Specific Acetylation of p53 by HDAC Inhibition Prevents DNA Damage-Induced Apoptosis in Neurons

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Histone deacetylase (HDAC) inhibitors have been used to promote neuronal survival and ameliorate neurological dysfunction in a host of neurodegenerative disease models. The precise molecular mechanisms whereby HDAC inhibitors prevent neuronal death are currently the focus of intensive research. Here we demonstrate that HDAC inhibition prevents DNA damage-induced neurodegeneration by modifying the acetylation pattern of the tumor suppressor p53, which decreases its DNA-binding and transcriptional activation of target genes. Specifically, we identify that acetylation at K382 and K381 prevents p53 from associating with the pro-apoptotic PUMA gene promoter, activating transcription, and inducing apoptosis in mouse primary cortical neurons. Paradoxically, acetylation of p53 at the same lysines in various cancer cell lines leads to the induction of PUMA expression and death. Together, our data provide a molecular understanding of the specific outcomes of HDAC inhibition and suggest that strategies aimed at enhancing p53 acetylation at K381 and K382 might be therapeutically viable for capturing the beneficial effects in the CNS, without compromising tumor suppression.

Introduction

Histone deacetylases (HDACs) and histone acetyltransferases (HATs) play important roles in the control of protein acetylation homeostasis and the regulation of basic cellular activities such as gene expression. Disruption of the balance between HAT and HDAC activities leads to a disequilibrium in acetylation and transcriptional dysregulation, two established features of neurodegeneration (Rouaux et al., 2004). Treatment with various HDAC inhibitors (HDACis) can adjust these deficiencies and has emerged as an attractive therapeutic approach for neurodegeneration in the last decade (Kazantsev and Thompson, 2008; Langley et al., 2009). In addition to neuroprotection, there is growing evidence that HDACis can also enhance learning and memory (Fischer et al., 2007) and promote axonal regeneration (Riveccio et al., 2009). Moreover, HDACis are also being developed for the treatment of various cancers, suggesting that they could treat chronic neurodegeneration without increasing the potential for tumorigenesis.

HDACis are known to selectively alter gene transcription by promoting histone acetylation (Grunstein, 1997). Moreover, they can increase the acetylation of many nonhistone proteins,

modifying their function or activity (Choudhary et al., 2009). A good example is the tumor suppressor p53, which drives many neurodegenerative processes (Morrison et al., 2003). p53 plays a central role in the DNA damage response, which contributes to neurodegeneration during cerebral ischemia, brain trauma, and age-related disease (Chang et al., 2012). After DNA damage, p53 accumulates and is activated to coordinate cellular responses that lead to DNA repair or the elimination of cells with extensive damage (Brooks and Gu, 2003; Culmsee and Mattson, 2005). The molecular basis underlying the choice between cell-cycle arrest and induction of apoptosis by p53 is not well understood. However, among the multiple posttranslational modifications of p53 that have been characterized, the acetylation of key lysine residues within the C-terminal region of p53 appears to be a determinant of activity (Olsson et al., 2007). Indeed, most studies suggest that acetylation stimulates p53 stabilization, sequence-specific binding activity, and activation of target genes (Gu and Roeder, 1997; Zhao et al., 2006). Nevertheless, other evidence indicates that p53 activity depends on the specific lysines that are acetylated (Knights et al., 2006).

Uo et al. (2009) have highlighted the protective role of HDACis against p53-dependent apoptosis in neurons. However, the molecular targets of HDAC activity remain to be identified. Here, we demonstrate for the first time that HDAC inhibition prevents DNA damage-induced death in neurons by specifically modifying the acetylation pattern of p53, which decreases its transcriptional activity and suppresses the expression of pro-apoptotic PUMA. Furthermore, using mutants of p53, we identify lysines 381 and 382 as critical acetylation sites that prevent p53 from associating with chromatin, activating transcription, and inducing apoptosis in neurons. Finally, we make the significant finding

Received Nov. 8, 2012; revised Feb. 22, 2013; accepted March 18, 2013.

Author contributions: C.B., R.R.R., and B.L. designed research; C.B., G.D., M.A.R., K.M., and G.C. performed research; C.B., G.D., M.A.R., G.C., and B.L. analyzed data; C.B. and B.L. wrote the paper.

This work was supported by the New York State Spinal Cord Injury Research Program (Grant C019772), the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation, and the Burke Medical Research Foundation. We thank Dr. Dianna Willis, Dr. Marijose Metcalfe, and Rebecca Meyer for help and support in the preparation of this manuscript.

The authors declare no competing financial interests.

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DOI:10.1523/JNEUROSCI.5214-12.2013

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that this outcome is specific to neurons by showing that acetylation at lysines 381 and 382 has diametric effects in tumor cells, where it induces p53-dependent activation of the *PUMA* promoter, and cell death. Together, the results presented herein suggest that p53 acetylation can be manipulated to prevent neuronal death without compromising tumor suppression.

Materials and Methods

Plasmids and general reagents. Plasmids encoding shRNA targeting Puma (shPUMA) and a random nucleotide sequence (shCTRL) were a kind gift from Dr. L. Aminova (University of Illinois, Urbana-Champaign, IL) (Aminova et al., 2008). Plasmids containing sequences for human p53 R175H DNA-binding mutant, p53 R175H K320R, p53 R175H K373R, and p53 R175H K382R double mutants were a kind gift from Dr. H.-G. Wang (Penn State College of Medicine, Hershey, PA) (Yamaguchi et al., 2009). pPUMA-Frag1 was obtained from Addgene (plasmid 16591; Yu et al., 2001). pEGFP-C3 was obtained from Clontech (catalog #6082-1).

The following HDAC inhibitors were purchased: trichostatin A (TSA; Enzo Life Sciences); sodium butyrate (Sigma-Aldrich); camptothecin (CPT; Sigma-Aldrich); etoposide (Enzo Life Sciences).

Antibodies. The following antibodies were obtained: anti- β actin (AC-74; Sigma-Aldrich); anti-ATM (ab78; Abcam); anti-phospho S1981 ATM (MAB3806; Millipore); anti-COX IV (4844; Cell Signaling Technology); anti-cytochrome C (cytC; 456100; Invitrogen); anti-histone H4 (04-858; Millipore); anti-acetyl histone H3 (06-599; Millipore); anti-H2A.X (ab11175; Abcam); anti-phospho S139 H2A.X (05-636; Millipore); Anti-MAP2 (AB5622; Millipore); anti-p21 (556430; BD Pharmingen); anti-human p53 (DO-1; Santa Cruz Biotechnology); anti-mouse p53 (M-19; Santa Cruz Biotechnology); anti-acetyl K317 p53 (ab62704; Abcam); anti-acetyl K373 p53 (06-916; Millipore); anti-acetyl K379 p53 (2570; Cell Signaling); anti-acetyl K381 p53 (ab61241; Abcam); anti-phospho S15 p53 (9284; Cell Signaling Technology); anti-PUMA (3041; ProSci); anti-Sp1 (17-601; Millipore); anti- α tubulin (DM1A; Sigma-Aldrich); anti- β III tubulin (TJ-1; Covance).

