

DNA Damage Promotes Histone Deacetylase 4 Nuclear Localization and Repression of G₂/M Promoters, via p53 C-terminal Lysines^{*§}

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Repression of G₂/M promoters after DNA damage is an active mechanism that requires the p53 tumor suppressor. We have recently found that histone deacetylase 4 (HDAC4) is recruited on NF- κ B-dependent repressed promoters. In this report, we describe the relationship between p53 and HDAC4 recruitment following DNA damage using immunofluorescence, chromatin immunoprecipitation, and transfection experiments. HDAC4 shuttles from the cytoplasm into the nucleus, following DNA damage, independently of the activation of p53 and becomes associated with promoters through a p53-dependent mechanism. The C-terminal lysines of p53, which are acetylated and methylated, are required for HDAC4 recruitment and transcriptional repression. Trichostatin treatment, but not HDAC4 functional inactivation, relieves the adriamycin-mediated repression of G₂/M promoters. Our results indicate that HDAC4 is a component of the DNA damage response and that post-translational modifications of p53 are important for repression of G₂/M genes.

The transcriptional response to DNA damage is regulated by specific genes under the control of the tumor suppressor p53. The p53 protein is normally present in low amounts in growing cells and is activated in response to external insults via a plethora of post-translational modifications (1). This activation results in binding to specific DNA sequences located within the promoter region or the first intron of the activated genes (2). Upon DNA damage, p53 can also function as a negative regulator of a variety of genes whose products are critical for the cell cycle progression; most of the promoters of these genes do not contain a typical consensus binding site for p53 (3–11). Many G₂/M promoters can be repressed through p53 induction (12). G₂/M promoters depend upon NF- κ B binding to CCAAT boxes (5, 6, 9, 10), and NF- κ B-p53 association is one of the mechanisms for transcriptional repression (11). In addition to DNA-binding proteins, other complexes are essential for repression. Among the co-repressors, the histone deacetylases (HDACs)³ play an important role in controlling transcription. In particular, repression of cell

cycle promoters results in histone deacetylation mediated by the recruitment of HDACs to the promoters (13, 14). HDACs fall into four major categories based on sequence homology and domain organization. Class I HDACs (HDAC1/2/3/8) are similar to the yeast transcriptional regulator Rpd3p (15–19). Class II deacetylases HDAC4/5/6/7/9/10 are more similar to yeast Hda1p (20–22). The third class of HDACs is composed of proteins similar to the yeast NAD⁺-dependent deacetylase Sir2 (23–26) and the fourth by HDAC11 (reviewed in Ref. 27). Many of the Class II HDACs are regulated by nuclear trafficking; the process involves post-translational modifications as well as proteolytic cleavage of specific domains. Many pathways activated by cell proliferation, differentiation, and apoptosis lead to nuclear localization of Class II HDACs (28–31). They repress genes involved in differentiation, and the nuclear localization can lead to the loss of differentiated phenotypes through transcriptional silencing. HDAC4 and -5 repress MEF2 activity through direct physical interaction (32–34), and their export from the nucleus may be involved in MEF2-dependent activation of many muscle-specific genes (32, 34–37). An additional level of regulation results from an interplay between the N-terminal nuclear localization signal and the C-terminal leucine-rich nuclear export sequence (38–40). Specific stimuli induce phosphorylation of conserved serine residues by calcium/calmodulin-dependent kinase, creating docking sites for the 14-3-3 family proteins (41–43). This results in export of HDACs from the nucleus and, consequently, in the derepression of HDAC target genes. The vast majority of the data available on the regulation of the cellular localization of HDAC has been gathered from experiments on overexpressed proteins. In C2C12 myocytes, transfected HDAC4 was found to localize in the nucleus, whereas few cells displayed staining onto the cytoplasmic inclusions (28). HDAC4/5 overexpressed in U2OS cells can be localized exclusively to either the cytoplasm or the nucleus, often aggregating in foci (41). Ectopic overexpression of HDAC4/5 in HeLa cells shows the formation of nuclear aggregates, named matrix-associated deacetylase bodies (44, 45). Finally, there is increasing evidence that correlates HDACs in processes of response to DNA damage (29, 46, 47). In human HeLa cells, endogenous HDAC4 is recruited to nuclear foci with kinetics similar to 53BP1, in response to DNA damage (29). The nuclear/cytoplasmic shuttling during apoptosis is also regulated by caspases, which provide HDAC4 cleavage (48). Following the addition of non-apoptotic doses of adriamycin (ADR) to mouse NIH3T3 fibroblasts, we have recently noticed that HDAC4 is recruited on promoters in a late phase, after HDAC1, concomitantly with promoter repression (11). To investigate the molecular interplay between p53, the fundamental effector of the DNA damage

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³ The abbreviations used are: HDAC, histone deacetylase; ADR, adriamycin; ChIP, chromatin immunoprecipitation; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TRITC, tetramethylrhodamine isothiocyanate; RT, reverse transcription;

FACS, fluorescence-activated cell sorter; wt, wild type; siRNA, small interfering RNA; NF, nuclear transcription factor; TSA, trichostatin; MEF, myocyte enhancer factor 2.

HDAC4 Recruitment by DNA Damage

response, and HDAC4, we used immunofluorescence on endogenous and transfected proteins, chromatin immunoprecipitation (ChIP) assays and transfection experiments in NIH and human HCT116 cell lines.

MATERIALS AND METHODS

Cell Culture and Treatments—NIH3T3 mouse fibroblasts were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. The HCT116 cell line, derived from a human colorectal carcinoma, was cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum. HCT116/E6 were grown with G418 (0.5 mg/ml). Adriamycin was added at 1 $\mu\text{g}/\text{ml}$ for 8 h (see Figs. 5 and 6) or for the indicated times as shown in Figs. 1–4. Cells were collected and DNA distribution analysis of propidium iodide-stained cells was performed by an Epics cytofluorometer (Coulter).

Antibodies and Immunofluorescence—Cells were washed twice in PBS at room temperature, fixed in cold methanol/acetone for 2 min, and permeabilized with 0.05% Triton X-100 in PBS for 5 min. Samples were preincubated with 1% BSA in PBS for 15 min and then incubated overnight at 4 °C with the following primary antibodies diluted 1:100 in PBS+BSA 1%: anti-HDAC4 (Active Motif catalog number 40969 for endogenous HDAC4; Cell Signaling catalog number 2072 for transfected HDAC4); anti-phospho-histone H2AX (Ser-139), clone JBW301 (Upstate catalog number 05-636); anti-enolase C19 (Santa Cruz Biotechnology catalog number Sc-7455), and anti-NF- κ B (49). The cells were then washed twice with PBS and incubated 15 min at room temperature in PBS+BSA 1% before a further incubation for 60 min at room temperature in the dark with Hoechst and the relative secondary antibodies: fluorescein isothiocyanate anti-rabbit 1:200 (Sigma catalog number F7512), TRITC anti-mouse 1:150 (Sigma catalog number T5393), and TRITC anti-sheep 1:200 (Upstate Biotechnology catalog number 12-511). After three washes with PBS, monolayers were mounted with Vectashield and examined with a Zeiss AxioSkop 40 fluorescence microscope (Carl Zeiss, Jena, Germany) and the images collected with an AxioCam HRc camera and the AxioVision version 3.1 software package. The same samples were analyzed by confocal microscopy (Leica DM IRE2).

