization (Brooks and Gu, 2003), we examined whether Cdk5 activity is also associated with p53 acetylation and subsequent transcriptional activation. Western blotting analyses revealed that p53 acetylation dramatically increased in SH-SY5Y cells treated with mitomycin C or SNP (Fig. 7A), and that this effect was suppressed by siRNA knockdown of Cdk5 (Fig. 7B). Because p300, a co-activator of p53, induces acetylation at the carboxyl terminus, resulting in activation and stabilization of p53 protein (Bode and Dong, 2004), and phosphorylation of p53 may regulate interactions with p300 and promote acetylation (Xu, 2003), we examined whether Cdk5-induced phosphorylation of p53 is related to the association of p53 with p300 and/or the acetylation of p53. SH-SY5Y cells were transfected with vectors encoding p53-WT or p53-SDM, and treated with mitomycin C or SNP. The acetylation induced by mitomycin C or SNP was significantly suppressed in the unphosphorylatable mutant p53, suggesting that Cdk5-mediated phosphorylation of p53 is related to its acetylation (Fig. 7C). To establish whether Cdk5-mediated p53 phosphorylation contributes to increased p300 binding, total p53 was immunoprecipitated and the amount of bound p300 was detected with a specific antibody. As shown in Fig. 7D, a significant increase in p300 binding to p53-WT was detected following mitomycin C treatment but not p53-SDM. Based on the results, we propose a novel regulatory mechanism in which Cdk5 modulates