Generation of p53 mutants by site-directed mutagenesis. Plasmids containing sequences for human p53 R175H DNA-binding mutant, p53 R175H K320R, p53 R175H K373R, and p53 R175H K382R were used as templates for site-directed PCR mutagenesis.

For each reaction, 50–100 ng of plasmidic template was mixed with 125 ng of each HPLC-purified primer, 10 μ M β ine (Invitrogen), 0.2 mM dNTP, and 2.5 units of Pfu Ultra High Fidelity (Stratagene). Cycling conditions were as follows: 3 min at 95°C, followed by 30 cycles of 30 s at 95°C, 1 min at 55°C and 1 min/kbp template at 65°C, and 5 min at 65°C. Mutagenesis products were immediately digested with 20 units of DpnI (BioLabs) for 90 min at 37°C. Two microliters of the reaction were used to transform heat shock competent Top10 bacteria following the manufacturer's protocol (Invitrogen), and positive clones were selected based on their resistance to ampicillin. Successful mutagenesis was confirmed by sequencing of the positive clones. Mutagenesis primers were designed as follows: K320Q (forward, CAGCCAAAGCAGAAACCACTGGATGGAGAATATTTCACCCCT; reverse, CAGTGGTTCTGCTTTGGCTGGGAGAGGAGCTGGTGTGTT); K373Q (forward, AAGTCCAAACAGGGTCAGTCTACCTCCGCCATAAAAACTC; reverse, AGACTGACCTGTTTGGACTTCAGGTGGCTGGAGTGAGCCCT); K381Q (forward, TCCCGCCATCAAAAATCATGTTCAAGACAGAAGGGCCTGAC; reverse, CATGAGTTTGTAGTGGCGGGAGGTAGACTGACCCCTTTTGA); K381R (forward, TCCCGCCATGAAAAATCATGTTCAAGACAGAAGGGCCTGAC; reverse, CATGAGTTTGTAGTGGCGGGAGGTAGACTGACCCCTTTT); K381R, K382R (forward, CGCCATAGAAGACTCATGTTCAAGACAGAAGGGCCTGAC; reverse, GAACATGAGTTGTTTATGGCGGGAGGTAGACTGACCCCTTTT); K381R, K382R (forward, CGCCATAGAAGACTCATGTTCAAGACAGAAGGGCCTGAC; reverse, GAACATGAGTTGTTTATGGCGGGAGGTAGACTGACCCCTTTT); K381R, K382R (forward, CGCCATAGAAGACTCATGTTCAAGACAGAAGGGCCTGAC; reverse, GAACATGAGTTGTTTATGGCGGGAGGTAGACTGACCCCTTTT).

Primary neurons and cell cultures. The HT22 murine hippocampal cell line was a kind gift from D. Schubert (Salk Institute, La Jolla, CA).

Figure 1. Pharmacological HDAC inhibition protects primary cortical neurons from DNA damage-induced death. **A**, Mouse p53^{+/+}, p53^{+/-}, and p53^{-/-} cortical neurons (E15.5) were treated with or without CPT (10 μ M) for 16 h. Neuronal viability was measured by MTT assay. Error bars indicate SD of mean from six independent experiments. ****** p < 0.01, significant protection compared with p53^{+/+} (one-way ANOVA followed by Dunnett's multiple-comparisons test). **B**, Neuronal viability measured by MTT assay after treatment of mouse primary cortical cultures (E15.5) with CPT (10 μ M) with or without TSA (0.67 μ M) for 16 h. Error bars indicate SD of mean from six independent experiments. ******* p < 0.001, significant difference between TSA- and non-TSA-treated groups; **###** p < 0.001, significant difference between CPT- and non-CPT-treated groups (two-way ANOVA followed by Bonferroni's test). **C**, Neuronal viability assessed by live/dead staining. Live cells are identified by green fluorescence (calceinAM); dead cells are identified by red fluorescence (ethidium homodimer, EthD-1). **D**, Neuronal viability measured by MTT assay after treatment of mouse primary cortical cultures (E15.5) with CPT (10 μ M) or etoposide (ETP; 10 μ M), with or without pan-HDAC inhibitor TSA (0.67 μ M) or the class-I HDAC inhibitor sodium butyrate (NaBu; 5 mM) for 16 h. Error bars indicate SD of mean from six independent experiments. ******* p < 0.001, significant difference between TSA- and non-TSA-treated groups or NaBu- and non-NaBu-treated groups; **###** p < 0.001, significant difference between CPT- and non-CPT-treated groups or ETP- and non-ETP-treated groups (two-way ANOVA followed by Bonferroni's test). **E**, Immunoblot analysis of phosphorylated H2AX (γ H2AX), total H2AX, phosphorylated ATM (pS1981-ATM), total ATM, phosphorylated p53 (pS15-p53), and total p53 (pan-p53) in neurons treated with CPT (10 μ M) with or without TSA (0.67 μ M) for 8 h. **F**, Immunocytochemical analyses of γ H2AX (red) and total H2AX (green) in neurons treated with CPT (10 μ M) with or without TSA (0.67 μ M) for 8 h. Anti-MAP2 (microtubule-associated protein 2; green) or anti-TUBB3 (Tubulin β -3; red) antibodies were used to define neurons. Nuclei were stained with DAPI (blue).

DLD-1 and HCT116 human cell lines were purchased from American Type Culture Collection. HT22 cells were propagated in DMEM, DLD-1 cells in RPMI-1640, and HCT116 cells in McCoy's 5a modified medium, all supplemented with 10% fetal calf serum and antibiotics (Invitrogen).

Figure 2. DNA damage and HDAC inhibition differentially regulate the transcription of known p53 target genes. **A**, Heat map depicting gene expression in mouse primary cortical neurons (E15.5) treated with CPT (10 μ M) with or without TSA (0.67 μ M) for 12 h. Shown are the 42 putative p53 gene targets that were significantly regulated in at least one condition compared with untreated control ($p < 0.05$). Expression profiles are displayed in descending order, based on induction level by CPT. **B–F**, Quantitative RT-PCR of select p53 gene targets in neurons from p53^{+/+} and p53^{-/-} mice treated with CPT (10 μ M) with or without TSA (0.67 μ M) for 8 h. Error bars indicate SD of mean from five independent experiments. ** $p < 0.01$, * $p < 0.05$, significant difference compared with untreated control; *** $p < 0.001$, significant difference compared with CPT alone (one-way ANOVA followed by Bonferroni's test); ### $p < 0.001$, significant difference compared with knock-out p53 (two-way ANOVA followed by Bonferroni's test).

