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Inhibition of nucleolar transcription as a trigger for neuronal apoptosis

Katarzyna Kalita^{*,^}, Denys Makonchuk^{*}, Cynthia Gomes^{*}, Jing-Juan Zheng^{*}, and Michal Hetman^{*,#,1}

^{*}Kentucky Spinal Cord Injury Research Center and the Department of Neurological Surgery, University of Louisville, Louisville, Kentucky 40292 [#]Department of Pharmacology and Toxicology, University of Louisville, Louisville, Kentucky 40292 [^]Institute of Experimental Biology, 3 Pasteur St., 02-093 Warsaw, Poland

Abstract

In postmitotic neurons, the mechanisms of the apoptotic checkpoint that is activated by DNA damage remain unclear. Here we show that in cultured cortical neurons, the DNA damaging agent camptothecin (CPT) reduced transcription of rRNA and disrupted nucleolar staining for B23/nucleophosmin suggesting DNA damage-induced nucleolar stress. Although CPT activated the pro-apoptotic protein p53, the CPT-induced nucleolar stress was unaffected by p53 inhibition. In addition, BDNF-mediated protection from CPT-induced apoptosis prevented neither nucleolar stress nor p53 activation. Therefore, inhibition of rRNA transcription might be upstream of the pro-apoptotic p53 activity. Indeed, shRNA-mediated inhibition of a RNA-Polymerase-I co-factor, TIF-IA, attenuated rRNA transcription causing nucleolar stress and p53-dependent neuronal apoptosis. The protein synthesis inhibitor cycloheximide blocked apoptosis that was induced by overexpressed shTIF-IA or active form of p53. Also, the general transcription inhibitor actinomycin D triggered nucleolar stress and activated p53. However, it did not induce apoptosis except at the low concentration of 0.05 µg/ml with stronger inhibitory activity against nucleolar than extranucleolar transcription. Hence, nucleolar stress-activated apoptosis requires extranucleolar transcription. This study identifies the nucleoli of postmitotic neurons as sensors of DNA damage coupling reduced rRNA transcription to p53-mediated apoptosis that requires *de-novo* expression of protein-coding genes. Thus, rDNA selectivity of DNA damage may determine its ability to induce neuronal apoptosis.

Keywords

nucleolus; p53; DNA-damage; apoptosis; camptothecin; DNA-topoisomerase-I

INTRODUCTION

DNA damage in postmitotic neurons is proposed to contribute to neuronal loss and/or functional deficits in several neurological disorders including Alzheimer's disease, stroke and neurotoxicity of anti-cancer chemotherapy (Noussipiel & Hanawalt 2003, Fishel *et al.* 2007). Reduced gene transcription has been observed after neuronal DNA damage and suggested to contribute to functional decline in the aging brain or neurodegenerative

¹To whom correspondence should be addressed: KY Spinal Cord Injury Research Center, University of Louisville, 511 S. Floyd St., MDR616, Louisville, KY 40292. Tel.: 502-852-3619; Fax: 502-852-5148; michal.hetman@louisville.edu.

diseases (Lu *et al.* 2004). On the other hand, *de-novo* gene expression including transcription is required for apoptotic cell death that is induced by neuronal DNA damage (Martin *et al.* 1990, Morris & Geller 1996). Apoptosis of DNA-damaged neurons is mediated by the tumor suppressor transcription factor/cell death regulator p53 (Morrison & Kinoshita 2000, Jacobs *et al.* 2006). It is unclear whether transcriptional deficits and the p53-mediated apoptotic pathway represent separate or interacting modes of neuronal response to DNA damage.

Inhibitors of DNA topoisomerases, including the DNA topoisomerase-I poison camptothecin (CPT), induce apoptosis in proliferating cells and in post-mitotic neurons (Kaufmann 1998, Morris & Geller 1996). In cycling cells, CPT has been proposed to induce cell death through generation of DNA strand breaks during DNA replication (Kaufmann 1998). DNA-topoisomerase-I is enriched at sites of active transcription including nucleolus, where RNA-Polymerase-I (RNA-Pol-I) transcribes ribosomal DNA (rDNA) accounting for the major portion of the cellular transcription output (Muller *et al.* 1985). Moreover, in the actively transcribed genes including rDNA loci, CPT induces strand breaks blocking elongation of the transcripts (Zhang *et al.* 1988). Thus, it has been proposed that in neurons, CPT triggers DNA damage and apoptosis by inhibiting the DNA topoisomerase-I activity assisting transcription (Morris & Geller 1996). As neuronal apoptosis requires *de-novo* gene expression, it has been suggested that the CPT-mediated transcription block itself is an unlikely trigger for apoptosis of CPT-treated neurons (Morris & Geller 1996). Conversely, it is possible that a selective transcriptional impairment that is limited to a group of genes, such as rDNA, is a trigger for gene expression-dependent neuronal apoptosis. The possibility that DNA damage induces neuronal death by arresting transcription at the specific loci serving as sensors of genomic integrity implies that interventions aiming at their repair may offer effective neuroprotection.

In cycling cells, p53 orchestrates responses to DNA damage including cell cycle arrest, DNA repair and/or apoptosis (Meek 2004, Chipuk & Green 2006). Several intra-cellular events may activate p53 in proliferating cells including DNA strand breaks, replicative stress, inhibition of proteasome, blocked nuclear export, transcriptional inhibition and/or disruption of the nucleolus (Meek 2004, Derheimer *et al.* 2007, Chen *et al.* 2000, Rubbi & Milner 2003). The latter process has been proposed as a p53-activating mechanism in response to the inhibition of RNA-Pol-I-mediated rDNA transcription (Yuan *et al.* 2005, Rubbi & Milner 2003). While nucleolar disturbances are documented in degenerating neurons, their contribution to activation of p53 is unclear (Mann *et al.* 1988, Tomiwa *et al.* 1986, Valero *et al.* 2006, Anamizu *et al.* 2005).

In this study we tested a possibility that in postmitotic neurons, the specific inhibition of RNA-Pol-I mediated nucleolar transcription but not the general transcriptional block is a sensor of CPT-induced DNA damage that activates p53-mediated neuronal apoptosis.