Chromatin Immunoprecipitation Assays—Chromatin immunoprecipitations were essentially performed as described previously (11), with the following modifications. Exponentially grown NIH3T3, HCT116wt, and HCT116/E6 cells were incubated for 10 min with 1% formaldehyde; after quenching the reaction with 0.1 M glycine, the cross-linked material was broken with Dounce (type B pestle) and sonicated to 500–800-bp fragments. Chromatin was frozen in liquid nitrogen and kept at –80 °C. Immunoprecipitation was performed with protein G-Sepharose (Kirkegaard & Perry Laboratories) and 3–5 μg of the indicated antibodies: NF- κ B-purified rabbit polyclonal, p53 Ab7 (Oncogene Science), anti-HDAC1 (Sigma catalog number H3284); anti HDAC4–5 (Active Motif) (see Ref. 50). The chromatin solution was precleared by adding protein G-Sepharose for 2 h at 4 °C, aliquoted, and incubated with the antibodies overnight at 4 °C. Before use, protein G-Sepharose was blocked twice at 4 °C with 1 $\mu\text{g}/\mu\text{l}$ salmon sperm DNA sheared at 500 bp length and 1 $\mu\text{g}/\mu\text{l}$ BSA for 2 h and overnight. PCRs on NIH3T3 chromatin were performed with the *Cdc2*, *Cdc25C*, *Cyclin B2*, and *PLK* primers described in Ref. 50. For the PCR amplification of Cyclin B2-luciferase oligonucleotides were: CycB2 (forward), 5'-CTAGCAAGCCAGCCAATCAACGTGC-3'; and luciferase, 5'-TTGCTCTCCAGCGTTCCAT-3'. PCRs on HCT116wt and HCT116/E6 chromatin were performed with primers: CCNB1 (forward), 5'-TGTCACCTCCAAAGGCCACTA-3'; CCNB1 (reverse), 5'-AGAAGAGCCAGCCTAGC-

CTCAG-3'; CCNB2 (forward), 5'-AGAGGCGTCCTACGTCTGCTTT-3'; CCNB2 (reverse), 5'-ATTCAAATACCGCGTGCCTTG-3'; *Cdc25C* (forward), 5'-GCTGAGGGAACGAGGAAAAC-3'; *Cdc25C* (reverse), 5'-CGCCAGCCCAGTAACCTATC-3'; *Cdc2* (forward), 5'-TAGCTTCTGCTCCGCTGGAC-3'; *Cdc2* (reverse), 5'-TCCCCTAGACAGACCCTGA-3'; TopoII α (forward), 5'-CTGCACACTTTTGCCCTCAG-3'; TopoII α (reverse), 5'-GACCAGCCAATCCCTGACTC-3'.

Plasmids and Transfections—10⁵ NIH3T3 cells were transiently transfected with Lipofectamine 2000 (Invitrogen) using the indicated doses of the HDAC4 vector, 0.1 μg of p53 vector, 0.1 μg of Cyclin B2-luciferase, 50 ng of β -galactosidase, and carrier plasmid to keep the total DNA concentration constant at 800 ng. Cells were recovered 24 and/or 36 h after transfection, resuspended in lysis buffer (1% Triton X-100, 25 mM, glycil-glycine, 15 mM MgSO₄, and 4 mM EGTA) for luciferase activities. β -galactosidase was assayed to control for transfection efficiency. Three independent transfections in duplicate were performed. The HDAC4 vector was obtained from Dr. S. Kochbin (Institute Albert Bonniot, Grenoble, France), p53 9KR was a kind gift of S. McMahon (Wistar Institute, Philadelphia, PA). The ChIP analysis performed in Fig. 6 was carried out on NIH3T3 cells (15-cm plates, 6 \times 10⁶ cells) transfected with the p53 plasmids (10 μg) and the Cyclin B2-luciferase vector (10 μg). After 24 h, one plate was treated with adriamycin for 8 h. ChIP analysis was then performed as described above.

Preparation of Cell Extracts and Western Blot Analysis—NIH3T3 nuclear extracts were prepared by collecting cells in Buffer A (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 1.5 mM dithiothreitol, protease inhibitors). Pelleted nuclei were then resuspended in ice-cold Buffer C (20 mM Hepes, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 1.5 mM dithiothreitol, protease inhibitors), rotated at 4 °C for 30 min, and cleared by high speed centrifugation at 4 °C. 25 μg of nuclear extracts were then separated by SDS-PAGE as previously described and immunoblotted with anti-HDAC4 antibody. Total extracts were prepared by resuspending the cell pellet in lysis buffer containing 50m mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, and protease inhibitors. Immunoblot analysis of acetylated histones was performed using HCT116 acid extracts. The cell pellet was suspended in lysis buffer (10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl) with the addition of hydrochloric acid at 0.2 N and incubated on ice for 30 min and cleared by high speed centrifugation for 10 min at 4 °C. Western blotting was performed with anti-acetyl histone H3 (Upstate Biotechnology catalog number 06-599) and anti-acetyl histone H4 (Upstate Biotechnology catalog number 06-866). The expression of transfected protein in Fig. 7 was confirmed by Western blotting analysis of equal amounts of total cellular extracts. The proteins were resolved by SDS-PAGE, electrotransferred to nitrocellulose membrane, and immunoblotted with anti-p53 (Ab7, Oncogene Science) and anti-HDAC4 (Active-Motif) antibodies.

Reverse Transcription (RT)-PCR Analysis—RNA was extracted, using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, from NIH3T3 and HCT116 cells not treated or treated with TSA (0.1 $\mu\text{g}/\text{ml}$) and adriamycin (1 $\mu\text{g}/\text{ml}$) for the times indicated in the results. For cDNA synthesis, 5 μg of RNA was retrotranscribed with a Moloney murine leukemia virus reverse transcriptase (Finzymes). Semiquantitative PCR was performed with oligonucleotides: mCycB2ex3, 5'-CTGTGAAACCAGTGCAGATG-3'; mCycB2ex6, 5'-ACTGGTGTAAGCATTATCTG-3'; hCycB2 (forward), 5'-GAGAA-TATTGACACAGGAG-3'; hCycB2 (reverse), 5'-CCCAACTAATTG-AAGCTT-3'; mCdc25C (forward), 5'-CAGTGGAGAGATGTCTGCTC-3'; mCdc25C (reverse), 5'-CTCCAGGGAACACTCCATTGG-

3'; hCdc25C (forward), 5'-CACTTCCTTTACCGTCTGTCC-3'; hCdc25C (reverse), 5'-CTGAAATCTTCTGCGCTGGTC-3'; mTopoII α (forward), 5'-CTGGAACATATACTGCTC-3'; mTopoII α (reverse), 5'-GTATAATCTTCTCCACTAAAGG-3'; mBax (forward), 5'-AGGGTTTCATCCAGGATCGAGCAG-3'; mBax (reverse), 5'-ATCTTCTCCAGATGGTGAGCCAG-3'; hBax (forward), 5'-CGACTCCTCGCAGATCGTCATC-3'; hBax (reverse) 5'-CTGGATGTGGTTCTTGGACTTC-3'; mMdm2 (forward) 5'-GTGCAATACCAACATGTCTGTGTC-3'; mMdm2 (reverse), 5'-TTTCCTGTGCTCCTTCACAGAGA-3'; hMdm2 (forward), 5'-GCTGTAACCCCTCACAGATTC-3'; hMdm2 (reverse), 5'-CAGATGTACTGAGTCCGATG-3'. Glyceraldehyde 3-phosphate dehydrogenase control RT-PCR was performed with standard oligonucleotides.

Small Interfering RNA (siRNA) Experiments—HCT116 cells (60% confluency) were transfected (Lipofectamine 2000, Invitrogen) with 100 and 200 nM of paired HDAC4 small interfering RNA: 5'-GACGGGCAGUGGUCACUG-3' (sense) and 5'-CAGUGACCACUGGCCGUC-3' (antisense). Total extracts were prepared after 24 and 48 h, and HDAC4 expression inhibition was confirmed by Western blotting analysis. RT-PCR was performed using RNA extracted from HCT116 cells transfected with siRNA at 200 nM for 48 h.