All animal surgeries and euthanasia were performed according to Institutional Animal Care and Use Committee guidelines under approved protocols. Male and female animals were used in this study. Embryonic day 15.5 (E15.5) pregnant wild-type (WT) C57BL/6J and CD-1 mice were obtained from Charles River. The B6.129S2-Trp53tm1Tyj/J (p53 deficient; C57BL/6J background) mouse line was obtained from The Jackson Laboratory and bred in house. The p53-null neurons were obtained from embryos derived from heterozygous breeding. Genotyping of each embryo was performed according to The Jackson Laboratory protocol for this strain. Neuronal cultures were prepared from E15.5 mice cortices by the papain dissociation method, as described previously (Langley et al., 2008). Cultures from the cortex of fetal mice at this stage of development are nearly 90% neuronal, the balance being predominantly glial. All cultures were plated at a density of 1×10^6 cells/ml of Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen)

and Glutamax (Life Technologies). There was no significant difference in the basal viability of the cultures prepared from wild-type or p53-null embryos. All experiments described here were initiated 24 h after plating unless stated otherwise.

MTT (Promega) assay was performed as described by Langley et al. (2008). One-way or two-way ANOVA followed by the Dunnett's or Bonferroni's *post hoc* tests, respectively, were used to measure statistical significance. $p < 0.05$ was considered to be statistically significant. The effectiveness of the MTT assays in measuring cell viability was confirmed by using the Live/Dead assay (Invitrogen) and fluorescence microscopy.

Electroporations. Electroporations were performed using the Amaxa Mouse Neuron Nucleofector kit as directed by the manufacturer (Lonza). Neuronal survival and transfection efficiency were determined in a series of experiments with different cell densities and amounts of a plasmid encoding eGFP, using the O-005 program on the Amaxa Nucleofector II (Lonza). Conditions were optimal for 4×10^6 cells and 4 μ g of plasmid per electroporation, with a survival and transfection efficiency of 70 and 40%, respectively. To study neuronal morphology, images of random fields were captured 48 h after electroporation with the Axiocam MRM CCD camera (Carl Zeiss). Neurite length and number of processes were measured on cells expressing nontoxic p53 mutants and analyzed by using MetaMorph (Molecular Devices). No significant change was detected in terms of mean outgrowth per cell (eGFP, $89.4 \pm 8.21 \mu$ m; R175H, $80.76 \pm 7.90 \mu$ m; K381Q, $81.38 \pm 4.91 \mu$ m; K382Q, $74.55 \pm 6.92 \mu$ m) and number of processes (eGFP, 5.40 ± 0.78 ; R175H, 4.71 ± 0.87 ; K381Q, 4.74 ± 0.98 ; K382Q, 3.70 ± 0.57).

Protein purification, Western blotting, and immunocytochemistry. Protein lysates were prepared from cell cultures using RIPA buffer (Boston Bioproducts). Subcellular fractionation to isolate mitochondrial and cytosolic fractions was performed as described previously (Galli et al., 2008). Briefly, cells grown in 10 cm dishes were rinsed twice with $1 \times$ ice-cold PBS and centrifuged for 10 min at 5000 rpm, and pellets were resuspended in 100 μ l of ice-cold MSHE buffer (225 mM mannitol, 70 mM sucrose, 2 mM HEPES, and 1 mM EGTA). Cell suspensions were then passed 60 times through a blunted 26G needle and centrifuged

10 min at 3300 rpm. Supernatants, containing cytosolic and mitochondrial fractions, were collected and centrifuged for an additional 10 min at 3300 rpm. Cleared supernatants were then centrifuged for 20 min at 10,000 rpm. After centrifugation, the supernatants, which contain the cytosolic fraction, were transferred to clean microtubes. The pellets, which contain the mitochondrial fraction, were washed once in 200 μ l of MSHE buffer, centrifuged for 20 min at 10,000 rpm, and resuspended in 20 μ l of MSHE buffer. Immunoblot analysis was performed using a Li-Cor Odyssey system as described by Langley et al. (2008).

For immunocytochemistry, primary antibodies were used in conjunction with AlexaFluor 488- or 594-conjugated secondary antibodies (Invitrogen) for detection. Slides were mounted with ProLong antifade Gold reagent with DAPI (Invitrogen). Immunostaining was examined under a Carl Zeiss Axiocvert 200M microscope.

Quantitative RT-PCR. Total RNA preparation from cultured cells was performed as described by Langley et al. (2008). RNA-to-Ct one-step real-time PCRs were performed on total RNA as a duplex reaction using 6-carboxyfluorescein-labeled *Puma*, *p21*, *Gadd45a*, *Bax* and *Noxa* gene expression assays, and a VIC-labeled β -actin gene expression assay (Applied Biosystems).

Chromatin immunoprecipitation. Chromatin immunoprecipitations (ChIPs) were performed as described by Sleiman et al. (2011). Quantitative PCR was conducted with primers designed to amplify regions of the *Puma* promoter or the *p21* promoter, using SYBRGreen PCR Master-mix (Applied Biosystems). Promoter occupancy was determined relative to input. ChIP primers were designed as follows: *p21* promoter, p53RE (forward, CCTTTCTATCAGCCCCAGAGGATAC C; reverse, GACCCCAAAATGACAAAGTGAC AA); Sp1BS (forward, GTCTGGCGCGGGCTTA GA; reverse, GTCGAGCTGCCTCCTTAT). The *PUMA* promoter was designed as follows: p53RE (forward, GACTCAGTGCACCCTGGCGTGC; reverse, GACAAGTCGGGGCTTGCACTCG).

Promoter activity assays. Cells were transfected with pPUMA-Frag1 (Yu et al., 2001) and p53 plasmids using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Lysates were prepared using Luciferase passive lysis buffer (Promega) 5 h (neuronal cultures) or 24 h (cell lines) after transfection. Luciferase activity was measured using a Luciferase reporter assay system (Promega) and an LMax II 384 luminometer (Molecular Devices). Values were normalized to total protein concentration (Bradford assay; Bio-Rad).

Microarray analysis. Total RNA was extracted from mouse primary cortical neurons (E15.5) after treatments with CPT (10 μ M) with or without TSA (0.66 μ M) for 12 h, when the cells treated with CPT are committed to death. Three replicates were run per sample category. RNA was hybridized on Illumina Chips, and the results were analyzed as described previously (Sleiman et al., 2011). Quality assessment was achieved looking at the inter-array Pearson's correlation, and overall data coherence was measured by clustering based on top variant genes. Contrast analysis of differential expression was performed using the LIMMA package (Smyth et al., 2005). After linear model fitting, a Bayesian estimate of differential expression was calculated, and the threshold for statistical significance was set at $p < 0.005$. Data analysis was aimed at assessing the effect of CPT treatment on control cells and cells treated with TSA. Since our preliminary data suggested a role for p53 in CPT-induced neuronal death, we focused our study on established p53 targets (Riley et al., 2008) that were spotted on the array and significantly regulated in at least one condition.

Statistical analyses. One-way or two-way ANOVA followed by the Dunnett's or Bonferroni's *post hoc* tests were used to measure statistical significance. $p < 0.05$ was considered to be statistically significant. All experiments were performed a minimum of three times.