MATERIALS AND METHODS

Materials

The following plasmids have been described previously: p53 DD (DN-p53) (Shaulian *et al.* 1992), p53 Val135 (TS-p53) (Michalovitz *et al.* 1990); pON260 (Cherrington & Mocarski 1989). Small hairpin RNA (shRNA) construct that was based on the pSUPER shRNA expression vector and targeting GFP was donated by Dr. Jacek Jaworski (International Institute of Molecular and Cell Biology, Warsaw, Poland). The following antibodies and reagents were obtained from commercial sources: anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA; and Novocastra Laboratories Ltd., Newcastle Upon Tyne, United Kingdom); anti-(phospho-Ser15)-p53 (Cell Signaling Technology, Danvers, MA); anti-B23

(Santa Cruz Biotechnology, Santa Cruz, CA); anti-BrdU (Sigma, St. Louis, MO); anti-phospho-Ser139-histone H2AX (γ -H2AX; Trevigen, Gaithersburg, MD), anti- β -galactosidase (anti- β -gal, MP Biomedicals, Solon, OH); anti- β -actin (Sigma, St. Louis, MO); anti-GAPDH (Chemicon, Temecula, CA); horse radish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG antibodies (Calbiochem, San Diego, CA); Alexa 488-, Alexa 555-conjugated secondary antibodies, and, Lipofectamine 2000 (Invitrogen, Carlsbad, CA); BDNF (Alomone, Haifa, Israel); camptothecin (CPT), actinomycin D (ActD), 5-Fluorouridine (5-FU), Dizocilpine maleate (MK-801), and, cycloheximide (CHX) (Sigma, St. Louis, MO).

Generation of shRNA expression constructs

To generate TIF-1A shRNA constructs, the rat TIF-1A mRNA (Rn3, gene bank accession number XM_001053401.1) sequence was analyzed using shRNA design software (<http://sonnhammer.cgb.ki.se/>). Three sequences corresponding to nucleotides 260–278; 260–278; 1619–1637, respectively, were selected. Comparison of mouse and rat mRNA sequences of TIF-1A demonstrated that the three sequences were completely conserved between these species. Oligonucleotides were designed (sequence 1: GATCCCCGACTTAGAGTTGTTGAAGATTCAA-GAGATCTTCAACAACCTCTAAGTCTTTTTGGAAA; sequence 2: GATCCCCGCACAGACTGTCT-TCCTTATTCAAGAGATAAGGAAGACAGTCTGTGCTTTTTGGAAA; sequence 3: GATCCCCGT-GTTCTGCTACACCATCATTCAAGAGATGATGGTGTAGCAGAACACTTTTTGGAA A) together with their complementary counterparts, annealed and subcloned into a pSUPER vector digested with BglII and HindIII (OligoEngine, Seattle, WA).

Cell Culture and Transfection

Cortical neurons were prepared from newborn Sprague-Dawley rats at postnatal day 1 as described (Habas *et al.* 2006). Briefly, the culture medium was Basal Medium Eagle (BME) supplemented with 10% heat-inactivated bovine calf serum (Hyclone, Logan, UT), 35 mM glucose, 1 mM L-glutamine, 100 U/mL of penicillin and 0.1 mg/mL streptomycin. Cytosine arabinoside (2.5 μ M) was added to cultures on the second day after seeding (day in vitro 2, DIV2) to inhibit the proliferation of non-neuronal cells. Cells were used for experiments at DIV 5–6 unless indicated otherwise. Transient transfections were performed on DIV3 or DIV4 using Lipofectamine 2000. Electroporation of freshly dissociated newborn rat cortical neurons was conducted using the rat neuron nucleofection reagents (Amaxa, Köln, Germany).

Drug Treatment

BDNF was diluted in phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) before addition to the cells. CPT, ActD, and MK-801 (dizocilpine) were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the medium was 0.2–0.4%.

Trophic Deprivation

Medium was removed from cultures and saved ("serum-containing conditioned medium"). Cells were washed twice with serum-free BME and then incubated in serum-free BME supplemented with an NMDA receptor blocker, dizocilpine maleate (MK-801, 10 μ M). Control cells were washed the same way and then incubated in the serum-containing conditioned medium.

RNA isolation and RT-PCR

RNA was isolated from 5×10^6 cells using TRI Reagent (Sigma). The remaining DNA was removed by digestion with DNase I (Promega). A 1 μ g aliquot of each DNA-free RNA preparation was reverse transcribed using AMV First-Strand cDNA Synthesis Kit (Invitrogen) with random hexamers or oligo dT and Avian Myeloblastosis Virus reverse transcriptase enzyme (RTase). As controls, mixtures containing all components except RTase were prepared and treated similarly. All cDNAs and control reactions were diluted 5 $\times$ with water before using as a template for Q-RT-PCR. Quantitative PCR was performed using Applied Biosystems 7900HT (Applied Biosystems, Foster City, CA). Briefly, cDNA or diluted control cDNA were added to master mix RT² Real-Time™ SYBR Green (SuperArray Bioscience Corporation, Frederick, MD). All cDNA samples were run in triplicates. Each run included negative controls of RNA processed without RTase (see above), to test for DNA contamination of RNA preparation. Oligonucleotide primers sequence used for rRNA analysis included 45S pre-rRNA forward: 5'-TGGGGCAGCTTTATGACAAC-3'; 45S pre-rRNA reverse: 5'-TAGCACCAAACGGGAAAACC-3'; 18S rRNA forward: 5'-GTTGGTTTTTCGGAAGTGGGC-3'; 18S rRNA reverse: 5'-GTCGGCATCGTTTATGGTCG-3'. Pre-rRNA and 18S rRNA levels were analyzed using $\Delta\Delta$ ct method ($2^{-\Delta\Delta ct}$). Expression values obtained from triplicate runs of each cDNA sample of 45S pre-rRNA were calculated relative to the triplicate value for the 18S rRNA from the same cDNA preparation.