RESULTS

Analysis of HDAC4 Cytoplasmic/Nuclear Shuttling after DNA Damage—We first tested the localization of HDAC4 in DNA-damaged cells not committed to apoptosis. Cycling mouse NIH3T3 fibroblasts were initially analyzed by FACS after adriamycin treatment for 8 h (Fig. 1A); the treated cells showed an increased in late G₂, but no apoptosis was induced. We next performed immunofluorescence assays with anti-HDAC4 as well as with anti-H2AX antibodies, the latter commonly used to monitor DNA damage caused by adriamycin (Fig. 1B). As expected, the phosphorylated histone variant H2AX was detected in the nuclei only after adriamycin treatment. In cycling cells, endogenous HDAC4 was localized mainly in the cytoplasm, whereas HDAC4 accumulation in the nuclei was evident in treated cells. A time course immunofluorescence was performed after the addition of adriamycin (Fig. 1C, left panel). Within 30 min, part of the cytoplasmic HDAC4 relocates to the nucleus, and maximal relocation was achieved by 8 h. As a control, we used an antibody against the cytoplasmic enolase; no effect on enolase localization was observed (Fig. 1C, right panel). NIH3T3 cells treated with adriamycin were further analyzed for the presence of nuclear HDAC4 by Western blotting (Fig. 1D); a progressive increase in the amount of nuclear HDAC4 is consistent with the immunofluorescence data. Actin and whole cell extracts were loaded as controls (Fig. 1D, lower panel). The anti-HDAC antibody that detects endogenous HDAC4 was not previously employed. To control our experiments, we performed immunofluorescence on transiently transfected cells with an HDAC4 expression vector (Fig. 2A). As shown by quantitative analysis (Fig. 2C), expression of HDAC4 is in the cytoplasm of 85% of transfected cells, whereas after DNA damage, HDAC4 cytoplasmic localization drops to 18% and nuclear localization increases to 53%. The effect of adriamycin treatment is specific for HDAC4, because NF-YC, a protein also found in the cytoplasm (51, 52), did not translocate to the nucleus upon DNA damage (Fig. 2B). Taken together, these results indicate that, in NIH3T3 cells, endogenous HDAC4 is mainly localized in the cytoplasm and that DNA damage rapidly relocates it to the nucleus.

HDAC4 Cytoplasmic/Nuclear Shuttling Is Not p53-dependent—The tumor suppressor p53 functions as a transcriptional activator to induce cell cycle arrest and apoptosis in response to DNA damage. As subcellular distribution plays an important role in the control of HDAC4 tran-

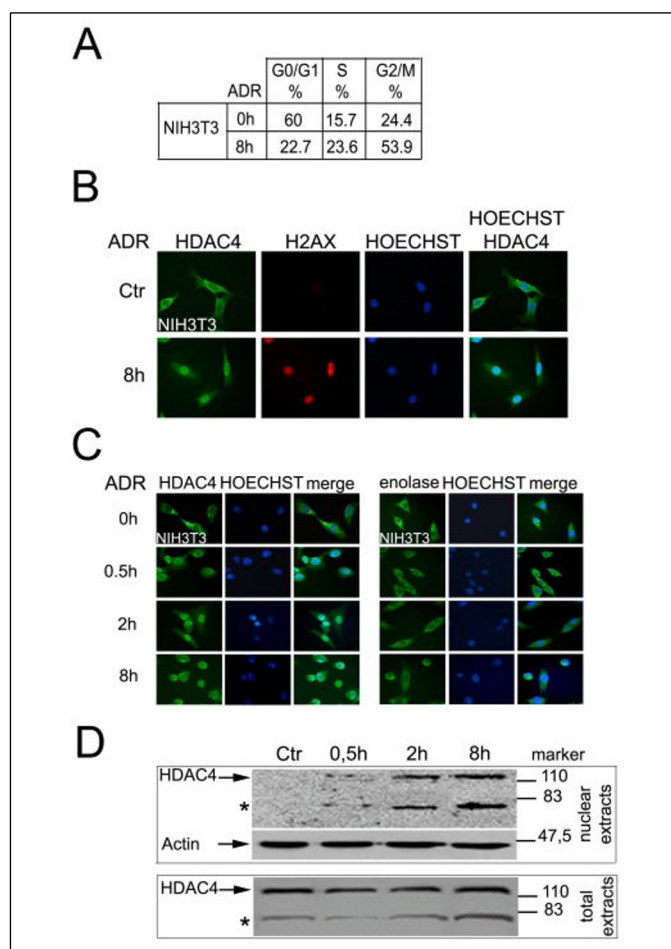


FIGURE 1. Endogenous HDAC4 cytoplasmic/nuclear shuttling after DNA damage in NIH3T3. A, NIH3T3 cycling cells were not treated or treated with 1 μ g/ml of adriamycin (ADR) for 8 h, and cell cycle distribution was analyzed via flow cytometry. Adriamycin addition showed an increased percentage in late G₂. Ctr, control. B, the top panels show on the left cytoplasmic HDAC4 staining in NIH3T3 asynchronous cells, followed by H2AX and Hoechst staining. On the right is shown Hoechst staining of cells was merged with HDAC4. The lower panels show 8-h adriamycin-treated cells. On the left is shown nuclear HDAC4 staining. C, the left panel shows HDAC4 and Hoechst fluorescence microscopy images following DNA damage by adriamycin for the indicated times. The right panel shows cytoplasmic enolase staining and Hoechst staining of the same time course-treated cells. Fluorescein isothiocyanate and Hoechst staining are merged on the right of each panel. D, upper panel, Western blot analysis of equal amounts of nuclear extracts from NIH3T3 cells treated with adriamycin for the indicated times. Anti-actin antibody was used for the detection of protein loading. Lower panel, equal amounts of total cell extracts were subjected to Western immunoblotting by using anti-HDAC4 antibody. The faster-migrating band (*) corresponds to the HDAC4-cleaved fragment.

scriptional activity, we asked whether nuclear import is p53-dependent. To address this, we examined the subcellular localization of HDAC4 in human HCT116 and HCT116/E6, expressing the viral E6 protein that induces p53 degradation (53–57). FACS analysis reported in Fig. 3A shows that adriamycin treatment increases the G₂/M ratio in HCT116. HCT116/E6 showed no block in the G₂ phase, as expected for cells lacking functional p53. As shown in Fig. 3B, HDAC4 is pan-cellular before adriamycin treatment, both in HCT116 and in HCT116/E6. Exposure to DNA damage significantly localizes HDAC4 to the nucleus. HCT116 and HCT116/E6 were transfected with an HDAC4 expression vector (Fig. 3C), and the percentage of HDAC4 cellular localization is shown in Fig. 3D. After 8 h, HDAC4 became nuclear in the majority of transfected cells, both in HCT116 and in HCT116/E6. These data confirm the NIH3T3 immunofluorescence experiments and indicate that the cytoplasmic/nuclear translocation of HDAC4 is not p53-dependent.