Results

HDAC inhibition protects primary cortical neurons from p53-dependent, DNA damage-induced apoptosis

HDACs are broadly neuroprotective *in vivo*, but the mechanisms underlying their beneficial effects remain unclear. Given the role of p53 in a number of neurodegenerative maladies, we used an *in vitro* model of neuronal DNA damage. In this model, cultured mouse primary cortical neurons are exposed to the topoisomerase-I inhibitor CPT, which results in the formation of double-strand breaks and p53-dependent apoptosis (Fig. 1A) (Morris and Geller, 1996). Treat-

Figure 3. Abrogation of PUMA induction by TSA is sufficient to prevent DNA damage-induced neurodegeneration. **A**, Immunoblot analysis of PUMA α and Cdkn1a (p21) in neurons treated with CPT (10 μ M) with or without TSA (0.67 μ M) for 8 h. **B**, Immunoblot analysis of CytC in cytosolic and mitochondrial fractions from neurons treated with CPT (10 μ M) with or without TSA (0.67 μ M) for 8 h. COX IV, a mitochondrial marker, was used to assess for fraction purity. **C**, Immunocytochemical analyses of CytC (red) in neurons treated with CPT (10 μ M) with or without TSA (0.67 μ M) for 8 h. Anti-MAP2 was used to detect neurons (green). Nuclei were stained with DAPI (blue). **D**, Immunoblot analysis of Puma to verify genetic knockdown in neurons transfected with plasmids encoding shPuma or shCTRL and treated with or without CPT (10 μ M) for 16 h. **E**, Neuronal viability in **D** was measured by MTT assay. Error bars indicate SD of mean from three independent experiments. *** $p < 0.001$, significant protection compared with shCTRL (one-way ANOVA followed by Bonferroni's test); ### $p < 0.001$, significant difference between CPT- and non-CPT-treated groups (two-way ANOVA followed by Bonferroni's test).

ment of neuronal cultures with CPT led to widespread cell death within 16 h (Fig. 1B,C). However, combined treatment with the pan-HDACi TSA stimulated the survival of nearly 80% of the culture (Fig. 1B,C; $p < 0.001$). TSA was also able to protect neurons from death induced by the structurally distinct DNA-damaging agent etoposide. Additionally, the class I HDAC-specific inhibitor sodium butyrate could protect neurons from CPT- and etoposide-induced apoptosis (Fig. 1D).

To identify the molecular events leading to protection, we examined the effect of HDAC inhibition at multiple steps of the DNA damage response. This approach revealed that cotreatment with TSA did not prevent CPT-induced phosphorylation of the histone variant H2AX (γ H2AX; Fig. 1E), which is known to occur within minutes of DNA damage and facilitate the accumulation of DNA repair and checkpoint proteins to the break site (Kinner et al., 2008). Furthermore, TSA had no effect on the chromatin distribution of γ H2AX or H2AX (Fig. 1F). Similarly, TSA did not prevent the activation of ataxia-telangiectasia mutated (ATM) by autophosphorylation on S1981 (S1987 in mouse; Bakkenist and Kastan, 2003), nor did it prevent the ATM-dependent phosphorylation of p53 on S15 (S18 in mouse; Fig. 1E). Consistent with the role of phosphorylation in p53 stabilization (Banin et al., 1998), TSA did not prevent p53 accumulation after CPT treatment (Fig. 1E). Together, these results suggest that HDAC inhibition mediates protection by regulating molecular events of the DNA damage response that are downstream of p53 stabilization.

TSA prevents the recruitment of p53 to Puma and p21 promoters in neurons

Since HDAC activity modulates gene expression by inducing or repressing transcription in a promoter-specific manner (Nusinzon and Horvath, 2005), we performed a microarray analysis of

Figure 4. HDAC inhibition by TSA abrogates DNA damage-induced binding of p53 to *Puma* and *p21* promoters, but it promotes the recruitment of Sp1 to the *p21* promoter. **A**, Schematic of *PUMA* gene. The human gene contains three coding exons (exons 2–4) and two noncoding exons (exons 1a and b), which are conserved in mouse. Two p53 response elements (p53RE) are found in the regulatory region of both human and mouse genes. The asterisk indicates PCR primer binding sites. **B**, **C**, ChIPs for p53 (**B**) and acetylated histone H3 (**C**) in extracts from mouse primary cortical neurons (E15.5) treated with CPT (10 μ M) with or without TSA (0.67 μ M) for 8 h. Promoter occupancy was determined by quantitative real-time PCR using primers specific for p53RE (defined in **A**). Error bars indicate SD of mean from three independent experiments. * $p < 0.05$, significant difference compared with untreated control (one-way ANOVA followed by Bonferroni's test). **D**, Schematic of *p21* (*Cdkn1a*) gene. The human gene contains two coding exons (exons 2 and 3) and one noncoding exon (exon 1), which are conserved in mouse. Two p53 response elements (p53RE) and six Sp1 binding sites (Sp1-BS) are found in the regulatory region of both human and mouse genes. Asterisks indicate PCR primer binding sites. **E**, **F**, ChIPs for p53, Sp1, and acetylated histone H3 in extracts from neurons treated with CPT (10 μ M) with or without TSA (0.67 μ M) for 8 h. Promoter occupancy was determined by quantitative PCR using primers specific for p53RE or Sp1-BS (defined in **D**). Error bars indicate SD of mean from three independent experiments. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, significant difference compared with untreated control; ### $p < 0.001$, significant difference compared with CPT (one-way ANOVA followed by Bonferroni's test).

the neuronal transcriptome after a 12 h treatment with CPT or CPT and TSA combined. A focused study on established p53-inducible targets (Riley et al., 2008) revealed 42 genes that were regulated in either condition, of which 20 were induced by CPT (Fig. 2A). Expression analyses by quantitative RT-PCR (qRT-PCR) in WT neurons confirmed the pattern of regulation identified in the microarray, whereby CPT induced the expression of the cell-cycle arrest genes *p21* and *Gadd45a*, which was not prevented by TSA (Fig. 2B,C; $p < 0.05$ and $p < 0.01$). CPT also induced two pro-apoptotic genes, *Puma* and *Bax* (Fig. 2D,E; $p < 0.01$). In contrast to *p21* and *Gadd45a*, CPT-induced *Puma* and *Bax* expression was abrogated by TSA. Interestingly, this effect of TSA was restricted to these two genes. Indeed, the CPT-induced expression of *Noxa*, an

other pro-apoptotic gene belonging to the BH3-only family, was enhanced by cotreatment with TSA (Fig. 2A,F; $p < 0.001$).

To confirm a direct role of p53 in the expression of *p21*, *Gadd45a*, *Puma*, *Bax*, and *Noxa*, qRT-PCRs were performed in p53-null cortical neurons treated with CPT with or without TSA (Fig. 2B–F). Surprisingly, the CPT-induced expression of *Noxa*, *Gadd45a*, and *Bax* was not altered in the absence of p53, which might reflect the contribution of other transcription factors of the p53 family, p63 and p73 (Flores et al., 2002). In contrast, the induction of *Puma* by CPT was completely lost in p53-null neurons ($p < 0.001$), and the expression of *p21* was significantly reduced ($p < 0.001$), although not suppressed, suggesting it may be coregulated by additional transcription factors. Immunoblot analysis of *Puma* and *p21* protein levels in WT neurons revealed the same regulation profile as their transcripts in response to DNA damage and HDAC inhibition (Fig. 3A).