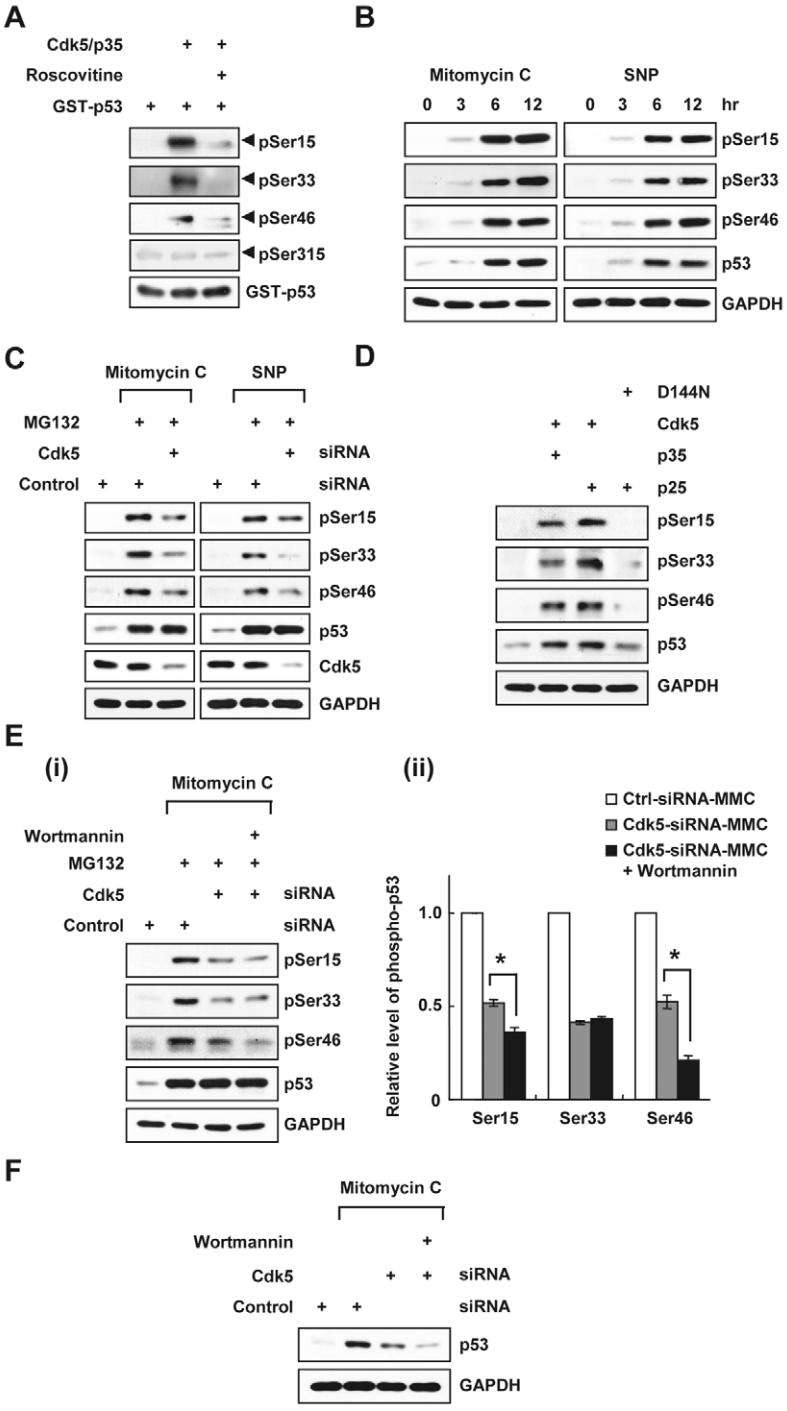

Fig. 5. Cdk5 activity induces phosphorylation of p53 at residues Ser15, Ser33 and Ser46. (A) GST-p53 protein was incubated with active Cdk5-p35 recombinant protein in a kinase reaction buffer, and phosphorylation of specific residues was determined by western blot analysis using the indicated phosphorylation-specific antibodies. The level of GST-p53 was assessed by immunoblotting with anti-GST. (B) SH-SY5Y cells were treated with 5 μM mitomycin C or 2 mM SNP for the indicated times, and cell lysates were subjected to western blotting with the indicated antibodies. (C) SH-SY5Y cells were transfected with Cdk5-targeting or scrambled control siRNA, and then treated with 5 μM mitomycin C or 2 mM SNP for 9 hours in the presence of 20 μM MG132. Total proteins were subjected to western blotting using the indicated antibodies. (D) SH-SY5Y cells were transiently transfected with mock vector or vectors encoding Cdk5-p35, Cdk5-p25 or Cdk5(D144N)-p25, and the amounts of the indicated proteins were determined by immunoblotting. (E) SH-SY5Y cells were transfected with Cdk5-targeting or scrambled control siRNA, treated with 15 μM wortmannin for 1 hour, and then treated with 5 μM mitomycin C for 6 hours in the presence of 20 μM MG132. (i) The amounts of site-specific p53 phosphorylation were determined by immunoblotting. (ii) The results displayed in the bar graphs represent quantitatively analyzed data from three independent experiments, and are given as the mean ± s.d. For the quantitative analysis, each blot was normalized with respect to the level of total p53. *P<0.05. (F) SH-SY5Y cells were transfected with Cdk5-targeting or scrambled control siRNA, treated with 15 μM wortmannin for 1 hour, and then treated with 5 μM mitomycin C for 6 hours. The amounts of p53 and GAPDH were determined by immunoblotting.

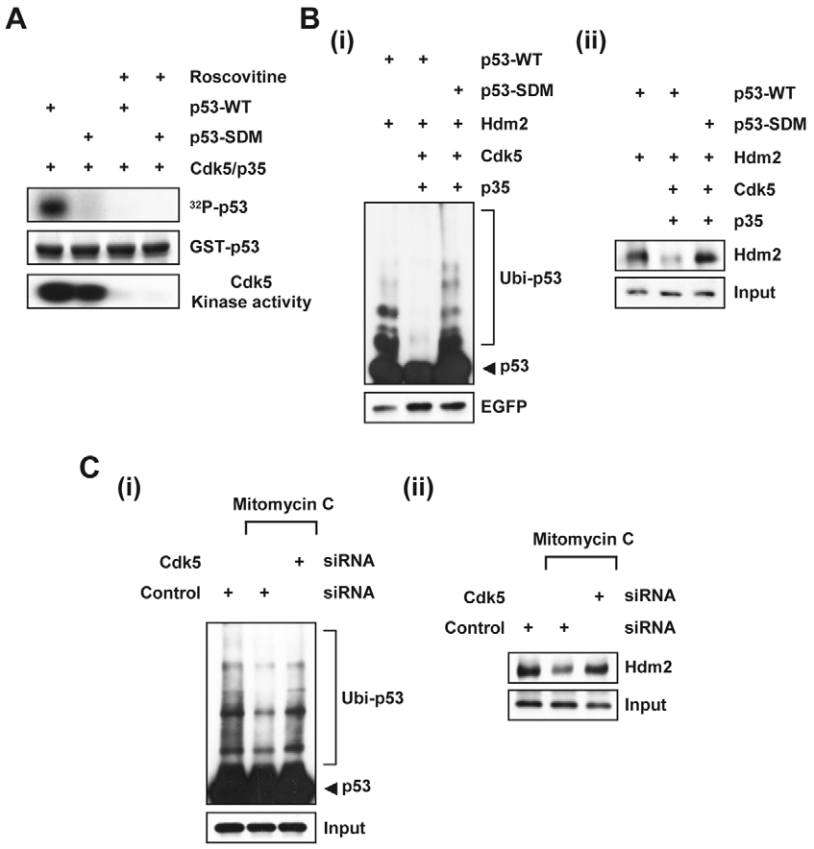

Fig. 6. Cdk5 induces p53 stabilization by phosphorylation. (A) GST-p53 or GST-p53-SDM (a mutant p53 with alanine substituted for Ser15, Ser33 and Ser46) proteins were incubated with active Cdk5-p35 recombinant protein in a kinase reaction buffer, with roscovitine added to inhibit Cdk5 activity. Levels of p53 phosphorylation and Cdk5 kinase activity were examined by autoradiography, and the amount of GST-p53 was assessed by Coomassie Blue staining. (B) H1299 cells were transfected with the indicated plasmids, as described in Fig. 3D,E. At 36 hours post-transfection 20 μM MG132 was added and cultures were incubated for an additional 8 hours. (i) Total p53 was immunoprecipitated with a polyclonal antibody and detected with a monoclonal antibody. The ubiquitylated form of p53 is indicated. EGFP was used as the transfection control. (ii) To detect the association of p53 with Hdm2, cell lysates were immunoprecipitated with anti-p53 and then immunoblotted with anti-Hdm2, with 5% of the cell lysates used as input controls. (C) SH-SY5Y cells were transfected with Cdk5-targeting or scrambled control siRNA, and then treated with 5 μM mitomycin C or an equal volume of vehicle for 8 hours in the presence of 20 μM MG132. (i) Total p53 was immunoprecipitated with a polyclonal antibody and detected with a monoclonal antibody. The ubiquitylated form of p53 is indicated, and 5% of the cell lysates was used as input controls. (ii) For detecting the association of p53 with Hdm2, cell lysates were immunoprecipitated with anti-p53 and then immunoblotted with anti-Hdm2, with 5% of cell lysates used as input controls.

p53 transcriptional activity through phosphorylation-mediated p300 binding and acetylation.