HDAC4 Recruitment by DNA Damage

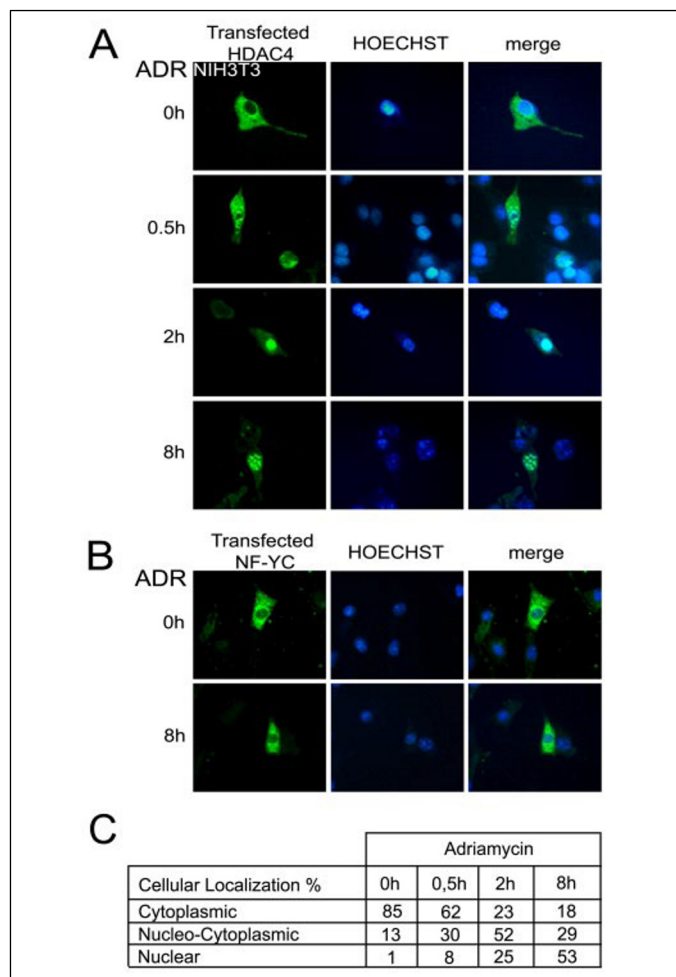


FIGURE 2. Overexpressed HDAC4 nuclear shuttling upon DNA damage in NIH3T3. *A*, immunofluorescence analysis of NIH3T3 cells overexpressing HDAC4. Transfected cells were incubated with adriamycin (ADR) for the indicated times. Hoechst and HDAC4 staining are merged on the right. *B*, immunofluorescence analysis of NIH3T3 cells overexpressing the NF-YC, treated or not with adriamycin for 8 h. *C*, cellular localization of overexpressed HDAC4 upon the time course of adriamycin was resumed in the table.

HDAC4 and p53 Colocalize in the Nucleus upon DNA Damage—As DNA-damaging agents induce post-translational modifications that activate p53, we reasoned that p53 can target HDAC4 on repressed promoters after DNA damage. We examined p53 and HDAC4 localization after 8 h of adriamycin treatment by immunofluorescence. As shown in Fig. 4, p53 is detected only upon DNA damage; it is clearly nuclear, both in NIH3T3 (Fig. 4A) and in HCT116 (Fig. 4B). The analysis of HDAC4 and p53 cellular localization of damaged cells suggested nuclear co-localization of the two proteins. To confirm these results, we carried out confocal microscopy analysis. Adriamycin-treated NIH3T3 and HCT116 cells revealed diffuse co-localization of endogenous HDAC4 and p53 in the nucleus, in particular in nuclear dots (Fig. 4C). Therefore, we conclude that DNA damage relocalized HDAC4 into p53-containing parts of the nucleus.

HDAC4 Recruitment on Cell Cycle Promoters Is p53-dependent—DNA damage induces the transcriptional repression of NF-Y-binding G₂/M promoters in NIH3T3 (11). The co-localization analysis suggests that p53 and HDAC4 might be targeting the same promoters. To verify this, we performed CHIP experiments with anti-HDAC4, anti-HDAC1, and control anti-NF-YB antibodies in NIH3T3 before and after 8 h of treatment (Fig. 5A). PCR amplification of the cell cycle-regulated *Cyclin B2*, *Cdc2*, *Cdc25C*, and *PLK* determined that

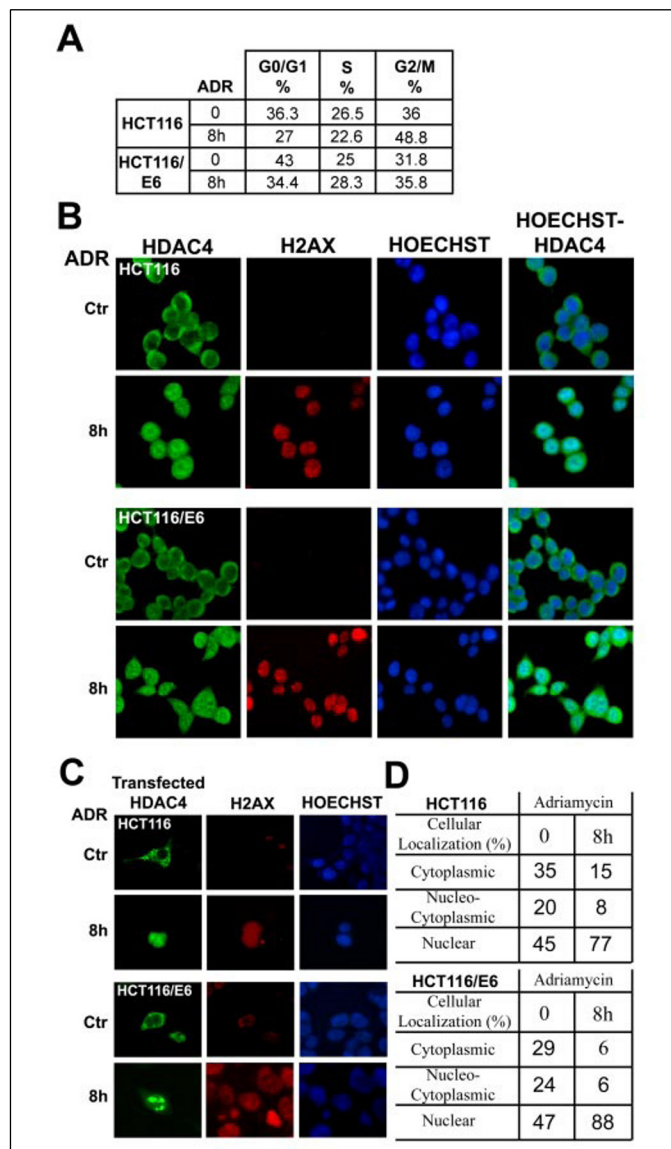


FIGURE 3. HDAC4 nuclear recruitment upon DNA damage is not p53-dependent. *A*, HCT116 and HCT116/E6 cycling cells were not treated or treated with adriamycin (ADR) for 8 h. Cell cycle distribution was analyzed by FACS. The addition of adriamycin showed an increased percentage in late G₂ in HCT116 but not in HCT116/E6 cells. *B*, HCT116 (upper panel) and HCT116/E6 (lower panel) were not treated or treated with adriamycin. Endogenous HDAC4 staining shows cytoplasmic localization in control (Ctrl) cells and nuclear localization upon DNA damage. DNA damage was detected with H2AX staining. Hoechst and HDAC4 staining are merged on the right. *C*, HCT116 (upper panel) and HCT116/E6 (lower panel) were transfected with HDAC4 expression vector and then treated or not with adriamycin. Both of the immunofluorescence images show HDAC4 nuclear localization upon DNA damage, as resumed in the tables (*D*).

the control NF-Y was bound before and after the addition of adriamycin. HDAC1 was bound before DNA damage, and HDAC4 was associated to promoters only after DNA damage. These data suggest that DNA damage induces the HDAC4 recruitment to G₂/M promoters. It was then of substance to investigate whether p53 association was required for HDAC4 recruitment. ChIPs were performed on HCT116/E6 and HCT116 chromatin with anti-NF-YB, anti-p53, and anti-HDAC1/4/5 antibodies. Fig. 5, *B* and *C*, shows that the control anti-NF-YB was positive before and after damage in HCT116 and HCT116/E6. In HCT116, p53 is weakly associated to *Cyclin B1* and *CDC25C* before DNA damage and increased significantly after damage to all promoters (Fig. 5B). The E6 cells were completely negative in both conditions (Fig. 5C). HDAC4 became associated to