The pro-apoptotic activity of *Puma* relies on its critical role in mitochondrial outer membrane permeabilization and cytochrome c release (Jabbour et al., 2009). Consistent with this, immunoblot and immunocytochemical analysis revealed that CPT induced the translocation of cytochrome c to the cytosolic fraction (Fig. 3B,C). As predicted by the finding that TSA abrogates *Puma* induction, cotreatment with TSA prevented the cytosolic translocation of cytochrome c (Fig. 3A–C). To confirm that *Puma* is a key mediator of apoptosis in neurons, cortical neurons were electroporated with plasmids encoding sh*Puma* or shCTRL and examined for survival after CPT treatment. Coinciding with *Puma* depletion (Fig. 3D), neurons were effectively protected from CPT-induced death (Fig. 3E; $p < 0.001$).

To understand how HDAC inhibition prevents the p53-dependent induction of *Puma* and *p21* in neurons after CPT treatment, ChIP analysis was performed. Determination of p53 binding at characterized

p53 response elements in *Puma* and *p21* promoters (Fig. 4A,D) demonstrated an increase in p53 occupancy after CPT treatment (Fig. 4B,E; $p < 0.05$ and $p < 0.001$). Consistent with promoter activation, ChIP analysis for acetyl-histone H3 showed higher levels of acetylation within these regions (Fig. 3C,F; $p < 0.05$ and $p < 0.01$). However, cotreatment with TSA disrupted the CPT-induced increase in p53 occupancy (Fig. 4B,E), as well as the increase in histone H3 acetylation (Fig. 4C,F). Given our observation that TSA treatment alone induced the expression of *p21* independent of p53 (Fig. 2B), we also examined Sp1 occupancy at well documented Sp1-binding sites (Fig. 4D). ChIP analysis showed that TSA enhanced Sp1 binding and histone H3 acetylation at the proximal region of the *p21* promoter (Fig. 4E,F; $p < 0.001$).

Together, our results suggest that the HDACi-mediated neuroprotection from DNA damage-induced death is mediated by the regulation of p53 transcriptional activity, whereby p53 binding to the *Puma* promoter is lost, leading to the suppression of *Puma* expression. Moreover, we show that whereas p53 induction of *p21* expression is similarly suppressed by HDAC inhibition, Sp1-dependent transcription was promoted.

DNA damage and HDAC inhibition differentially modulate p53 acetylation in neurons

Specific acetylation of p53 within its C-terminal region is known to regulate its stability and transcriptional activity, thereby facilitating active transcription of cell-cycle arrest, survival, or apoptotic gene programs (Olsson et al., 2007) (Fig. 5A). However, the effects of acetylation and deacetylation on p53 activity seem to be cell-type dependent, and little is known about their consequence in neurons.

To examine whether changes in p53 acetylation by HDACi might account for the loss of *Puma* expression, we examined acetylation changes in the C-terminal region of p53 in neurons treated with CPT and TSA (Fig. 5B). Immunoblot analysis determined that CPT treatment increased the level of p53 acetylated at K373 and decreased the level of p53 acetylated at K381. In contrast, TSA treatment increased the levels of p53 acetylated at K320, K381, and K382. When neurons were treated with both CPT and TSA, p53 acetylation levels were increased at all sites. Together, these results indicate that K373 acetylation and K381 deacetylation in p53 correlate with the DNA damage response and apoptosis in neurons, whereas acetylation of p53 at K320, K381, and K382 correlates with HDAC inhibitor-mediated neuroprotection.

The nuclear import or retention of p53 is essential for its normal function in growth inhibition (Knippschild et al., 1996) and induction of apoptosis (Ryan and Clarke, 1994). It is facilitated by the nuclear localization signal (NLS) located in the C-terminal part of the protein, between amino acids 305 and 322 (Liang et al., 1998). Since K320 lies within the NLS (Fig. 5A), we used immunocytochemistry to determine whether TSA alters the distribution of p53 in neurons (Fig. 5C). Consistent with p53 activation, the level of p53 phosphorylated at S15 was increased in the nucleus after treatment with CPT. TSA neither changed the level of phosphorylated p53 nor its localization. Instead, increased immunoreactivity for p53 acetylated at K320, K381, or K382 was localized to the nuclei of neurons treated with CPT and TSA, confirming that acetylation of these lysines does not prevent nuclear localization of p53.

Figure 5. CPT-induced DNA damage and HDAC inhibition by TSA differentially modulate p53 acetylation. **A**, Schematic of p53 protein. Human p53 contains 393 amino acids, organized into three functional domains, that are conserved in mouse. The N terminus includes the transactivation domain (TAD) and the proline-rich domain (PRD). The central region contains the DNA-binding domain (DBD). The C-terminal region includes the tetramerization domain of p53 (4D), the C-terminal regulatory domain (CTD), and nuclear localization and export signals (NLS and NES). Lysines in the C-terminal region can be acetylated, affecting p53 protein stability and function. **B**, Immunoblot analysis of acetylated K320-p53, acetylated K373-p53, acetylated K381-p53, and acetylated K382-p53 (respectively, K317, K370, K376, and K379 in mouse) in mouse primary cortical neurons treated with CPT (10 μ M) with or without TSA (0.67 μ M) for 8 h. **C**, Immunocytochemical analyses of phosphorylated p53 (pS15), acetylated K320-p53, acetylated K381-p53, and acetylated K382-p53 (Ack320, Ack381, Ack382; green) in neurons treated with CPT (10 μ M) with or without TSA (0.67 μ M) for 8 h. TUBB3 (Tubulin β -3; red) was used to define neurons. Nuclei were stained with DAPI (blue). **D**, Scheme showing the lysine-to-arginine (deacetylation-mimic) and lysine-to-glutamine (acetylation-mimic) human p53 mutants generated and used in this study. The circle represents lysine substitution with arginine or glutamine. **E**, Immunoblot analysis of human p53 shows expression of wild-type p53 (WT), DNA-binding mutant p53 (R175H), or the different p53 acetylation mutants in mouse cortical neurons. eGFP was used as an electroporation control.