Cdk5 induces transactivation of p53 target genes and subsequent mitochondria-mediated neuronal death

A consequence of p300 binding and acetylation of p53 is the activation of transcription (Brooks and Gu, 2003). Given that p53 enhances transcription of its target genes (e.g. *Bax*, *Puma* and *Noxa*) in response to cellular stress (Morrison et al., 2003; Tan et al., 2005), we investigated whether Cdk5 could affect transactivation of p53 in response to DNA damage. Quantitative real-time RT-PCR analysis revealed that cells treated with mitomycin C exhibited increased mRNA levels of *Bax*, *Puma* and *Noxa*. However, suppression of Cdk5 expression with siRNA significantly impaired the induction of these target genes (Fig. 8A). Similar results were obtained in p53 knockdown cells, indicating that p53 indeed mediates induction of target genes in response to DNA damage (supplementary material Fig. S4B). Moreover, transcriptional activation of p53 was markedly suppressed by downregulation of Cdk5 activity in response to oxidative stress, similar to the effect observed during DNA damage (data not shown). These findings indicate that Cdk5 regulates p53 transcriptional activity in response to genotoxic and oxidative stress. Because *Bax*, *Noxa* and *Puma* are pro-apoptotic genes that play essential roles in mitochondria-mediated apoptosis (Culmsee and Mattson, 2005; Morrison and Kinoshita, 2000), we examined whether Cdk5 also regulates mitochondrial release of cytochrome C, a crucial event in the mitochondrial response to cellular stress. Immunofluorescence analysis showed that cytochrome C was mitochondrially distributed in control cells, as shown by its colocalization with MitoTracker green dye. Following mitomycin C treatment, mitochondrial cytochrome C almost disappeared, instead showing a scattered distribution throughout the cell, including the nucleus. By contrast, inhibition of Cdk5 activity was markedly associated with retention of cytochrome C in the mitochondria, indicating that Cdk5 regulates the p53-mediated mitochondrial pathway (Fig. 8B). Similar results were obtained under conditions of SNP-induced oxidative stress (data not shown). Moreover, immunocytochemistry using cleaved caspase-3 antibody showed that EGFP-Cdk5(D144N)-transfected cells were negative for cleaved caspase-3, whereas approximately 55% and 48% of the mitomycin C- and SNP-treated GFP-transfected control cells, respectively, were positive for cleaved caspase-3 (Fig. 8C). Consistent with these findings, western blotting analyses revealed that downregulation of Cdk5 activity by Cdk5(D144N) transfection markedly inhibited mitomycin C- or SNP-induced caspase-3 cleavage (Fig. 8D). Based on this, we investigated the effect of Cdk5 on DNA damage- or oxidative stress-induced neuronal death by abrogating its activity. We found that expression of Cdk5(D144N) significantly increased cell viability in mitomycin C- or SNP-treated SH-SY5Y cells, compared with mock-transfected controls (Fig. 8E). Our observations collectively indicate that transcriptional activation of p53 and subsequent mitochondria-mediated neuronal apoptosis are a consequence of Cdk5-induced accumulation and acetylation of p53.

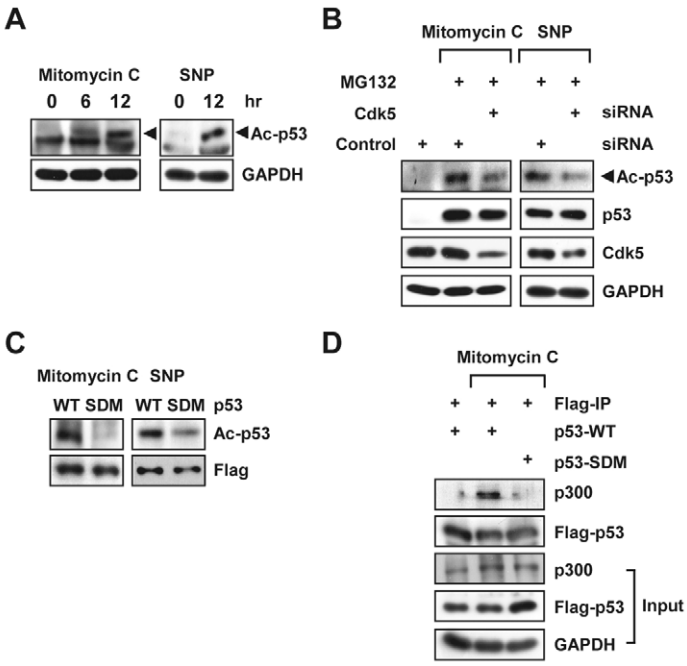

Fig. 7. Cdk5 induces acetylation of p53 and its cooperation with p300. (A) SH-SY5Y cells were treated with 5 μM mitomycin C or 2 mM SNP for the indicated times. The amounts of acetyl-p53 (◀) and GAPDH were determined by immunoblotting. (B) SH-SY5Y cells were transfected with Cdk5-targeting or scrambled control siRNA, and then treated with 5 μM mitomycin C or 2 mM SNP for 9 hours in the presence of 20 μM MG132. Total proteins were subjected to western blotting using the indicated antibodies. (C) SH-SY5Y cells were transiently transfected with vectors encoding Flag-p53-WT or Flag-p53-SDM (a mutant p53 substituted with alanine at Ser15, Ser33 and Ser46), and then treated with 5 μM mitomycin C or 2 mM SNP for 9 hours. Total proteins were subjected to western blotting using the indicated antibodies. (D) SH-SY5Y cells were transfected with vectors encoding Flag-p53-WT or Flag-p53-SDM and then treated with 5 μM mitomycin C or vehicle for 6 hours. Cell lysates were immunoprecipitated with antibodies against Flag-p53, and membranes were immunoblotted with anti-p300 and/or anti-Flag, with 5% of the cell lysates used as input controls.