Immunodetection of Nascent RNA

In situ labeling with 5-FU was performed using described methodology (Boisvert *et al.* 2000) with several modifications. For labeling experiments, neurons were cultured on glass coverslips in Neurobasal A/B27 medium (Invitrogen, Carlsbad, CA). At DIV5, cells were washed with culture media followed by placement in culture media containing with 5 mM 5-FU for 30 minutes. Cells were fixed with 4% paraformaldehyde for 20 min. Subsequently cells were permeabilized in PBS containing 0.5% Triton X-100 for 5 min followed by incubation with mouse anti-BrdU antibody (B-2351; Sigma; 1:200 in 5% donkey serum in PBS, 1hr). The anti-mouse IgG conjugated with Alexa 488 was used as a secondary antibody. To visualize nuclei, cells were counterstained with Hoechst-33258. After washing in PBS, coverslips were mounted in glycerol gelatin (Sigma) containing p-phenyldiamine (Sigma) to reduce photobleaching.

Immunofluorescence and immunohistochemistry

Immunostaining for β -gal was performed as described (Hetman *et al.* 1999). For γ -H2AX immunostaining, rabbit anti- γ -H2AX was used (Trevigen, Gaithersburg, MD). After fixation and 60-min. treatment with blocking solution (10% normal goat serum (NGS) in PBS-0.2% Triton-X100), primary antibody (1:300) was added in 1% NGS/PBS/0.2% Triton-X100 and incubated overnight at 4°C. For staining of endogenous B23 (1:500, Santa Cruz), neurons were fixed with ice-cold 4% paraformaldehyde in PBS for 20 min. After fixation, cells were incubated in 0.5 % IPEGAL for 5 min. then with blocking solution (3% BSA in PBS) for 1h. Primary antibody was applied in blocking solution overnight at 4°C. Following primary antibody incubations, standard indirect immunofluorescence protocol was used. To visualize nuclei, cells were counterstained with Hoechst-33258. After washing in PBS, coverslips were mounted in glycerol gelatin (Sigma) containing p-phenyldiamine glycerol.

Quantification of Apoptosis

To evaluate nuclear morphology, cells were stained with Hoechst-33258 and observed using fluorescent microscopy. Cells with condensed or fragmented nuclei were scored as

apoptotic. At least 150 transfected or 300 non-transfected cells were analyzed for each condition in each experiment.

Western Analysis

Western blotting was performed using standard procedures. The primary antibodies were as follows: anti-p53 (1:500, Santa Cruz Biotechnology, or, 1:500, Novocastra Laboratories Ltd); anti-phospho-Ser15-p53 (1:1000, Cell Signaling Technology), anti- β -actin (1:1000; Sigma); secondary antibodies were HRP-conjugated.

Image acquisition and analysis

Cells were visualized with a Nikon D-Eclipse C1 confocal microscope using oil immersion 20 $\times$ or 60 $\times$ lenses and digitized pictures were captured using EZ-C1 software (Nikon) followed by conversion to TIFF files. The figures were assembled and labeled using Adobe Photoshop and Corel software. Fluorescence intensity of B23 staining was quantified using the Image J software. The strength of the B23 staining, limited to the nucleolus, was automatically selected with the "Threshold" function of the Image J program. Fluorescent intensity of nucleolar B23 was measured by multiplying the mean nucleolar gray value by the nucleolar area and presented as a fold of vehicle-treated neurons. At least 40 cells were analyzed for each condition.

Statistical Analysis

Statistical analysis of the data was performed using one-way analysis of variance (ANOVA) followed by *post hoc* comparison.

RESULTS

CPT-induced DNA strand break response, apoptosis and inhibition of RNA-Pol-I-mediated transcription

To test the possibility that the genotoxic drug CPT induces neuronal apoptosis by transcriptional inhibition, primary cortical neurons from newborn rats were treated with 5 μ M CPT. Ninety min. after adding CPT, a focal intranuclear increase of histone-2AX phosphorylation at Ser-139 residue (γ -H2AX) was observed (Fig. 1A). The γ -H2AX-positive foci indicate repair response at the sites of DNA strand breaks (Rogakou *et al.* 1999) suggesting that CPT induced DNA damage in postmitotic neurons. In concert with previously published results with cultured cortical neurons from newborn rats, the apoptotic response was well established after 24- but not 4- or 8 hour treatment with CPT (Fig. 1B) (Hetman *et al.* 1999).

To study effects of CPT on transcription an *in-situ* run-on assay was performed using a halogenated RNA precursor, 5-fluorouridine (5-FU). Living neurons were pulse labeled with 5-FU and its incorporation into nascent RNA was visualized by immunostaining with an anti-bromo-deoxyuridine (BrdU) antibody. In control cells, a 30 minute pulse labeling with 5-FU resulted in a fine-granular staining scattered throughout the nucleus (Fig 1C). The most intense staining was found in larger intranuclear structures whose rounded shape and number (1–3 per nuclear profile) suggested nucleolar identity (Fig 1C). This is consistent with nucleoli being the sites of the most active RNA synthesis (Boisvert *et al.* 2000). The concentrated nucleolar stainings was absent from neurons that were treated with a general transcription inhibitor, actinomycin D (ActD, 0.05 μ g/ml) for 1, 4 or 8 hours prior to 5-FU pulses (Fig. 1C, D). The inhibition of extranucleolar labeling increased over these time points. At 8 hours, the 5-FU labeling was almost undetectable (Fig. 1C). Hence, at the low concentration of 0.05 μ g/ml, ActD inhibits RNA-Pol-I more potently than RNA-Pol-II/III.

These results indicate specific labeling of newly synthesized RNA at the sites of RNA-Pol-I or RNA-Pol-II/III-mediated transcription.

Neurons that were treated with 5 μ M CPT for 1, 4 or 8 hours prior to 5-FU pulses demonstrated reduced nucleolar RNA synthesis (Fig. 1C, D). At each of these time points, at least 80% of vehicle-treated neurons displayed newly synthesized RNA in nucleoli. Conversely, the nascent nucleolar RNA was found in less than 31% of CPT-treated cells (Fig. 1D). Unlike ActD, CPT did not appear to block the extranucleolar RNA synthesis (Fig. 1C). Hence, inhibition of neuronal DNA topoisomerase I produced relatively selective impairment of RNA-Pol-I-driven transcription.