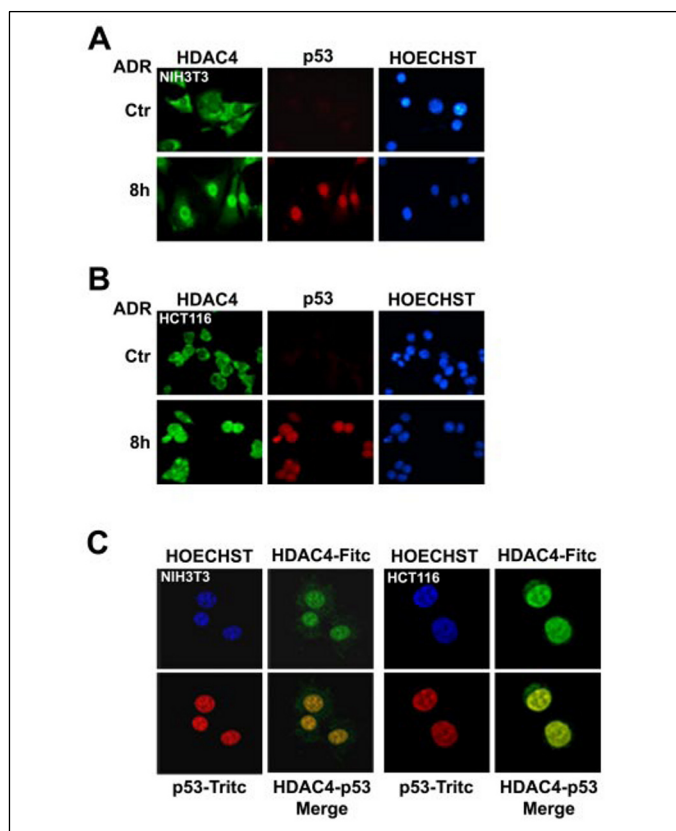


FIGURE 4. **HDAC4 and p53 co-localization after DNA damage.** NIH3T3 (A) and HCT116 (B) cells, treated or untreated with adriamycin, were analyzed by immunofluorescence with anti-HDAC4 and anti-p53 antibodies. DNA-damaged cells show nuclear localization of endogenous HDAC4 and p53. *Ctrl*, control. *C*, confocal microscopy images of NIH3T3 (left panel) and HCT116 (right panel) cells. The cells were stained with anti-HDAC4 and anti-p53 antibodies and Hoechst.

G_2/M promoters only after damage in HCT116 but not in the E6 expressing cells. HDAC1 decreased after damage (Fig. 5B). In HCT116/E6 cells, HDAC1 was bound to all targets, indicating that p53 is not required for its association (Fig. 5C). After DNA damage, HDAC5 was bound to some promoters (Fig. 5, B and C). Note that lack of p53 in E6 cells did not prevent its binding. The PCR of each ChIP was quantitated estimating the fold enrichment of the indicated antibodies compared with the nonspecific antibody (see supplemental data Fig. 5b). Taken together, these results indicate that the dynamic association of HDAC4, not HDAC1, is consistent with p53 binding, indicating a key role of p53 in HDAC4 recruitment upon DNA damage. They also highlight recruitment of HDAC5.

Role of p53 C-terminal Lysines in HDAC4 Recruitment—Post-translational modifications, in particular acetylation and methylation, are essential for p53 activation. Adriamycin treatment induces p53 acetylation at different residues in the C-terminal domain through p300/CREB-binding protein and PCAF/hGCN5 histone acetyl-transferases and methylation through the Set9 methyltransferase (58). We showed that p53 C-terminal lysines play a critical role in repression (11). To investigate the role of lysines in HDAC4 recruitment on G_2/M promoters, we performed ChIP experiments by co-transfecting a Cyclin B2-luciferase reporter together with wild-type p53 or a p53 mutant, p53-9KR, in which all lysines at the C terminus were mutated to arginines (59). As expected, both wild-type p53 and p53-9KR were similarly capable of binding to the transfected reporter before and after adriamycin treatment (Fig. 6B). NF-YB and p53 were also associated with the endogenous Cyclin B2 promoter (Fig. 6A), HDAC1 binding was evident

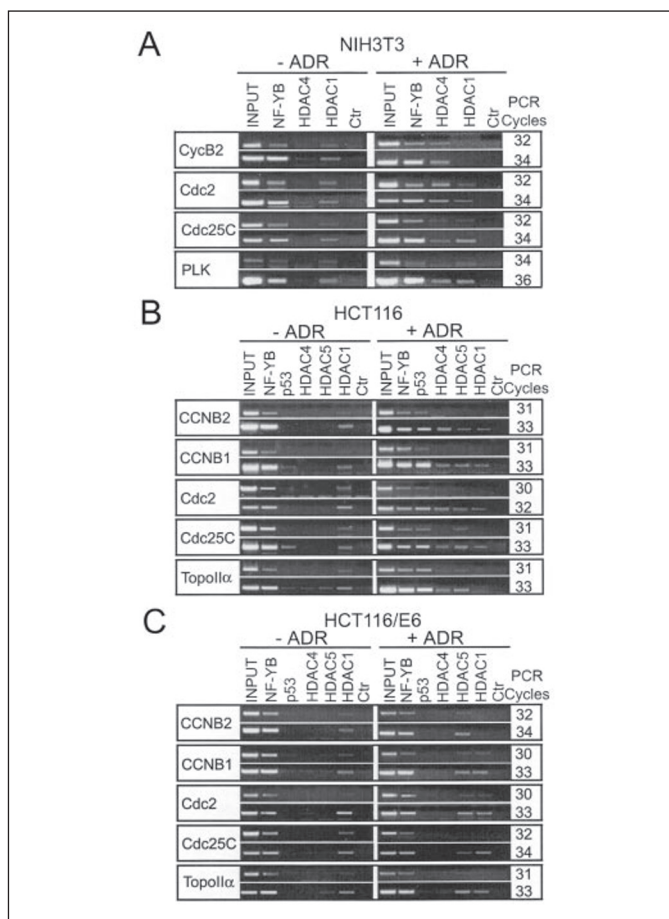


FIGURE 5. **HDAC recruitment to G_2/M -regulated promoters upon DNA damage.** A, NIH3T3 chromatin was immunoprecipitated with the indicated antibodies. HDAC4 is recruited on the indicated promoters upon adriamycin treatment. HCT116 (B) and HCT116/E6 (C) cells were damaged with adriamycin, and ChIP analysis was performed with antibodies against NF-YB, p53, HDAC4, HDAC5, and HDAC1. For each promoter, we show two amplifications at different PCR cycles. *Ctrl*, control; *PLK*, Polo-like kinase.

before DNA damage, and HDAC4 was subsequently recruited. HDAC1 and HDAC4 were similarly bound to Cyclin B2-luciferase when wild-type p53 was transfected (Fig. 6B, left panels). With p53-9KR, HDAC4 was not recruited after the addition of adriamycin. These results suggest that p53 C-terminal lysines modified in response to DNA damage play an essential role in HDAC4 recruitment.