Discussion

In the present study we provide the first description of the mechanisms by which Cdk5 regulates the stability and nuclear accumulation of transcriptionally active p53 protein through posttranslational, not transcriptional, modulation of p53. We observed that Cdk5 activity was correlated with changes in p53 phosphorylation and stability, suggesting that regulation of p53 probably occurs as a consequence of Cdk5 kinase activity. Phosphorylation at multiple sites is the main posttranslational modification of p53; these events result in stabilization of p53 through inhibition of its interaction with Hdm2, subsequently leading to transactivation of p53 and increased transcription of various target genes depending on cell types and external stimuli (D'Orazi et al., 2002; Tibbetts et al., 1999; Xu, 2003). The present study shows that Cdk5 associates with p53 and induces phosphorylation at diverse residues, including Ser15, Ser33 and Ser46. Phosphorylation of p53 by Cdk5 attenuates its interaction with Hdm2 and affects Hdm2-mediated p53 ubiquitylation, thus inducing nuclear localization and accumulation of p53 protein. Putative sites for phosphorylation by Cdk5, a proline-directed protein kinase, are present at Ser33, Ser46 and Ser315 of p53. As expected, Cdk5-mediated phosphorylation was observed at Ser33 and Ser46. However, Cdk5-induced phosphorylation of Ser315 was not observed in an in vitro kinase assay or under in vivo conditions, whereas Cdk5-induced phosphorylation was observed at Ser15, which does not conform to the Cdk5 consensus sequence. This may suggest that Cdk5 phosphorylates at non-consensus sites or targets another phosphorylation sequence, or it could indicate that Cdk5 activates other kinases that subsequently phosphorylate one or more of these residues. A recent study showed that deficiency of ATM attenuated but did not completely abrogate p53 accumulation during DNA damage-evoked neuronal death (Keramaris et al., 2003). Moreover, phosphorylation at Ser15, a well-known ATM phosphorylation site, was not completely abolished, suggesting that other factors are likely to account for the stability and transactivational potential of p53 in this system. Thus, our current results suggest that Cdk5 might act as another important factor for regulating p53 in these circumstances, especially in neurons.

In addition to phosphorylation of p53, it is also possible that Cdk5 induces p53 accumulation by directly disrupting the function of Hdm2 either by posttranslational modification or through regulating interactions of Hdm2 with other cellular proteins. The expression levels of Hdm2, its association with p53 and its activation of E3 ubiquitin ligase activity are known to be regulated by phosphorylation, nucleolar localization and binding to other factors such as p19-p14ARF, Mdmx and c-Abl (Bernardi et al., 2004; Goldberg et al., 2002; Isaacs et al., 2001; Kulikov et al., 2005). It has been demonstrated that p53 stabilization can occur without phosphorylation at multiple N-terminal sites, suggesting that direct disruption of Hdm2 function might be another mechanism underlying the accumulation of p53 (Ashcroft et al., 2000). Furthermore, other studies have suggested that N-terminal phosphorylation of p53 (Ser15 and Ser20) does not directly disrupt the Hdm2-p53 interaction, but rather leads to the recruitment of other factors, such as p300, CBP and TATA-binding protein (TBP)-associated factors (TAFs), which compete with Hdm2 for binding, leading to subsequent accumulation of p53 (Dumaz and Meek, 1999; Lambert et al., 1998; Xu, 2003). Consistent with these reports, the present study shows that Cdk5-mediated p53 phosphorylation induces recruitment of p300, implying that Cdk5 indirectly suppresses Hdm2 function through phosphorylation of p53 (Fig. 7D). In addition, we found that Hdm2 was phosphorylated by Cdk5 in an in vitro kinase assay, and could be destabilized by Cdk5 in response to DNA damage (our unpublished data), indicating that Cdk5 can directly modulate Hdm2 function through posttranslational modifications. Thus, Cdk5 may stabilize p53 through two different mechanisms, namely phosphorylation of p53, which leads to recruitment of other factors and subsequent attenuation of the p53-Hdm2 complex, and direct inhibition of Hdm2 function by posttranslational modification. In addition, Cdk5 could act on other p53 ubiquitin ligases, such as COP1 and Pirh2 (Dornan et al., 2004; Leng et al., 2003), or other negative regulators, thus stabilizing p53 by inhibiting its proteasome-