To further support the possibility that in neurons, CPT inhibits transcription mediated by RNA-Pol-I, we determined CPT effects on the levels of 45S pre-rRNA. Since 45S pre-rRNA is rapidly processed into mature 5S, 18S and 28S rRNAs, the levels of 45S pre-rRNA indicate activity of RNA-Pol-I (Mayer *et al.* 2005). Indeed, we observed a dramatic reduction of pre-rRNA expression in ActD-treated neurons (Fig. 1E). Decreased levels of pre-rRNA were also found in neurons that were treated with 5 μ M CPT (0.57-, 0.28- or 0.18- fold of controls at 4, 8 or 14 hours of CPT exposure, respectively, $p < 0.001$, Fig. 1E). Thus, the CPT-induced decrease of pre-rRNA expression further supports the notion that the inhibition of DNA topoisomerase-I disrupts nucleolar transcription in neurons.

Nucleolar stress in CPT-treated neurons

In non-neuronal cells, inhibition of RNA-Pol-I-driven transcription disrupts morphological and functional organization of the nucleolus resulting in a state of nucleolar stress (Rubbi & Milner 2003). Loss of B23/nucleophosmin from the nucleolus is a marker of nucleolar stress following RNA-Pol-I inhibition (Rubbi & Milner 2003, Yuan *et al.* 2005). In our hands too, strong nucleolar staining for B23 in controls disappeared after neuronal exposure to ActD (Fig. 2A, B). In ActD-treated neurons, diffused B23 staining appeared throughout the nucleus indicating translocation from the nucleoli to the nucleoplasm (Fig. 2A). Thus, disruption of nucleolar B23 is a marker of nucleolar stress in neurons.

In neurons that were treated with 5 μ M CPT for 4, 8, or 14 hours, nucleolar B23 staining was reduced (Fig. 2A, B). While B23 disappeared from the nucleoli, an increase of uniformly distributed B23 immunoreactivity was observed in the nuclei suggesting translocation to the nucleoplasm (Fig. 2A–C). Importantly, the reduction of nucleolar B23 preceded CPT-induced apoptosis as nuclear counterstaining with Hoechst 33258 revealed that at 4, 8 or 14 hours, loss of nucleolar B23 occurred in cells without apoptotic rearrangements of the chromatin (Fig. 2C and data not shown). Finally, in neurons exposed to trophic deprivation (TD), B23 remained in the nucleoli (Fig. 2A, B). Therefore, the DNA-damaging CPT but not TD induced nucleolar stress in pre-apoptotic neurons.

CPT-induced nucleolar stress is independent of p53

The transcription factor p53 is an important transducer of DNA damage responses both in proliferating cells and in postmitotic neurons (Morrison & Kinoshita 2000, Jacobs *et al.* 2006). In concert with previous reports from other neuronal populations, the p53 was activated in CPT-treated cultured cortical neurons from newborn rats (Fig. 3A). The maximum activation was observed at the pre-apoptotic time points of 4 and 8 hours coinciding with nucleolar stress (Fig. 3A and Fig. 2). The association of p53 activation with nucleolar dysfunction is further supported by the absence of p53 activation following TD (data not shown). Therefore, we used a dominant-negative mutant form of p53 (DN-p53) to determine whether p53 is required for the CPT-induced nucleolar stress. We employed human p53 lacking amino acids 15–301 (p53-DD). Upon overexpression, this protein

inhibits p53 activity while stabilizing the endogenous wild type p53 (Shaulian et al. 1992). Indeed, neurons transfected with p53-DD had increased levels of endogenous p53 (Fig. 3B). However, this DN-p53 did not prevent CPT-induced disruption of nucleolar B23 indicating that p53 activation was not necessary for RNA-Pol-I inhibition/nucleolar stress (Fig. 3C). On the other hand, DN-p53 protected CPT- but not TD-treated neurons from apoptosis (Fig. 3D–F). Thus, activation of p53 may be downstream of CPT-induced nucleolar stress. Alternatively, p53 and nucleolus may be involved in unrelated signaling pathways.

BDNF-mediated neuroprotection does not prevent nucleolar stress

The neurotrophin BDNF suppresses CPT-induced apoptosis of primary cortical neurons (Hetman et al. 1999) (Fig. 4A). The mechanism(s) of BDNF-mediated neuroprotection against CPT remains unclear. Therefore, we determined whether BDNF can prevent CPT-induced RNA-Pol-I inhibition/nucleolar stress. At 8 hours after CPT exposure, the loss of nucleolar B23 was not affected by BDNF (Fig. 4B). Also, BDNF neuroprotection did not reverse the inhibition of RNA-Pol-I transcription caused by CPT (Fig. 4C). Finally, BDNF did not inhibit the CPT-mediated, pro-apoptotic activation of p53 (Fig. 4D). These data suggest that in CPT-treated neurons, BDNF inhibits apoptosis downstream of p53 and nucleolar stress. Also, our results indicate that CPT-induced RNA-Pol-I inhibition/nucleolar stress are not the effects of DNA damage-induced apoptosis. Therefore, CPT-triggered transcriptional inhibition and/or nucleolar dysfunction appear as candidate initiators of the p53-mediated apoptosis.

Transcriptional inhibition as a trigger of nucleolar stress and p53-mediated neuronal apoptosis

To directly test the interesting possibility that, in neurons, a block of RNA-Pol-I-driven transcription is an activator for the p53-mediated cell death, we developed three shRNA expression plasmids to target a specific co-factor for RNA-Pol-I, TIF-IA (shTIF-IA). To validate these constructs, we determined their effects on nucleolar transcription and nucleolar staining for B23. Neurons were transfected with the equimolar mix of shTIF-IA plasmids or the control shRNA (shGFP) together with the expression plasmids for β -gal and the DN-p53. The DN-p53 was included because previous studies in proliferating cells demonstrated that the interference with RNA-Pol-I-driven transcription induces p53-mediated cytotoxicity that could prevent the conclusive validation of the shTIF-IA activity (Rubbi & Milner 2003, Yuan et al. 2005). After 48 hours, newly synthesized rRNA was found in 80% or 10% of shGFP- or shTIF-IA-transfected neurons, respectively ($p < 0.001$, Fig. 5A, B). These results indicate that shTIF-IA specifically disrupted neuronal RNA-Pol-I activity. Consistently with this notion, most shTIF-IA but not shGFP-transfected neurons displayed negative nucleolar staining for B23 (Fig. 5C). If the pooled shTIF-IA plasmids were expressed for 48 hours without DN-p53, they induced apoptosis whose morphological features included chromatin condensation and fragmentation (Fig. 5D). Also, transfection of the individual shTIF-IAs induced similar apoptotic responses at 48 or 72 after plasmid delivery (Fig. 5E). For instance, at 48 hours, there was 19% or 50% apoptosis in neurons receiving control shRNA (shGFP) or the shTIF-IA-1, respectively (Fig. 5E). The shTIF-IA-induced apoptosis was blocked by the DN-p53 (9% or 60% with or without DN-p53, respectively, $p < 0.001$, Fig. 5F). Thus, the selective inhibition of RNA-Pol-I-driven transcription is sufficient to initiate p53-mediated apoptosis of cultured cortical neurons.