Repression of Cyclin B2 by HDAC4—To detail the role of HDAC4 in promoter repression, we co-transfected NIH3T3 cells with Cyclin B2-luciferase reporter and p53 and HDAC4 vectors. Dose response assays indicated that HDAC4 is a modest repressor when expressed alone (Fig. 7A). As shown previously, overexpression of wild-type p53 also repressed the promoter. Interestingly, the effect was additive when p53 and HDAC4 were co-transfected, whereas upon co-transfection of the p53-9KR mutant, which has no repression capacity, only the modest effect of HDAC4 is scored. It should be noted that this experiment was performed at relatively low, suboptimal levels of p53 proteins to avoid the frank repressive effect of higher concentrations of p53. Note that p53-9KR is as efficient as wild-type p53 in activation (11, 59). Equivalent levels of wild-type p53 and p53-9KR and HDAC4 were visualized in Western blots of transfected cells (Fig. 7B). These results show that HDAC4-p53 co-repressive activity requires p53 C-terminal lysines. The lack of HDAC4 displacement on endogenous *Cyclin B2* promoter following expression of p53 9KR suggests that the latter does not act in a dominant negative way on repression of endogenous p53, once acti-

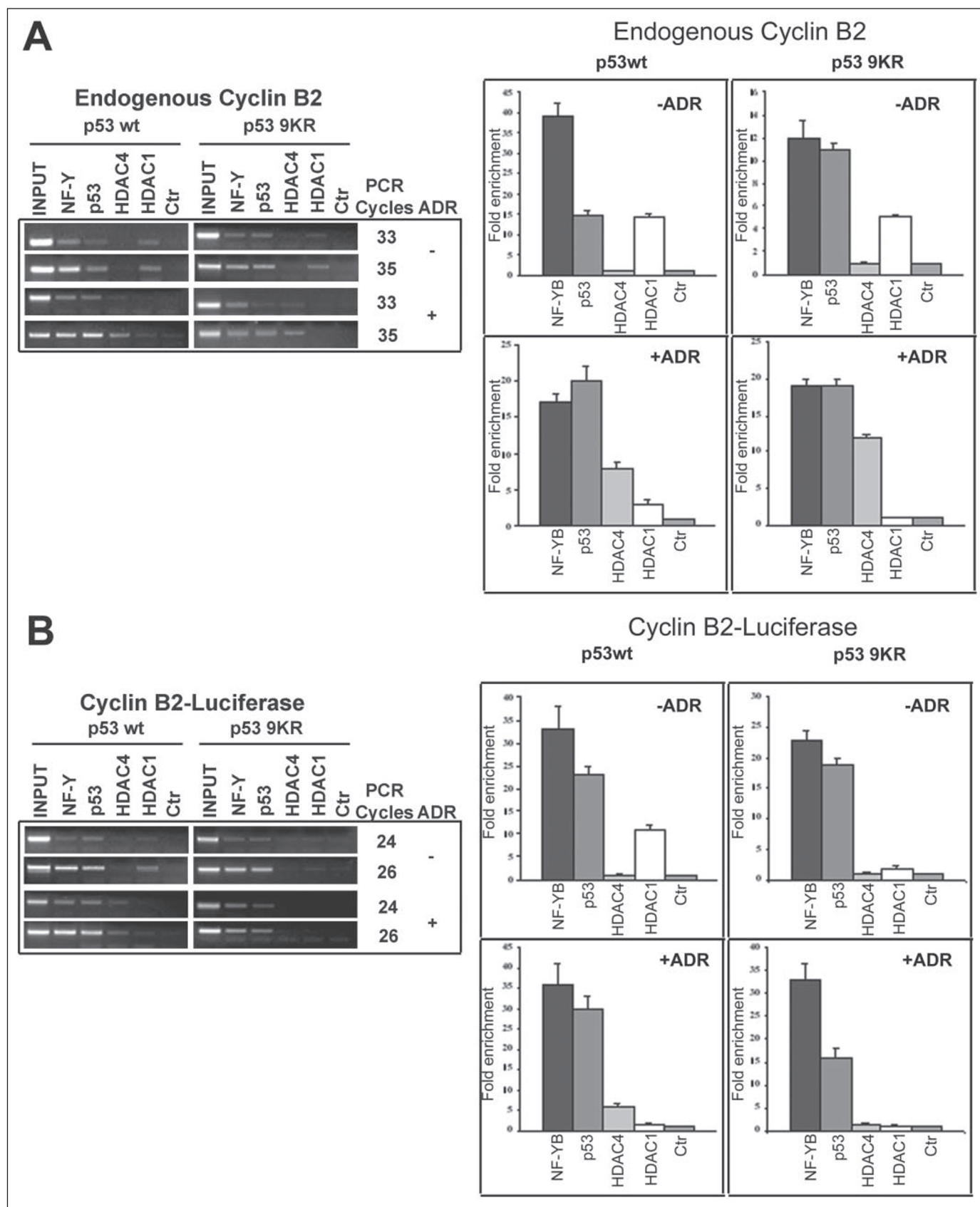


FIGURE 6. **Role of p53 C-terminal lysines in HDACs recruitment.** *A* and *B*, chromatin of NIH3T3 cells transfected with wild-type p53 or mutant p53-9KR, together with the CyclinB2-luciferase construct, was immunoprecipitated with the indicated antibodies, before (–) and after (+) adriamycin (ADR). PCRs with the endogenous Cyclin B2 gene are shown in *A*, and DNA amplification with the transfected template is shown in *B*. Two sets of PCRs are shown; fewer cycles are required for the transfected templates. Relative fold enrichment compared with the control antibody are plotted in the *right panels*. Ctr, control.

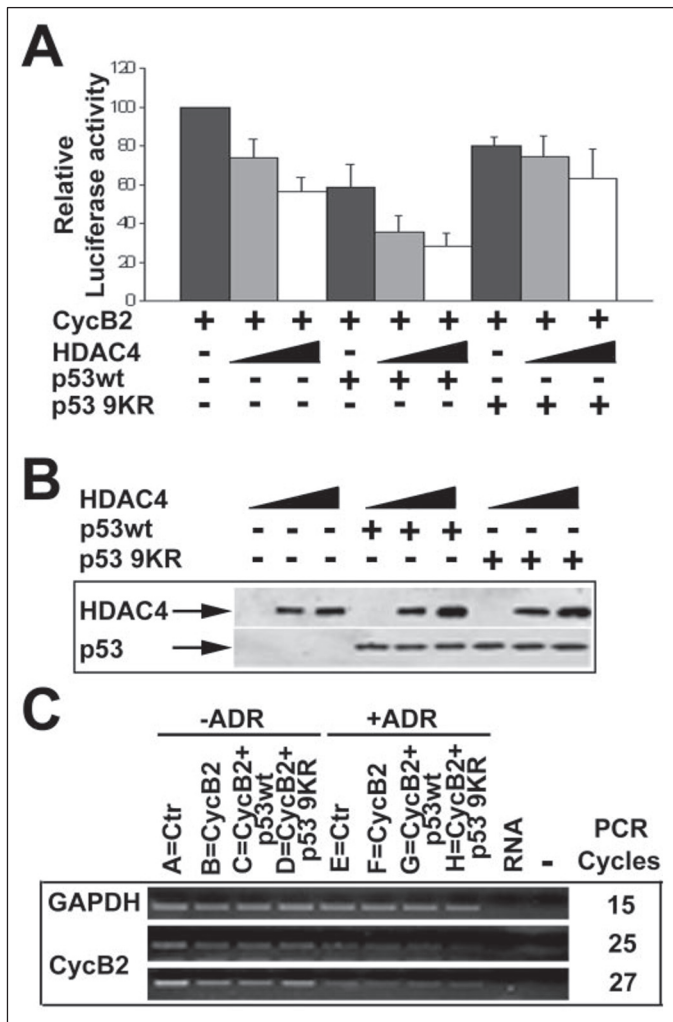


FIGURE 7. Repression of Cyclin B2 by HDAC4. *A*, dose response analysis of HDAC4 expression vector with wild-type and mutant p53-9KR in NIH3T3 on the Cyclin B2 reporter. Data represent arithmetic means \pm S.D. for three independent experiments. *B*, levels of expression of the transfected p53 (wild-type and 9KR) and HDAC4 are shown in the Western blot. *C*, RT-PCR analysis of mRNA extracted from NIH3T3 cells transfected with the indicated plasmids, untreated and treated with adriamycin (ADR) for 8 h.

vated by adriamycin. To verify this point, we co-transfected wild-type p53 and the 9KR mutant in NIH3T3 and assayed gene function by RT-PCR analysis, either with or without ADR treatment. As indicated by Fig. 7C, the effect of p53 overexpression, either wild type or mutant, without ADR treatment on *Cyclin B2* is minimal. As expected, in ADR-treated cells, we observed repression due to endogenous p53 activation, which was largely untouched by the presence of the 9KR mutant. This clearly indicates that the latter does not act as a dominant negative factor on repression of the endogenous promoters.