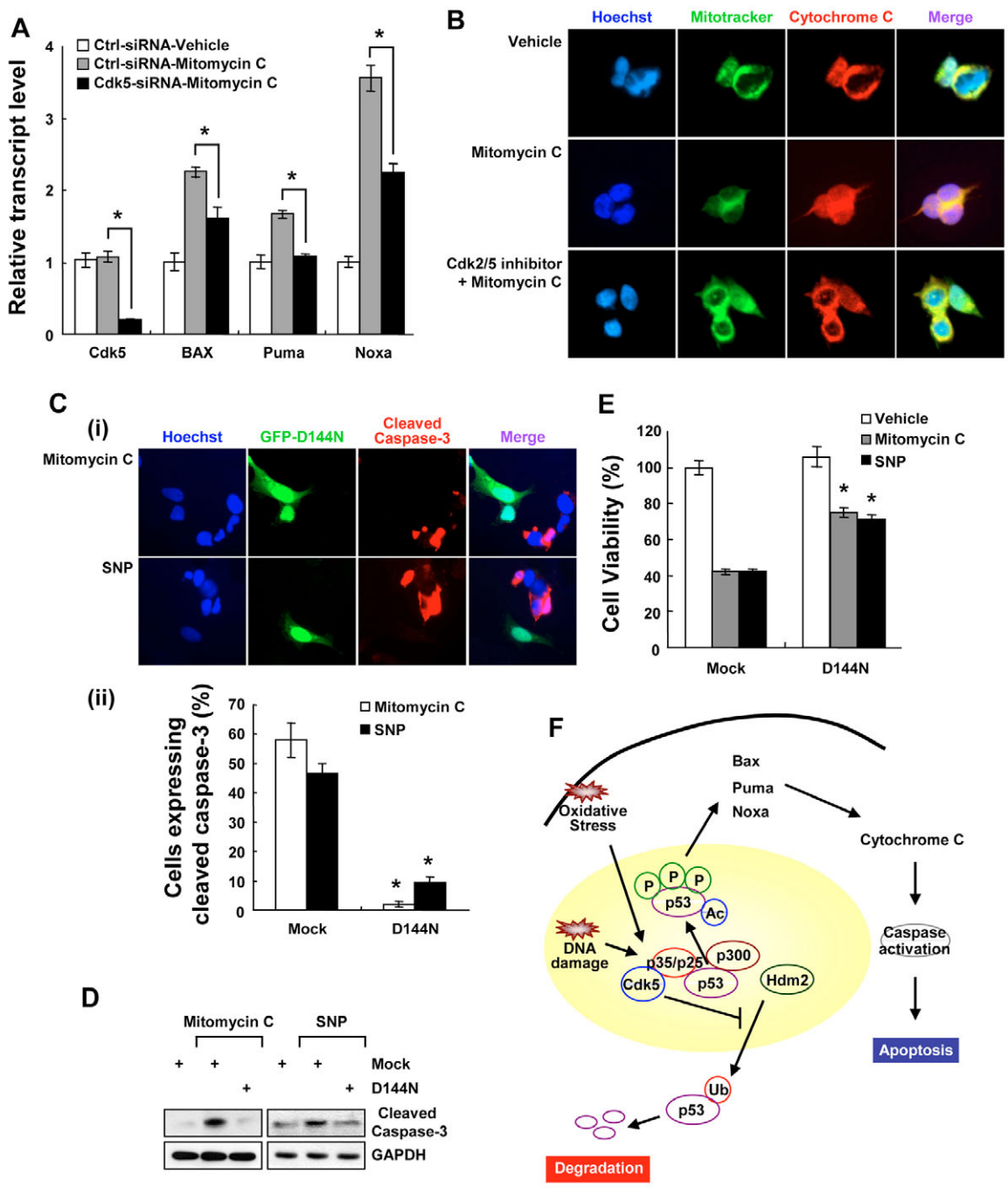

Fig. 8. Cdk5-mediated p53 transactivation induces mitochondria-mediated neuronal apoptosis in response to cellular stress. (A) SH-SY5Y cells were transfected with Cdk5-targeting or scrambled control siRNA, and then treated with 5 μM mitomycin C for 9 hours. Total RNA was isolated, and quantitative real-time RT-PCR was performed. The presented results are from three independent experiments and are given as the mean ± s.d. Each experiment was performed in triplicate. **P*<0.005. (B) SH-SY5Y cells were pretreated with 10 μM Cdk2/5 inhibitor or an equal volume of vehicle for 30 minutes and then incubated with 5 μM mitomycin C for 12 hours. Cells were triple-labeled with Hoechst (blue), anti-cytochrome C (red) and MitoTracker (green) and then analyzed by fluorescence microscopy. (C) SH-SY5Y cells were transiently transfected with vectors encoding EGFP-Cdk5(D144N) or EGFP, treated with 5 μM mitomycin C or 2 mM SNP for 12 hours and fixed and stained with antibodies against cleaved caspase-3. (i) Representative image of EGFP-Cdk5(D144N)-positive cells. (ii) Percentage of EGFP- or EGFP-Cdk5(D144N)-expressing cells positive for cleaved caspase-3. The data represent the mean ± s.d. from three independent experiments. **P*<0.0001. (D) SH-SY5Y cells were transiently transfected with mock vector or vectors encoding Cdk5(D144N), and then treated with 5 μM mitomycin C or 2 mM SNP for 12 hours. Cell lysates were subjected to immunoblotting with the indicated antibodies. (E) SH-SY5Y cells were transiently transfected with mock vector or vectors encoding Cdk5(D144N), and then treated with 5 μM mitomycin C, 2 mM SNP or an equal volume of vehicle. Cell viability was measured 24 hours later by MTT assay. The data represent the mean ± s.d. from quadruplicate determinations. Similar results were obtained in three independent experiments. **P*<0.0001. (F) Proposed model for Cdk5-mediated regulation of p53 and subsequent mitochondria-mediated apoptosis.

mediated degradation. Further studies will be warranted to clarify how Cdk5 affects Hdm2 function.