Apoptosis induced by transcriptional inhibition requires de-novo expression of protein-coding genes

Our data suggest that inhibition of RNA-Pol-I-driven transcription contributes to the activation of p53-regulated apoptosis in DNA-damaged neurons. The pro-apoptotic activity of p53 in neurons has been proposed to be a consequence of a p53-regulated gene expression

program (Morrison & Kinoshita 2000, Jacobs et al. 2006). On the other hand, p53 may also induce apoptosis in a gene/protein expression-independent manner (Chipuk & Green 2006). Therefore, we applied a translation inhibitor, cycloheximide (CHX) to test if p53-regulated apoptosis of RNA-Pol-I-inhibited neurons requires protein synthesis.

Twenty-four hours after transfection with shTIF-1A, neurons were treated with CHX for the next 24 hours. CHX reduced the shTIF-1A-induced apoptosis indicating its dependence on new protein synthesis (55% or 21% or 20% apoptosis with 0 or 1 or 3.5 μ M CHX, respectively, Fig. 6A).

To directly assess whether protein synthesis is needed for p53-induced neuronal apoptosis, neurons were transfected with an expression vector for a temperature-sensitive mutant form of p53 (TS-p53). For the next 48 hours, cells were kept at the non-permissive temperature of 37°C which favored the dominant-negative conformation of TS-p53. Then, the temperature was lowered to 32°C restoring the wild type activity of the overexpressed TS-p53. After 8 hours at 32°C, the expression of TS-p53 was detected by western blotting (Fig. 6B). After 24 hours at 32°C, neurons receiving the empty vector or TS-p53 displayed 20% or 50% apoptosis, respectively, indicating that the selective activation of p53 is sufficient to induce neuronal apoptosis (Fig. 6C). If the temperature shift was in the presence of CHX, apoptosis of TS-p53-transfected neurons was reduced (20 or 10% apoptosis after 1 or 3.5 μ M CHX treatment, respectively; Fig. 6C). These results suggest that p53-regulated neuronal apoptosis requires *de-novo* protein synthesis to express p53-regulated killer genes.

To test whether extranucleolar transcription may be involved in nucleolar stress-induced neuronal apoptosis we used the general transcriptional inhibitor ActD. While ActD is a non-selective transcriptional blocker, it is relatively more potent against RNA-Pol-I-driven transcription. For instance, at the low concentration of 0.05 μ g/ml, the complete inhibition of rRNA transcription appeared before the shutdown of extranucleolar transcription (Fig. 1C). Also, ActD induced nucleolar stress and activated p53 (Fig. 2A–B, and, 6D, respectively). The pattern of p53 activation was similar to that caused by CPT (Fig. 6D and 3A). We also determined whether ActD can induce neuronal apoptosis. After 24 hour treatment with several concentrations of ActD, apoptosis was moderately increased at the low concentration of 0.05 μ g/ml (15% vs. 32% with 0 or 0.05 μ g/ml ActD, respectively, $p < 0.01$; Fig. 6E). No apoptotic response was found after 24- or 48 hour ActD exposure at any higher concentration tested indicating that ActD-mediated inhibition of extranucleolar transcription blocked pro-apoptotic effects of ActD-induced nucleolar stress (Fig. 6E and data not shown). Therefore, inhibition of RNA-Pol-I-driven transcription activates a p53-dependent death program that likely involves RNA-Pol-II-mediated induction of genes encoding pro-apoptotic proteins (Fig. 6F).

DISCUSSION

We showed that in postmitotic primary cortical neurons, the DNA topoisomerase-I inhibitor CPT blocked RNA-Pol-I-mediated transcription resulting in nucleolar stress and the activation of p53-mediated apoptosis. We also demonstrated that inhibition of RNA-Pol-I was sufficient to activate p53-mediated neuronal death. Therefore, we identified a novel mechanism that, in postmitotic neurons, links neuronal DNA damage to the pro-apoptotic activity of p53.

Block of RNA-Pol-I-mediated transcription as a mechanism of p53 activation

Our results indicate that inhibition of RNA-Pol-I-mediated transcription is sufficient to activate p53 in postmitotic neurons. This is consistent with several observations in proliferating cells. In those systems, either exposure to the non-specific inhibitor of RNA-

Pol-I, ActD or microinjection of an antibody against an essential co-factor of RNA-Pol-I, UBF or, genetic deficiency of another RNA-Pol-I co-factor, TIF-IA induced accumulation of p53 (Rubbi & Milner 2003, Yuan *et al.* 2005). Therefore, the link between RNA-Pol-I-driven transcription and p53 activation is a common feature of proliferating cells and terminally differentiated postmitotic neurons. In addition, our results further support the existence of a nucleolar checkpoint that activates p53 after transcriptional inhibition (Rubbi & Milner 2003).

Several features of RNA-Pol-I-driven transcription make it a good candidate for a genome integrity checkpoint. The RNA-Pol-I-driven transcription provides a major contribution to the total transcriptional output of the cell (Grummt 2003). The rate of rDNA transcription is reduced by various forms of DNA damage and recovers after the damage is repaired (Kruhlak *et al.* 2007, Zhang *et al.* 1988). The 45S pre-rRNA is encoded by similarly organized and regulated rDNA loci whose high copy number (several hundreds/genome) and constitutively high transcription rate provide a consistent indicator of genomic integrity (Grummt 2003). Lastly, as rRNA is expressed in both terminally postmitotic and actively dividing cells, the nucleolar transcription checkpoint may be independent of the ability to enter the cell cycle (Grummt 2003).