TSA Prevents Repression of DNA Damage on G₂/M Promoters—Trichostatin A is known to inhibit all HDACs. It has been shown recently that TSA increases the efficacy of several anti-cancer agents, including adriamycin (60). To shed light on the role of HDACs on promoter repression following DNA damage, we performed RT-PCR analysis of cell cycle-regulated genes under several experimental conditions outlined in Fig. 8. As checked by FACS analysis (Fig. 8A), treatment of cells for 8 h with adriamycin and TSA, or for 2 h only with the latter, partially reversed the G₂/M blocking effect of the DNA-damaging drug. Few apoptotic NIH3T3 cells were scored, whereas the degree of apoptosis increased over the controls in HCT116, when both drugs were given for 8 h. The doses of TSA employed here were responsible for a large

increase in H3 and H4 acetylation, as checked by Western blotting with anti-acetyl antibodies (Fig. 8B). In RT-PCR analysis, adriamycin increased *Bax* and *Mdm2* mRNA, both p53 targets, as expected (Fig. 8C) and decreased *Cyclin B2*, *Topoisomerase II α* , and *CDC25C* levels (Fig. 8C, lane B). The addition of TSA for 8 h had minimal effects on *Mdm2* and *Cyclin B2*, although up-regulating *Bax* (Fig. 8C, lanes C and D). Co-incubation of TSA and adriamycin blocked the G₂/M promoter repression but not *Mdm2* and *Bax* activation (Fig. 8C, lane E). The effect is also visible when TSA was incubated for 2 h, cells washed, and incubated for 8 additional h with adriamycin alone (Fig. 8C, lane F). Note that the effect of TSA is somewhat gene-dependent, as *CDC25C* shows the larger recovery of activity. Moreover, the degree of recovery by TSA treatment was somewhat different for the promoters analyzed, depending upon the length of the TSA addition; for *CDC25C*, 8 h gave a complete recovery, whereas for *Cyclin B2*, the 2-h treatment was more effective. In summary, the activity of HDACs is collectively required for transcriptional repression mediated by DNA damage.

HDAC4 Inactivation Is Insufficient to Revert Adriamycin-mediated Repression—To ascertain whether HDAC4 is sufficient to bring the ADR-mediated repression on G₂/M promoters, we treated HCT116 with an HDAC4-specific siRNA. Initial immunofluorescence performed as in Figs. 1–3 confirmed a clear negativity of cells in HDAC4 staining (Fig. 9A). To quantify this effect, we ran Western blots. Fig. 9B indicates that, although clearly diminished, HDAC4 expression is not completely ablated in siRNA-treated HCT116. RT-PCR of control and siRNA-treated cells before and after ADR failed to reveal a recovery of activity following HDAC4 inactivation in ADR-treated cells (Fig. 9C). Thus, additional mechanisms are operative in repression of G₂/M genes.

DISCUSSION

We have reported here on the behavior of HDAC4 following DNA damage. Three relevant findings have been presented. (i) HDAC4 shuttles from the cytoplasm into the nucleus following DNA damage, becoming associated to promoters that are being shut off through a p53-dependent mechanism. The relocation is independent of the activation of p53. (ii) The C-terminal lysines of p53, which are modified by acetylation and methylation, are required for the recruitment of HDAC4 on repressed promoters, both in ChIPs and in functional experiments. (iii) Inhibition of HDACs, but not of HDAC4 alone, relieves the adriamycin-mediated repression of G₂/M promoters.

Regulation of HDAC4 Localization—In the majority of cell types, Class II HDACs are located in the cytoplasm, and their nuclear shuttling has been detailed (30, 37, 38, 61). In particular, the myocytes-myotubes system of C2C12 has been exploited to understand the events that lead to nuclearization of HDAC4 (and HDAC5) following induction to differentiation. 14-3-3 σ retains HDAC4 in the cytoplasm (39, 41). Interestingly, 14-3-3 σ also stabilizes p53 by antagonizing the ubiquitination functions of Mdm2 and nuclear export (62). MEF2, a transcription factor that activates muscle-specific genes, promotes nuclearization of HDAC4 (32–34, 36, 40, 42, 61). This can also be promoted, in neurons, by depolarization (63). In one of the few studies performed with antibodies recognizing the endogenous HDAC4, irradiation of HeLa cells modified the localization within nuclei, promoting the formation of nuclear dots, or repair foci, together with a p53-interacting protein, p53BP1 (29). The discrepancy of our data with those of HeLa cells is only apparent because cellular localization of HDAC4 varies among different cell types (29, 32, 33, 40, 42, 43, 48). Moreover, upon long incubation times, indeed we do see the emergence of HDAC4 and p53-containing dots, whose nature was not further determined Figs. 2 and 4. A relevant