Another candidate mechanism for the action of Cdk5 on p53 is the mediation of p300 protein. The p300 coactivator and its homolog, CBP, which are well known for their acetyltransferase activities, are capable of stabilizing p53 and functioning as transcriptional coactivators (Bode and Dong, 2004; Dornan et al., 2003). Phosphorylation at the N-terminal region of p53 facilitates binding of p300, which then acetylates lysine residues in the C-terminus, resulting in increased stabilization and transcriptional activity of p53 (Dumaz and Meek, 1999; Vega et al., 2004). The present study shows that Cdk5 regulates the acetylation of p53 (Fig. 7B), and unphosphorylatable p53 by Cdk5 lacks p300 binding (Fig. 7D), implying that Cdk5-mediated phosphorylation may enhance the formation of stable p53-p300 complexes, subsequently increasing acetylation of p53. These results also suggest a mechanism by which Cdk5 might stabilize and induce p53 transcriptional activity. As shown in Fig. 8A, and supplementary material Fig. S4B, the mRNA levels of the p53 target genes *Bax*, *Puma* and *Noxa* are increased as a consequence of p53 modulation by Cdk5. Numerous p53-inducible proapoptotic genes, including *Bax*, *Puma*, *Noxa* and *Bid*, function in neuronal apoptosis by disrupting mitochondrial membrane potential, releasing cytochrome c and activating caspases (Culmsee and Mattson, 2005; Morrison and Kinoshita, 2000; Polster and Fiskum, 2004; Tan et al., 2005). Consistent with previous observations, the present study showed that Cdk5-dependent p53 regulation promoted apoptosis through the mitochondrial pathway, prompting us to speculate that Cdk5 is one of the key factors facilitating neuronal apoptosis through phosphorylation and activation of p53. However, we cannot rule out the possibility that additional signaling pathways are involved in regulating p53 transactivation and turnover. It is plausible that the concerted actions of inducible modifications at diverse sites, along with the cooperative actions of Cdk5 and other factors, combine to yield maximal activation and stabilization of p53.

Posttranslational modification, subcellular redistribution of p53 and inhibition of Hdm2 activity have all been associated with the rapid accumulation of p53 in cells (Bernardi et al., 2004; Schneiderhan et al., 2003), with different mechanisms utilized depending on the type of cell and/or extracellular stimulus (Ashcroft et al., 2000). Consistent with this, we herein present several lines of evidence suggesting that p53 is regulated by different mechanisms in response to genotoxic or oxidative stress. First, DNA damage led to increased p53 transcript levels, whereas oxidative stress was not accompanied by elevation of p53 mRNA (Fig. 1E, supplementary material Fig. S2B), but both stimuli triggered increases in p53 protein, suggesting that SNP-evoked increases in p53 are induced solely by posttranslational mechanisms. However, Cdk5-mediated phosphorylation and stabilization of p53 were seen in response to both oxidative and genotoxic stress, suggesting that Cdk5 activity might be a point of convergence in the regulation of p53 under these stress conditions. Second, our findings suggest that oxidative and genotoxic stresses are likely to involve different signaling pathways mediating the posttranslational modification of p53 in addition to Cdk5. The phosphorylation of p53 is determined by the particular set of available kinases, which depend on the involved cell type and extracellular stimulus (Bode and Dong, 2004; Brooks and Gu, 2003). This may explain why phosphorylation at Ser15 was marginally regulated by Cdk5 in SNP-treated cells, compared with mitomycin C-treated cells. Previous studies have shown that PKC-δ and p38 mediate Ser15 phosphorylation and subsequent stabilization of p53 in response to SNP-induced oxidative stress (Kim et al., 2002; Lee et al., 2006). Because SNP-induced phosphorylation at Ser15 was only marginally suppressed by Cdk5 siRNA, it is likely that PKC-δ, p38 and/or other signaling molecules are more involved in SNP-induced p53 phosphorylation at Ser15. PI3-kinase family members, including ATM, ATR and DNA-PK, are well-known for inducing N-terminal phosphorylation and stabilization of p53 in response to DNA damage (Appella and Anderson, 2001). Here, we clearly demonstrate that p53 is stabilized through collaboration of Cdk5 and members of the PI3-kinase family in response to mitomycin C. Our results showed that PI3-kinase family members mediated p53 phosphorylation on residues Ser15 and Ser46 in cooperation with Cdk5 (Fig. 5E,F), suggesting that p53 may be maximally activated and stabilized in response to DNA damage through collaboration of these kinases. Further studies will be warranted to clarify the cooperative roles played by Cdk5 and additional factors in terms of cell damage-induced regulation of p53. Finally, we show that the activity of Cdk5 itself is differentially regulated in response to DNA damage and oxidative stress. Mitomycin C treatment enhanced Cdk5 activity as a consequence of increased p35 protein levels, whereas SNP increased Cdk5 activity by inducing proteolytic cleavage of p35 to p25 (Fig. 2A, supplementary material Fig. S2C). We recently showed that the ERK pathway is associated with p35 expression and subsequent Cdk5 activity upon mitomycin C treatment (Lee and Kim, 2007), and the calcium-dependent cysteine protease, calpain, is known to induce cleavage of p35 to p25 in response to oxidative stress, leading to increased Cdk5 activity (Dhavan and Tsai, 2001; Shelton and Johnson, 2004). These findings collectively indicate that, depending on the extracellular stimulus, different mechanisms may be utilized to activate Cdk5 and different signaling pathways may cooperate with Cdk5 to stabilize and activate p53, resulting in neuronal death.