Currently, it is unclear how nucleolar stress activates p53 in neurons. The levels of p53 are regulated through nuclear ubiquitination by MDM2, followed by nuclear export and proteasome-mediated degradation (Meek 2004, Jacobs *et al.* 2006). Several nucleolar proteins including ARF, B23, or L11 may disrupt MDM2-mediated degradation of p53 (Pomerantz *et al.* 1998, Colombo *et al.* 2002, Lohrum *et al.* 2003). The nucleolar transcription block may trigger the release of these inhibitors to the nucleoplasm where MDM2/p53 interactions occur (Yuan *et al.* 2005). Alternatively, MDM2 may be trapped in the nucleolus and/or nucleolar disorganization may result in inhibition of MDM2-regulated p53 export to the cytosol (Rubbi & Milner 2003). Finally, the DNA damage-activated ATM that directly regulates p53 may also directly inhibit RNA-Pol-I (Kruhlak *et al.* 2007). As in neurons, ATM activates p53 (Keramiris *et al.* 2003), nucleolar stress may act as an amplifier for the ATM-p53 signaling to initiate neuronal apoptosis. It is unclear, whether other modulators of neuronal p53 activity including calpains or NFκ-B contribute to the apoptotic checkpoint at the nucleolus (Sedarous *et al.* 2003, Aleyasin *et al.* 2004). The mechanistic links between the DNA damage-induced disruption of neuronal nucleoli and p53 remain to be investigated.

Transcriptional inhibition as a trigger for p53-mediated neuronal apoptosis

Neuronal apoptosis including the p53-mediated apoptotic response to CPT is blocked by transcription or translation inhibitors (Morris & Geller 1996, Hetman *et al.* 1999). Therefore, *de-novo* expression of protein coding genes, whose transcription is mediated by RNA-Pol-II, is required for DNA-damage-induced neuronal apoptosis. Consistent with this notion, we demonstrated that neuronal DNA damage selectively blocked RNA-Pol-I-driven transcription while activating p53-dependent apoptosis that was sensitive to protein synthesis inhibition. The p53-mediated neuronal death has been associated with p53 ability to induce several killer genes including *Bax*, *Puma*, *Noxa* or *APAF1* (Morrison & Kinoshita 2000, Jacobs *et al.* 2006). On the other hand, the transcription-independent killer activity of p53 has also been identified but its contribution to neuronal apoptosis remains to be investigated (Chipuk & Green 2006).

The critical role of gene expression for neuronal apoptosis that is induced by inhibition of RNA-Pol-I transcription indicates the requirement for ongoing RNA-Pol-II-driven transcription. Therefore, DNA damage that activates apoptosis through this mechanism would be relatively selective for rDNA loci sparing the RNA-Pol-II-regulated genes. In the

case of topoisomerase inhibitors, such selectivity may be explained by the nucleolar enrichment of DNA topoisomerase I and II (Muller et al. 1985, Tsutsui *et al.* 2001). In our hands, CPT blocked nucleolar, but not extranucleolar transcription (Fig. 1C). In addition, ActD which caused general transcriptional inhibition induced nucleolar stress and activated p53, while inducing moderate apoptosis at the low concentration of 0.05 $\mu\text{g/ml}$ but not at any higher concentration tested (Fig. 1C, Fig. 2A–B, and, Fig. 6D–E). Also, at the pro-apoptotic concentration, ActD completely inhibited nucleolar transcription before it shut off extranucleolar transcription (Fig. 1C). Therefore, in neurons, the apoptotic response to DNA damage may be determined by the relative selectivity of a damaging agent towards rDNA. Only such a selective injury would permit the execution of p53-regulated death program that is dependent on *de-novo* gene expression.

The mechanisms linking RNA-Pol-I transcriptional inhibition/nucleolar stress to neuronal death may also engage p53-independent events including direct activation of the effector killer protein Bax (Kerr *et al.* 2007). It is possible that this or a similar mechanism may play a role in the non-apoptotic death of DNA damaged-neurons with inhibited p53 (Lang-Rollin *et al.* 2003).

Role of nucleolar stress in neurodegeneration

Our results indicate that the DNA topoisomerase-I poison CPT and the transcriptional inhibitor ActD induce RNA-Pol-I inhibition and nucleolar stress. Our results are in good agreement with previous reports of nucleolar dysfunction/disorganization observed in neurons from the peripheral nervous system in rodents that were treated with the DNA intercalating agent, cisplatin (Tomiwa et al. 1986). In addition, the nucleolar stress has been documented in degenerating neurons of mouse *pcd* or *klotho* mutants (Valero et al. 2006, Anamizu et al. 2005). Finally, reduced nucleolar volume indicative of RNA-Pol-I inhibition has been reported at the early stages of Alzheimers's disease (Mann et al. 1988). While neither the cause nor the consequences of nucleolar malfunction in degenerating neurons are clear, our results suggest the interesting possibility that DNA damage and p53-dependent cell death may be upstream and downstream of neurodegeneration-associated nucleolar dysfunction, respectively.

In addition to p53-mediated apoptosis, there may be other possible effects of nucleolar dysfunction. For instance, reduced biogenesis of ribosomes and impaired translation would likely follow prolonged nucleolar stress. Interestingly, at the early stages of Alzheimer's disease a marked decrease in translation capacity has been observed (Ding *et al.* 2005). Thus, it is tempting to speculate that in degenerating neurons, nucleolar stress might contribute to the impairment of protein synthesis.

Taken together, we showed the existence of the nucleolar transcription checkpoint that activates p53-mediated neuronal apoptosis of DNA topoisomerase-I-inhibited neurons. Hence, we identified a novel mechanism that controls survival of postmitotic neurons challenged by DNA damage. Our results also indicate that, similarly to telomers (Cheng *et al.* 2007), rDNA loci may be a good target for future neuroprotective strategies that employ genomic repair. Finally, as nucleolar stress is not prevented by apoptotic inhibition, an important question for future studies is that of the non-apoptotic consequences of nucleolar dysfunction in neurons.