Acetylation at lysines 381 and 382, but not 373, is sufficient to prevent the pro-apoptotic activity of p53 in neurons

To investigate a causative role of p53 acetylation on neuronal survival, we generated a series of p53 mutants bearing glutamine (Q; which mimics acetylated lysine) or arginine (R; which mimics deacetylated/nonacetylatable lysine) substitutions at lysines 320, 373, 381, and 382 (Fig. 5D). The ability of the p53 acetylation mutants to induce neuronal death was compared with a WT p53 and a DNA-binding-deficient p53 (R175H). All p53 constructs showed similar levels of expression (Fig. 5E), and all were detected in the nuclei of neurons (data not shown). As expected,

Figure 6. Acetylation of p53 on lysines 381 and 382 abrogates its ability to induce apoptosis in neurons. **A**, Neuronal viability measured by MTT assay of cortical neurons prepared from p53^{-/-} mice (E15.5) 48 h after electroporation with plasmid DNA encoding the DNA-binding mutant (R175H), WT, or different acetylation mutants of p53. Neurons expressing WT p53 were treated with or without TSA (0.67 μ M). eGFP was used as an electroporation control. Error bars indicate SD of mean from three independent experiments. *** p < 0.001, ** p < 0.01, * p < 0.05, significant difference compared with eGFP; ### p < 0.001, ## p < 0.01, # p < 0.05, significant difference compared with wild-type p53 (WT) (one-way ANOVA followed by Bonferroni's test). **B**, Viability of p53-null neurons assessed by live/dead staining under the conditions described in **A**. Live cells are identified by green fluorescence (calceinAM), whereas dead cells are identified by red fluorescence (ethidium homodimer, EthD-1). **C**, Viability of p53^{-/-} neurons measured by live/dead staining after electroporation with plasmid DNA encoding DNA-binding mutant (R175H), WT, or K381/382Q acetylation-mimic of p53 and treated with CPT (10 μ M). eGFP was used as a control.

overexpression of R175H was not toxic when compared with neurons expressing an eGFP control (Fig. 6A,B). In contrast, neurons overexpressing exogenous WT p53 displayed a significant 68% death (Fig. 6A; p < 0.001), which could be prevented by TSA treatment (p < 0.001). Consistent with our findings that DNA damage induces p53 acetylation at K373, the overexpression of the K373Q acetyl-mimic showed significant toxicity (Fig. 6A,B; p < 0.001), whereas the overexpression of the K373R non-acetylatable mutant was significantly less toxic than WT p53 (Fig.

Figure 7. Acetylation of lysines 381 and 382 abrogates the transactivating activity of p53 in neurons. **A**, Luciferase reporter assay of plasmid DNA encoding a fragment of the human PUMA proximal promoter (PUMA Frag1-Luc) in p53^{-/-} primary cortical neurons. Promoter reporter plasmid was cotransfected with DNA-binding mutant (R175H), WT, or different acetylation mutants of p53. WT p53-transfected neurons were treated with or without TSA (0.67 μ M). Error bars indicate SD of mean from five independent experiments. *** p < 0.001, significant difference compared with R175H; ### p < 0.001, # p < 0.05, significant difference compared with wild-type p53 (WT) (one-way ANOVA followed by Bonferroni's test). **B**, **C**, Chromatin immunoprecipitations for human p53 in extracts from mouse primary cortical neurons electroporated with p53 plasmids: R175H, WT, or the acetyl-mimics K381Q and K382Q. Promoter occupancy was determined by quantitative PCR using primers specific for p53RE (defined in Fig. 4A). Error bars indicate SD of mean from three independent experiments. *** p < 0.001; ** p < 0.01; * p < 0.05, significant difference compared with R175H (one-way ANOVA followed by Dunnett's multiple-comparisons test). **D**, **E**, Quantitative RT-PCR of Puma (**D**) and p21 (**E**) expression in neurons electroporated with p53 plasmids: R175H, WT or the acetyl-mimics K381Q and K382Q. Error bars indicate SD of mean from three independent experiments. * p < 0.05, significant difference compared with R175H (one-way ANOVA followed by Dunnett's multiple-comparisons test).

6A,B; p < 0.001). In contrast to K373, acetyl-mimic mutations of K320, K381, and K382, all of which are acetylated with TSA treatment and correlate with protection, significantly reduced the toxicity associated with p53 overexpression. Indeed, K320Q and

K381Q overexpression showed significantly less toxicity than that of WT p53 (Fig. 6A,B; $p < 0.01$). Furthermore, K382Q overexpression was not toxic, suggesting that acetylation at this site is sufficient to prevent p53 pro-apoptotic activity in neurons. Consistent with this, mutation of these lysines to the deacetylation-mimic arginine (K320R, K381R, and K382R) did not abrogate the induction of cell death (Fig. 6A,B). To study the effects of combinatorial acetylation, two additional mutants were generated, one bearing substitutions at K381 and K382 (K381/382R/Q) and one bearing substitutions at all of the C-terminal lysines (6KR/Q; Fig. 5D). Neurons overexpressing the K381/382 mutants displayed the same survival profile as those overexpressing the K382 mutants. Importantly, 6KQ overexpression was significantly less toxic than that of WT p53 (Fig. 6A,B; $p < 0.001$), indicating that K320Q, K381Q, and K382Q substitutions could overcome the pro-death activity of K373Q. Overexpression of the converse mutant 6KR was not different from that of the single mutant K373R, which highlights the role of K373 acetylation in p53-dependent neuronal death.

To study the role of p53 acetylation in the context of DNA damage-induced neuronal death, we studied the ability of the K381/382Q double mutant to restore apoptosis of p53-null neurons treated with CPT (Fig. 6C). Although significant death was apparent in WT p53-expressing neurons after CPT treatment, neither the DNA-binding mutant R175H nor the acetylation-mimic K381/382Q could mediate CPT-induced death, indicating the importance that specific acetylation of these lysines plays in TSA-mediated neuroprotection.

Given the role of *Puma* induction in CPT-induced neuronal death (Fig. 3E), the p53 acetylation mutants were screened for their ability to transactivate a *PUMA* promoter upstream of the firefly Luciferase gene. In p53-null neurons, overexpressed WT p53 activated the *PUMA* promoter by more than threefold compared with the DNA binding-deficient R175H (Fig. 7A; $p < 0.001$), which could be prevented by TSA treatment. The nonacetylatable mutants K320R, K373R, K381R, K382R, K381/382R, and 6KR, showed a decreased capacity to activate the *PUMA* promoter. Consistent with our survival data (Fig. 6A,B), the K373Q mutant showed a transactivation activity similar to that of WT p53. In contrast to this, K381Q, K382Q, or K381/382Q, which comprise the sites acetylated by HDAC inhibition, abolished the transactivating activity of p53. Likewise, the 6KQ acetylation-mimic was not able to transactivate the *PUMA* promoter (Fig. 7A), supporting the observation that K381Q and K382Q substitutions could overcome the pro-death activity of K373Q (Fig. 6A,B).

Figure 8. Acetylation of lysines 381 and 382 promotes the pro-apoptotic functions of p53 in proliferating tumor cell lines. **A**, HCT116, DLD1, and HT22 cells were transfected with an equal amount of plasmid DNA encoding wild-type human p53 (WT), DNA-binding mutant p53 (R175H), or the p53 acetylation mutants K373Q and K381/382Q. Immunoblot analysis was performed on total cell lysates 24 h after electroporation using antibodies specific for human p53. Plasmid DNA encoding eGFP was used as a transfection control. **B**, Luciferase reporter assay of plasmid DNA encoding a fragment of the human *PUMA* proximal promoter (*PUMA* Frag1-Luc) in human colorectal carcinoma cell lines HCT116 and DLD-1 and in mouse hippocampal cell line HT22. Promoter reporter plasmid was cotransfected with DNA-binding mutant p53 (R175H), wild-type p53 (WT), K373Q, or K381/382Q acetylation mutants of p53. Error bars indicate SD of mean from three independent experiments. *** $p < 0.001$, significant difference compared with R175H; ## $p < 0.01$, * $p < 0.05$, significant difference compared with WT (one-way ANOVA followed by Bonferroni's test). **C**, Cell viability measured by MTT assay of HCT116, DLD-1, and HT22 cultures 24 h after transfection with R175H, WT, K373Q, or K381/382Q acetylation mutants of p53. Error bars indicate SD of mean from three independent experiments. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, significant difference compared with R175H; ## $p < 0.01$, significant difference compared with WT (one-way ANOVA followed by Bonferroni's test). **D**, HCT116 cell death assessed by ethidium homodimer staining (EthD-1; red) 24 h after transfection with R175H, WT, K373Q, or K381/382Q acetylation mutants of p53.