Cdk5 has received considerable attention as a regulator of neuronal death (Guo, 2003; Weishaupt et al., 2003), and Cdk5 is involved in the responses of neurons treated with excitotoxins, β-amyloid and oxidative stress, as well as in animal models of stroke and Parkinson's disease (Monaco, 3rd, 2004; O'Hare et al., 2005; Shelton and Johnson, 2004). Although Cdk5 activity has primarily been associated with neurons, accumulating evidence indicates that Cdk5 activity and function also affect non-neuronal cells. For example, Cdk5 activation is reportedly required for caspase-3 activation and subsequent cAMP-induced apoptosis in rat leukemia cells (Sandal et al., 2002), and correlations between increased Cdk5 activity and apoptosis have been identified in the embryonic limb, a well-characterized system for studying apoptosis during development (Zhang et al., 1997). It has been demonstrated that accumulation of p25, and subsequent Cdk5 activity, causes digoxin-triggered cell death of prostate cancer cells (Lin et al., 2004). However, although aberrant Cdk5 activity appears to play an essential role in apoptosis of extraneuronal cells, the mechanisms by which active Cdk5 facilitates apoptosis are not yet well understood. Thus, the

present finding of Cdk5-mediated p53 stabilization and activation in neuronal apoptosis may provide important new insights into the molecular mechanisms underlying apoptosis in non-neuronal cells.

Apoptosis is crucial for neuronal development, and is associated with neuronal death in many neurological disorders. Because both Cdk5 and p53 play crucial roles in neuronal apoptosis (Culmsee and Mattson, 2005; Guo, 2003; Polster and Fiskum, 2004), our current finding may provide important evidence for the convergence of signaling molecules in neuronal apoptosis. Based on our findings, we propose a model for the action of Cdk5 on p53 (Fig. 8F). Under physiological conditions in the absence of stress, Hdm2 maintains p53 at low levels by ubiquitylation-dependent nuclear export and degradation. In response to cellular stress, the activated Cdk5-p35 or Cdk5-p25 complex associates with p53, inducing phosphorylation and subsequent acetylation. Activation of Cdk5 attenuates the inhibitory action of Hdm2, resulting in nuclear accumulation and transcriptional activation of p53, which then exerts its apoptotic activity by transactivating proapoptotic target genes, leading to mitochondria-mediated apoptosis. Thus, the present findings facilitate our understanding of the molecular mechanisms underlying neurodegenerative diseases.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and penicillin-streptomycin were purchased from Gibco (Grand Island, NY). Bovine calf serum and fetal calf serum were obtained from HyClone Laboratories (Logan, UT). Mitomycin C was purchased from Sigma Chemical Co. (St Louis, MO). SNP was from ALEXIS Biochemicals (Lausen, Switzerland). The Cdk5 inhibitor, roscovitine, the Cdk2/5 inhibitor, N^4-(6-aminopyrimidin-4-yl)-sulfanilamide, MG132 and histone H1 were purchased from Calbiochem (La Jolla, CA). Rabbit polyclonal antibodies raised against Cdk5 (C-8) and p35 (C-19) and GST-p53 protein were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mono- and polyclonal p53 antibodies, polyclonal antibodies raised against phospho-p53 (Ser15, Ser33, Ser46 and Ser315) and polyclonal acetylated-p53 (Lys382) antibodies were obtained from Cell Signaling (Beverly, MA). Recombinant Cdk5-p35 proteins were obtained from Upstate Biotechnology (Lake Placid, NY). siRNA against Cdk5 and p53, SMARTpool™ containing four pooled SMARTselected siRNA duplexes were purchased from Dharmacon (Lafayette, CO). The western blot detection reagent (SUPEX) was obtained from NEURONEX (Pohang, Korea). [α-^{32}P]dATP was purchased from NEN (Boston, MA).

Cell culture

Human neuroblastoma SH-SY5Y cells and the human lung cancer cell line H1299 (*p53*$^{-/-}$) were grown in DMEM supplemented with 10% heat-inactivated bovine calf serum and RPMI supplemented with 10% fetal calf serum, respectively, with 1% penicillin-streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. Before experiments, SH-SY5Y cells were cultured in low-serum medium (DMEM supplemented with 1% heat-inactivated bovine calf serum) for 12 hours, and treated with the indicated drugs. All experiments were performed under low-serum conditions. The immortalized fibroblasts from double-knockout p53/Mdm2 mice, a gift from Jaewhan Song (Sungkyunkwan University, Korea), were grown in DMEM supplemented with 10% fetal calf serum.

Plasmids, transfections and siRNA experiments

Expression vectors encoding mouse wild-type Cdk5, a dominant-negative mutant of Cdk5(D144N), and mouse p35 were as described elsewhere (Lee and Kim, 2004). Mouse p25 was cloned into the *Bam*HI/*Hin*dIII restriction sites of the pcDNA3.1 vector. p53-SDM, a mutant version of p53 with alanine substituted at residues Ser15, Ser33 and Ser46, was generated by PCR-based mutagenesis and subcloned into the *Eco*RI/*Bam*HI restriction sites of the N-terminal pFLAG expression vector. The DsRed-p53 expression construct was generated by cloning the p53 cDNA into the *Eco*RI/*Bam*HI restriction sites of the pDsRed1-C1 plasmid. EGFP-Cdk5 and EGFP-D144N expression constructs were generated by cloning the cDNAs encoding Cdk5 and D144N into the *Hin*dIII/*Sal*I restriction sites of the pEGFP-N1 plasmid. The expression construct for Hdm2 was kindly provided by Bernhard Brüne (University of Kaiserslautern, Germany). Transient transfection of plasmids, siRNA targeting Cdk5 and p53, or scrambled control siRNA was performed using Lipofectamine 2000 (Gibco, Grand Island, NY), according to the manufacturer's instructions. The transfection medium was replaced with fresh low-serum medium 24 hours post-transfection, and then cells were collected for immunoblotting or treated with the indicated drugs for further experiments.