Abbreviations

ActD	actinomycin D
ATM	ataxia telangiectasia mutated

BDNF	brain-derived neurotrophic factor
β -gal	β -galactosidase
CPT	camptothecin
CHX	cycloheximide
DMSO	dimethyl sulfoxide
DIV	day in vitro
5-FU	5-fluorouridine
Q-RT-PCR	quantitative real time PCR
rDNA	ribosomal DNA
RNA-Pol-I	RNA-Polymerase-I
shRNA	short hairpin RNA
TD	trophic deprivation
TIF-IA	transcription initiation factor IA
γ -H2AX	phospho-Ser139-histone H2AX

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Figure 1. CPT inhibits RNA-Pol-I-driven transcription in primary neurons

A, DNA stand breaks in CPT-treated neurons. Cortical neurons (DIV 5) were treated for 90 min. with 5 μ M camptothecin (CPT) or its vehicle (0.2% DMSO). The phosphorylation of histone H2AX at Ser-139 (γ H2AX) was analyzed by indirect immunofluorescence. The nuclear foci of γ H2AX immunoreactivity indicated induction of a DNA strand break response in CPT-treated neurons. **B**, The kinetics of the apoptotic response to CPT. Averages of duplicate determinations from a representative experiment $\pm$ SD are presented. **C**, **D**, Inhibition of new rRNA synthesis in CPT-treated neurons at the time points preceding apoptosis. Transcription of nascent RNA was detected using an *in situ* run on assay. Neurons were treated with vehicle (0.2% DMSO) or 0.05 μ g/ml ActD or 5 μ M CPT for the

indicated times followed by a 30 min. pulse labeling with a halogenated RNA precursor, 5-FU. Indirect immunofluorescence with an anti-BrdU antibody was used to detect halogenated RNA. In vehicle treated neurons, the 5-FU labeling was detected in nucleoli (arrows) and throughout the nuclei. Lack of nucleolar 5-FU incorporation (arrowheads) indicates inhibition of RNA-Pol-I-mediated transcription at all time points after ActD or CPT. In ActD-treated neurons, extranucleolar transcription was initially reduced (1 hr) and then abolished (4 and 8 hr). The percentage of neurons displaying nascent RNA in nucleoli is presented on the graph (D). Data represent mean of duplicate determinations $\pm$ SD. Similar trends were observed in several independent experiments. **E**, Reduced levels of 45S pre-rRNA in ActD- or CPT-treated neurons. At the indicated times after ActD (0.05 μ g/ml) or CPT (5 μ M) treatments, the levels of 45S pre-rRNA were determined by quantitative real time PCR and normalized against the levels of 18S rRNA. The decreases of unstable 45S pre-rRNA indicate transcriptional inhibition at rDNA loci. Graphs represent the means of three independent experiments $\pm$ SEM.

Figure 2. Loss of nucleolar B23/nucleophosmin in CPT-treated neurons

Cortical neurons (DIV 5) were treated with vehicle (0.2% DMSO) or 0.05 $\mu\text{g/ml}$ ActD or 5 μM CPT or trophic deprivation (TD). At the indicated times, localization of a nucleolar marker, B23 was analyzed by immunofluorescence. In **C**, nuclei were counterstained with Hoechst 33258. Confocal images are presented. **A**, **B** Reduction of nucleolar B23 staining in ActD- or CPT-treated neurons indicates nucleolar stress. Simultaneous increase of B23 signal in nuclei suggests possible nucleoplasmic translocation. Arrows indicate neurons with nucleolar localization of B23; arrowheads identify cells with disrupted nucleoli. Note that TD does not alter nucleolar B23 staining. In **B**, changes of nucleolar B23 immunofluorescence intensity are presented as a fold of vehicle-treated controls (Veh). Each

bar represents at least 40 cells $\pm$ SEM from one representative experiment; statistically significant differences as compared to vehicle-treated neurons are indicated (***, $p<0.001$). **C**, Neurons with CPT-induced nucleolar stress demonstrate normal nuclear morphology. Thus, nucleolar stress is an early, pre-apoptotic response to CPT-induced DNA damage. Similar results were obtained in several independent experiments.

Figure 3. CPT-induced apoptosis but not nucleolar stress is p53-dependent

A, Activation of p53 by CPT. P53 phosphorylation at Ser-15 residue (pp53) and total levels of p53 (p53) were analyzed by western blotting. The numbers under the blots indicate relative levels of pp53 or p53 after normalization against β -actin that was detected by re-probing the blots with an anti- β -actin antibody. Note that 5 μ M CPT increased levels of pp53 and p53 indicating its activation. Similar results were obtained in 2 additional experiments. Trophic deprivation did not affect pp53 or p53 levels (not shown). **B**, Freshly isolated cortical neurons were co-electroporated with an empty expression vector (pcDNA3.1, vector) or the CMV-driven dominant negative p53 construct (DN-p53, 3 μ g plasmid DNA/ 5×10^6 neurons). The DN-p53 used here was the deletion mutant DD-p53 that

upon overexpression inactivates and stabilizes wt-p53 (Shaulian et al. 1992). Therefore, the increased levels of endogenous p53 (p53) that were detected 4 days post-transfection indicate efficient neuronal expression and activity of DN-p53. The blot was re-probed for GAPDH to ensure equal protein loading. **C-F**, Cortical neurons were co-transfected with expression plasmids for β -gal (pON260) and the DN-p53 (0.2 + 0.5 μ g of plasmids DNAs/ 500,000 neurons, respectively). An empty cloning vector (pcDNA3.1) was used as a control for the DN-p53 construct. Two days after transfection, neurons were treated with CPT or trophic deprivation (TD) for 8 (**C**), or 24 hours (**D, E, F**), as indicated. Sham-treated controls were used in TD experiments (control; see Materials and Methods for details). Transfected cells were identified by the presence of β -gal; nuclear morphology was visualized by counterstaining with Hoechst 33258 (Hoechst); B23 was detected by immunofluorescence. **C**, The 8 hr treatment with CPT induced nucleolar stress despite p53 inhibition. Representative images depicting B23 staining in DN-p53-transfected neurons are shown; arrows indicate transfected neurons containing B23-positive nucleoli; arrowheads indicate transfected neurons with disruption of nucleolar B23. **D**, Representative images of vector- or DN-p53-transfected neurons after the 24 hr CPT treatment; arrows indicate transfected neurons with normal nuclear morphology; arrowheads indicate transfected neurons with apoptotic nuclei as defined by chromatin fragmentation and/or condensation. **E, F**, Quantification of apoptosis in the transfected neurons revealed that DN-p53 abolished apoptotic response to CPT but not TD. Data represents averages of duplicate determinations from 3 independent experiments $\pm$ SEM; ***, $p < 0.001$; ns, $p > 0.05$.