To confirm these findings, which use a nonchromatinized promoter-reporter, we used ChIP analysis to examine R175H, WT-p53, K381Q, and K382Q binding at the endogenous *Puma* promoter. WT p53 showed greater occupancy compared with the DNA-binding deficient R175H mutant (Fig. 7B; $p < 0.001$). In contrast, K381Q and K382Q mutations prevented the association of p53 with the *Puma* promoter. Consistent with p53 binding, qRT-PCR analysis revealed a similar pattern, whereby WT p53 increased endogenous *Puma* expression relative to R175H, but K381Q and K382Q mutations prevented *Puma* upregulation (Fig. 7D; $p < 0.05$). Interestingly, and supporting our previous observation that HDAC inhibition prevents p53 occupancy at the *p21* promoter in neurons (Fig. 4E), these mutations also prevented p53 binding and subsequent induction of endogenous *p21*

Figure 9. HDAC inhibition does not prevent PUMA induction and DNA damage-induced death in HCT116 cells. **A**, Immunoblot analysis of pan-p53, phosphorylated p53 (pS15-p53), and acetylated p53 (AcK381-p53 and AcK382-p53) in extracts from HCT116 cells treated with CPT (20 μ M) with or without TSA (1.3 μ M) for 8 h. **B**, Quantitative RT-PCR of *PUMA* expression in HCT116 cells treated with CPT (20 μ M) with or without TSA (1.3 μ M) for 8 h. Error bars indicate SD of mean from three independent experiments. *** p < 0.001, significant difference compared with untreated control; ## p < 0.01, significant difference compared with CPT (one-way ANOVA followed by Bonferroni's test). **C**, HCT116 cell viability measured by MTT assay after treatment with CPT (20 μ M) with or without TSA (1.3 μ M). Error bars indicate SD of mean from three independent experiments. *** p < 0.001, ** p < 0.01, significant difference compared with untreated control; # p < 0.05, significant difference compared with CPT (one-way ANOVA followed by Bonferroni's test). **D**, HCT116 cell death assessed by ethidium homodimer staining (EthD-1; red) after treatment with CPT (20 μ M) with or without TSA (1.3 μ M).

expression, suggesting that they alter global, rather than specific, p53 transcriptional program in neurons (Fig. 7C,E).

Acetylation at lysines 381 and 382 promotes p53 pro-apoptotic activity in cancer cells

Our findings, thus far, demonstrate that when acetylated at lysines 381 and 382, p53 is severely defective for DNA binding, transcriptional activation, and induction of apoptosis in neurons. However, although p53 loss of function might be beneficial for the prevention of neuronal death, it increases the potential for cell transformation and cancer. This led us to investigate the consequence of acetylation at lysines 381 and 382 on the tumor suppressor function of p53 in two colorectal cancer cell lines, HCT116 and DLD-1, in which the p53-dependent induction of *PUMA* was initially characterized (Yu et al., 2001). We also used the immortalized murine HT22 cell line, which was derived from a primary embryonic hippocampal culture, as a model of proliferative cells of neuronal origin (Frederiksen et al., 1988).

The cell lines were transfected with wild-type, DNA-binding deficient, or acetyl-mimic mutants K373Q and K381/382Q (Fig. 8A). In all of the cell lines, promoter-reporter studies demonstrated that, similar to neurons, the overexpression of WT p53 and K373Q significantly activated the *PUMA* promoter compared with R175H (Fig. 8B; p < 0.001). However, overexpression of K381/382Q, which did not transactivate the *PUMA* promoter in neurons (Fig. 7A), increased reporter expression levels in all cell lines by threefold to fourfold compared with WT p53 (Fig. 8B; p < 0.001). These results suggest that acetylation at these sites, including K381/382, may also facilitate p53-induced apoptosis in HCT116, DLD-1, and HT22 cells. As expected, the overexpression of WT p53, K373Q, and K381/382Q induced death in all cell lines (Fig. 8C,D). Indeed, the overexpression of K381/382Q displayed significantly more toxicity than that of WT p53 (Fig. 8C; p < 0.01). Together, these results demonstrate that in proliferat-

ing cells, unlike postmitotic neurons, the acetylation of lysines 381 and 382 enhances the pro-apoptotic function of p53.

The opposing outcomes of acetylated p53 in neurons versus tumor cells are reminiscent of the neuroprotective versus anticancer properties of HDAC inhibitors. Thus, we examined the effect of HDAC inhibition on the stabilization and acetylation pattern of p53 in a cancer cell line. HCT116 cells express wild-type p53 and have been widely used as a model system to study the induction of *PUMA* and apoptosis by DNA damage-activated p53 (Yu et al., 2001). In agreement with our observations in neuronal cultures, treatment with CPT induced p53 stabilization and phosphorylation, which was not prevented by TSA (Fig. 9A). Conversely, TSA treatment alone did not induce stabilization or phosphorylation of p53 but increased the levels of p53 acetylated at K381 and K382 (Fig. 9A). TSA had no effect on K320 acetylation (data not shown). Since TSA treatment results in the acetylation of K381 and K382, we examined its effect on *PUMA* expression and survival in HCT116 cells cotreated with CPT. Quantitative RT-PCR analysis

showed that, like in neurons, CPT treatment increases *PUMA* expression in HCT116 cells (Fig. 9B; p < 0.001). However, unlike in neurons, cotreatment with TSA potentiated this induction (Fig. 9B; p < 0.01). In agreement with this, CPT-induced HCT116 cell death was not prevented, but enhanced, by cotreatment with TSA (Fig. 9C,D; p < 0.05).