Western blotting, immunoprecipitation and Cdk5 kinase assay

Cells were treated with the indicated drugs, washed with chilled PBS and then lysed for 45 minutes on ice, using Triton lysis buffer (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 25 mM beta-glycerophosphate, 2 mM sodium pyrophosphate, 2 mM EDTA, 1 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.5 mM DTT, and protease inhibitors). The lysates were clarified by centrifugation at 18,000 ***g*** for 15 minutes, and the supernatants were stored at –80°C until use. The proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and the following procedures were performed as previously described (Lee and Kim, 2004). For western blot analysis, bands were visualized using the western blot detection kit (NEURONEX). For immunoprecipitation, anti-p53 and anti-Cdk5 antibodies coupled to protein A-agarose beads were incubated with solubilized lysates. The beads were washed four times with Triton lysis buffer, and the bound proteins were resolved by SDS-PAGE and analyzed by western blotting. For the Cdk5 kinase assay, active recombinant Cdk5-p35 proteins (Upstate Biotechnology, Lake Placid, NY) or immunoprecipitated endogenous Cdk5 was mixed with 8 μg histone H1 peptide as a substrate in kinase reaction buffer containing 25 mM HEPES, pH 7.4, 25 mM beta-glycerophosphate, 25 mM $MgCl_2$, 100 μM Na_3VO_4, 500 μM DTT and 1 mM [γ-^{32}P]ATP. The reaction was allowed to proceed at 30°C for 30 minutes as described previously (Harada et al., 2001), and radioactivity was measured by autoradiography.

Immunocytochemical analysis

Transfected H1299 (*p53*$^{-/-}$) cells and SH-SY5Y cells were fixed with 4% paraformaldehyde and incubated with blocking solution (2.5% bovine serum albumin and 2.5% equine serum in PBS) for 1 hour at room temperature. The samples were then incubated overnight at 4°C with the indicated antibodies, followed by incubation with rhodamine-conjugated anti-rabbit or anti-mouse IgGs. Slides were mounted, and the results were visualized by fluorescence microscopy (Axioplan2; Zeiss, Oberkochen, Germany).

Cell viability and TUNEL assays

To evaluate cell survival, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed with a modification (Kubo et al., 1996). In brief, cultures were incubated with a final concentration of 1 mg/ml MTT tetrazolium salt at 37°C for 2 hours. The reaction was stopped by the addition of lysis buffer containing 20% sodium dodecyl sulfate and 50% N,N-dimethyl formamide, pH 4.5, samples were incubated overnight at 37°C and absorbance was measured photometrically at 570 nm. Apoptotic cells were further visualized using the modified TUNEL (TdT-mediated dUTP nick-end labeling) assay offered by the DeadEnd colorimetric detection system (Promega, Madison, WI), according to the manufacturer's instructions.

RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted using the Trizol reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. For quantitative real-time RT-PCR, total RNA was reverse transcribed using the iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The MyiQ™ real-time PCR detection system (Bio-Rad) was used for detection and quantification, and GAPDH was amplified as an internal control. PCR amplification was performed using the iQ™ SYBR Green Supermix kit (Bio-Rad), in a final volume of 20 μl containing 0.5 μM of each primer, cDNA and 10 μl of the supplied enzyme mixture containing the DNA double-strand-specific SYBR Green I dye for detection of PCR products. The cycling conditions consisted of 3 minutes of preincubation at 95°C followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C. PCR products were verified by melting curve analysis, agarose gel electrophoresis and DNA sequencing. The following primers were used: GAPDH, forward 5′-GCCATCAATGACCCCTTCATT-3′, reverse 5′-GCTCCTGGAAGATGGTGATGG-3′; p53, forward 5′-GAGCGCTTCG-AGATGTTCCGAGAGC-3′, reverse 5′-GTCTGAGTCAGGCCCTTCTGTCTTG-3′; Bax, forward 5′-AGAGGATGACAACCACCCTGGTCTTGGATCCAGC-3′; Puma, forward 5′-GAGACAAGAGGAGCAGCAGCGGCAC-3′, reverse 5′-CTA-ATTGGGCTCCATCTCGGGGGCTC-3′; Noxa, forward 5′-AAGAAGGCGCGC-AAGAACGCTCAAC-3′, reverse 5′-GGTTCCTGAGCAGAAGAGTTTGGAT-3′; Cdk5, forward 5′-GGCCAAGCTGTACTCCACGT-3′, reverse 5′-CTTATAGTC-TGGCAGCTTGG-3′. Relative gene expression levels were analyzed using the $2^{-\Delta\Delta C_T}$ method (Lee and Kim, 2004).

Preparation of nuclear and cytoplasmic extracts

Cells were harvested, washed and resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 30 mM NaCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, 0.05% NP-40 supplemented with protease inhibitors). The nuclei

were spun down at 2000 ***g*** for 4 minutes, and the supernatants were transferred to fresh tubes and centrifuged at 18,000 ***g*** for 10 minutes for recovery of the cytosolic fractions. The nuclei were washed three times with 50 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 0.1 mM EDTA and 40% glycerol, and then lysed at 4°C for 30 minutes in a solution containing 10 mM HEPES, pH 7.9, 350 mM NaCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, 1.5% Triton X-100 and protease inhibitors. The nuclear lysates were centrifuged at 18,000 ***g*** for 10 minutes, and the supernatants were recovered as the nuclear fractions.

Statistical analysis

All experiments, including the immunoblots and kinase assays, were independently repeated at least three times. All assays and blots presented are representative of more than three separate experiments. All quantitative data are presented as the mean ± s.d. Comparisons between two groups were analyzed using Student's *t*-test, and values of $P<0.05$ were considered statistically significant.

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