Figure 4. BDNF protects against CPT-induced apoptosis without restoring RNA-Pol-I-mediated transcription and blocking p53 activation

At DIV5, cortical neurons were treated with CPT in the presence or absence of BDNF. **A**, BDNF protection from apoptosis following the 24 hour treatment with CPT. **B**, BDNF did not inhibit CPT-induced B23 translocation after 8 hr CPT treatment. **C**, In the absence of CPT, 8 hr treatment with BDNF increased the levels of 45S pre-rRNA. Upon co-treatment with CPT, BDNF did not affect the pre-rRNA levels suggesting that the CPT-induced inhibition of RNA-Pol-I is not sensitive to BDNF. Q-RT-PCRs were performed and analyzed as described for Fig. 1E. **D**, BDNF did not prevent CPT-mediated activation of p53. The levels of p53 phosphorylation at the Ser-15 residue (pp53) were analyzed as described for Fig. 3A. The numbers under the blot indicate relative levels of pp53 after normalization against β -actin. In A, and C, the means of three independent experiments $\pm$ SEM are shown; ***, $p < 0.001$; *, $p < 0.05$; ns, $p > 0.05$.

Figure 5. Specific inhibition of RNA-Pol-I-driven transcription induces p53-dependent apoptosis of primary neurons

A–C, Cortical neurons were co-transfected with expression plasmids for β -gal (pON260) together with an equimolar mix of three shTIF-IA plasmids to knockdown a RNA-Pol-I-specific co-factor, TIF-IA (0.2 + 1 μ g plasmid DNAs/500,000 neurons, respectively). The pSUPER-cloned shRNA targeting GFP (shGFP) was used as a control; DN-p53 (0.5 μ g plasmid DNA/500,000 cells) was also added to enhance survival of shTIF-IA-transfected neurons. Forty eight hours after transfection, cells were metabolically labeled with 5-FU as described for Fig. 1C and/or fixed followed by immunofluorescence to detect β -gal + 5-FU (A, B), or β -gal + B23 (C). A, The shTIF-IA (arrowhead) but not shGFP (arrow) inhibited

nucleolar incorporation of 5-FU indicating loss of RNA-Pol-I activity. **B**, Eighty % of shGFP- and 10% of shTIF-IA-transfected neurons demonstrated active nucleolar transcription. **C**, The shTIF-IA (arrowheads) but not shGFP (arrows) disrupted nucleolar B23 immunostaining. Thus, shTIF-IA specifically inhibited RNA-Pol-I activity inducing nucleolar stress in primary cortical neurons. **D, E**, Neurons were transfected as in A–C except DN-p53 was omitted. In **E**, the individual shTIF-IA plasmids were used as indicated. Apoptosis was evaluated in transfected neurons as described for Fig. 3D–F. In **D**, representative photomicrographs of transfected neurons at 48 hr post-transfection are shown; arrows indicate viable neurons; arrowheads indicate neurons with nuclear alterations indicating apoptosis (nuclear shrinkage and condensation with or without fragmentation). **E**, Quantification of apoptosis in transfected neurons revealed that each shTIF-IA construct individually induced apoptotic cell death at 48 and 72 hours after transfection (shGFP vs. each of shTIF-IAs, $p < 0.001$). **F**, Cortical neurons were transfected as in A–C. In addition, an empty cloning vector (pcDNA3.1, vector) was used as control for the DN-p53. Forty eight hours post-transfection, apoptotic response to shTIF-IA was blocked by DN-p53. In **B, E**, and **F**, averages of duplicate determinations from three independent experiments $\pm$ SEM are shown; ***, $p < 0.001$.

Figure 6. Role of gene expression in p53-regulated apoptosis of RNA-Pol-I-inhibited neurons

A, Neurons were co-transfected with shTIF-1A or shGFP together with an expression plasmid for β -gal as in Fig 5D. After 24 hr, cycloheximide (CHX) was added to the media. At 48 hr post-transfection, apoptotic response to shTIF-1A was reduced by CHX. **B**, Freshly isolated cortical neurons were co-electroporated with an empty expression vector (pcDNA3.1, vector) or a TS-p53 together with the pON260 β -gal expression construct (3 + 1 μ g plasmid DNAs/ 5×10^6 neurons, respectively). After 3 days at 37°C, neurons were placed at 32°C for 8 hours. Western blotting with anti-human p53 antibody followed by re-probing for β -gal revealed efficient expression of the TS-p53. **C**, Neurons were co-transfected with expression plasmids for β -gal and either a temperature sensitive mutant of p53 (TS-p53) or an empty expression vector (pcDNA3.1, vector; 0.2 + 0.5 μ g of plasmid DNAs/500,000 neurons, respectively). For next 48 hr neurons were kept at 37°C. At this temperature, TS-p53 remained in an inactive conformation. Then, neurons were placed at 32°C restoring wild type p53 conformation of TS-p53. In addition, cells were treated with CHX as indicated. At 24 hr after temperature shift, TS-p53-induced apoptosis was reduced by CHX. **D–E**, At DIV5, cortical neurons were treated with the general transcription inhibitor Actinomycin D (ActD) as indicated. **D**, ActD increased p53 phosphorylation at the Ser-15 (pp53) and induced accumulation of total p53 (p53). The numbers under the blots indicate relative levels of phospho-p53 or total p53 after normalization against β -actin. Similar results were obtained in 3 independent experiments. **E**, Neuronal apoptosis after 24 hr treatment with increasing concentrations of ActD. At 0.05 μ g/ml but not at any higher concentration tested, ActD moderately induced apoptosis. In **A**, and **C**, averages of duplicate determinations $\pm$ SD are shown; similar trends were observed in another set of independent experiments. In **E**,

averages of 4 independent experiments $\pm$ SEM are depicted; **, $p < 0.01$; ns, $p > 0.05$. **F**, The p53-regulated neuronal apoptosis that is induced by RNA-Pol-I-inhibition is sensitive to the translation blocker CHX and the general transcription blocker ActD suggesting apoptotic requirement of *de-novo* gene expression. The p53-mediated gene expression program resulting in *de-novo* synthesis of killer proteins is likely executed by RNA-Pol-II-driven transcription.