Discussion

Together, neurological disease and injury represent the leading cause of disability worldwide. A number of studies suggest that p53 plays a central role in neuronal death associated with neurodegenerative disorders. Although there are clear data showing the involvement of p53 in the acute neuronal apoptosis observed in experimental models of epilepsy (Sakhi et al., 1996), ischemic stroke (Culmsee et al., 2003; Endo et al., 2006), and traumatic brain injury (Napieralski et al., 1999), the evidence implicating p53 in chronic neurodegeneration is mostly indirect. For instance, p53 levels have been found to be upregulated in the brain of patients suffering from Alzheimer's disease and Parkinson's disease (Esposito and Cuzzocrea, 2010), and neuronal DNA damage is one of the major features of degeneration in Huntington's disease (Jeon et al., 2012). Supporting the idea that p53 mediates neurodegeneration, deletion of p53 or administration of pifithrin α , a p53 inhibitor, confers neuronal protection in various models of brain injury and disease (Culmsee et al., 2003; Endo et al., 2006; Esposito and Cuzzocrea, 2010). Interestingly, a converging line of inquiry has similarly described *in vitro* and *in vivo* neuroprotection associated with inhibition of HDAC activity. Connecting this with the idea that p53 might be an appropriate target for neuroprotective strategies, Uo et al. (2009) have shown that HDACi prevents p53-dependent apoptosis in neurons. Using two models of p53-mediated neuronal death, DNA damage and p53 overexpression, the present data describe the mechanisms of HDACi-mediated neuroprotection. In primary

Figure 10. The acetylation pattern of p53 determines the fate of neurons in response to DNA damage and HDAC inhibition. **A**, In unstressed neurons, p53 C terminus is in a steady state of acetylation, resulting from the balance of the dose and activity of histone acetyltransferases, zinc-dependent HDACs, and NAD-dependent histone deacetylases (SIRT). **B**, DNA damage induced by camptothecin triggers p53 acetylation at lysine 373, which, along with p53 stabilization and phosphorylation at serine 15, promotes p53 binding to the pro-apoptotic *PUMA* promoter and leads to neuronal death. **C**, When DNA damage occurs in the presence of an HDAC inhibitor, DNA damage-induced lysine 373 acetylation occurs as in **B**, and lysines 320, 381, and 382 are acetylated. This acetylation pattern abrogates the ability of p53 to bind to the *PUMA* promoter and induce apoptosis in neurons but not in cancer cells.

mouse cortical cultures, which are nearly 90% neuronal, we show that global HDAC inhibition with TSA can prevent DNA damage-induced death by modifying the acetylation pattern of p53, which regulates its activity (Fig. 10).

The transcriptional potency and function of p53 are finely regulated by an integrated set of highly coordinated posttranslational modifications (Yang and Seto, 2008). It has been suggested that, in response to DNA damage, the phosphorylation of N-terminal serines (S15, S33, S37) promotes p53 stabilization and recruits p300/CBP-associated factor and PCAF to induce p53 acetylation at C-terminal lysine residues (Lambert et al., 1998; Sakaguchi et al., 1998). In agreement with this, CPT treatment of primary cortical cultures induced p53 stabilization, S15 phosphorylation, and K373 acetylation (Figs. 1E, 5B). Interestingly,

these events still occurred in the presence of an HDAC inhibitor, but their death-inducing effect was neutralized in neurons by the acetylation of additional lysine residues, suggesting a complex mode of regulation where precise modifications of p53 can counteract the effects of its stabilization and phosphorylation in a cell-type-specific fashion.

Heterologous expression of high levels of p53 has been shown to induce neuronal apoptosis (Uo et al., 2007), which can be prevented by HDAC inhibition (Fig. 6A,B). We used this model to identify the specific sites critical to the regulation of p53's activity. By overexpressing a collection of p53 acetylation mutants in neurons, we show that specific acetylation at lysine 382, and to a lesser extent at lysine 381, is sufficient for suppressing the transactivation of p53-dependent gene targets and promoting the survival of neuronal cultures exposed to DNA damage or death-inducing levels of p53 (Figs. 6A–C, 10). Furthermore, it had no measurable outcome on neuronal morphology (Fig. 6B). Interestingly, we show that the inhibitory effect of acetylation on p53 function is not promoter specific but seemingly disrupts the global transcriptional program of p53 by decreasing its DNA-binding affinity at both degenerate (*PUMA*) and highly conserved (p21) response elements (Weinberg et al., 2005; Figs. 4, 7). Remarkably, our observation that acetylation at K382 abrogates p53-dependent transcription in neurons is contrary to the current idea that acetylation at this site promotes p53's activity as a transcription factor. Indeed, acetylation of p53 at lysine 382 has been characterized to stimulate the recruitment of transcriptional coactivators, such as CBP (Mujtaba et al., 2004), TFIID (Li et al., 2007), or PC4 (Debnath et al., 2011), and enhance p53 transcriptional activity. Consistent with this, our studies in tumor cell lines show that acetylated p53, or K381/382 acetylmimic p53, displays enhanced *PUMA* promoter transactivation and proapoptotic activity (Figs. 8B,C, 10).

The finding that, in neurons, the same acetylation pattern of p53 at lysines 381 and 382 has opposite effects suggests that identical posttranslational modifications can have diametric outcomes in different cell types. In the context of our findings, it is possible that the acetylation status of K381 and K382, or even K382 alone, could regulate the recruitment of a neuron-specific, p53-interacting protein. For instance, apoptosis repressor with caspase recruitment domain, an endogenous inhibitor of apoptosis that is expressed primarily in terminally differentiated cells such as cardiac myocytes and neurons (Koseki et al., 1998), interacts directly with p53 to prevent tetramerization and inhibit p53-dependent gene expression (Foo et al., 2007). Another possibility is that the effect acetylation has on p53 differs depending on the presence of other modifications that make up p53's posttranslational code (Yang and Seto, 2008). In this instance, such modifications may be present or absent in postmitotic versus proliferating cells. Indeed, several recent studies have demonstrated a significant degree of cross talk between p53, E2F family members, and cell-cycle checkpoint kinases, which are active during the cell cycle (Polager and Ginsberg, 2009).

The fact that the pro-survival effects of p53 acetylation at lysines 381 and 382 are limited to neurons makes it an attractive target for neuroprotective strategies. Certainly, inhibiting p53 function for the treatment of neuronal injury or disease is thought to be a double-edged sword as p53 deficiency increases spontaneous tumor rates and enhances susceptibility to carcinogens. In fact, p53 inactivation is estimated to occur in over 50% of tumors, and many strategies in cancer therapy aim at reactivating p53 (Polager and Ginsberg, 2009). Remarkably, our study reveals that a single lysine, K382, in the C-terminal region of p53 can be

acetylated to provide all of the neuroprotective effects of p53 inactivation in neurons, without affecting its role as a tumor suppressor in cancer cells. Moreover, our findings suggest that acetylation at this site actually potentiates the pro-apoptotic activity of p53 in cancer cells (Fig. 8C), which is consistent with the multiple studies that describe enhanced cytotoxic effects of DNA-damaging agents when combined with HDACis such as TSA or Vorinostat (Piacentini et al., 2006; Sarcar et al., 2010).

In summary, our data support the rationale for the use of small-molecule inhibitors of HDAC activity as therapeutic tools for protection in neurological injury and disease. Importantly, it provides a molecular understanding of why HDAC inhibition can promote both neuronal survival and cancer cell death; two seemingly disparate processes. Finally, it suggests that strategies aimed at enhancing p53 acetylation at lysines 381 and 382 might foster the development of compounds that would display beneficial therapeutic effects in the CNS, without compromising tumor suppression.

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