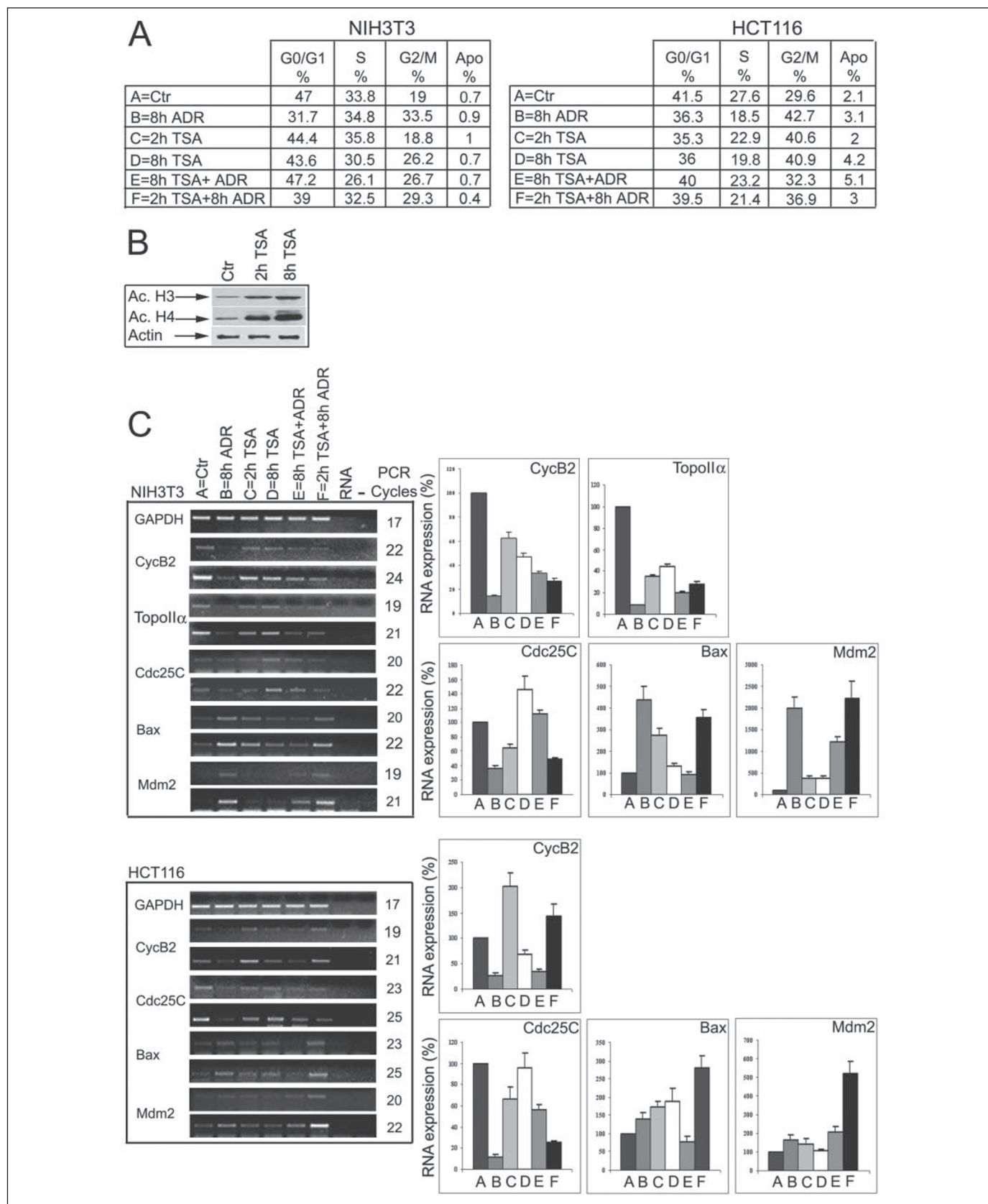


FIGURE 8. TSA prevents the adriamycin repression effect on G₂/M promoters. *A*, cell cycle distribution analysis via flow cytometry of NIH3T3 (*left panel*) and HCT116 (*right panel*), treated with the indicated drugs. *B*, HCT116 cells were treated with TSA for 2 and 8 h. Acid cell extracts were subjected to Western immunoblotting using anti-acetyl H3, anti-acetyl H4, and actin antibodies. *C*, RT-PCR analysis of mRNA extracted from NIH3T3 cells (*upper panel*) and HCT116 cells (*lower panel*), untreated and treated with TSA/adriamycin (ADR) for the indicated times. The *right panels* show RNA expression levels of the indicated target genes relative to RNA levels at 0 h after drug treatment. Ctr, control. APO, apoptosis.

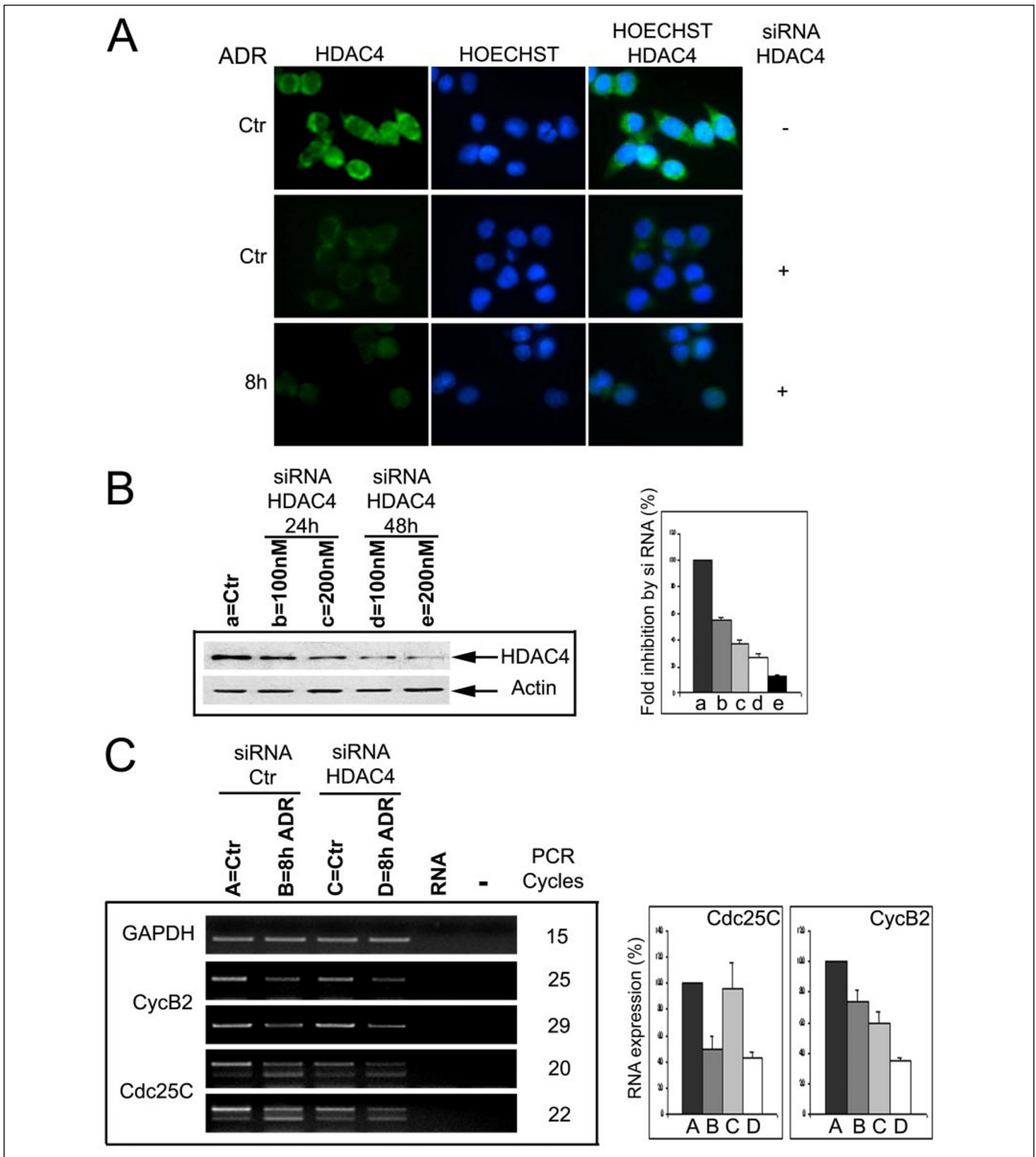


FIGURE 9. RNAi inactivation of HDAC4- and ADR-mediated repression. *A*, immunofluorescence analysis of HCT116 cells with anti-HDAC4 antibody before and after HDAC4 siRNA and adriamycin (ADR) treatment. *B*, HCT116 cells were transfected with HDAC4 siRNA, and total cellular extracts were Western immunoblotted with anti-HDAC4 and anti-actin antibodies. HDAC4 expression levels relative to control cells are plotted in the right panel. *C*, RT-PCR analysis of mRNA extracted from HCT116 cells transfected with control siRNA and HDAC4 siRNA and treated with adriamycin as indicated. RNA expression levels of the indicated target genes, relative to RNA levels at 0 h after drug treatment, are plotted in the right panels. Ctr, control.

point is that HDAC4 nuclear localization, or retention, does not depend on p53, as in E6 overexpressing HCT116, shuttling into the nuclei is observed, if anything, with higher efficiency (Fig. 3).

HDAC4, p53, and G₂/M Promoter Repression—A direct role of HDAC4 in cell cycle progression was documented by the finding that siRNA interference of HDAC4 (but not HDAC6 or HDAC2) abolishes

HDAC4 Recruitment by DNA Damage

an irradiation-mediated G₂ block in HeLa cells (29). p53, in addition to being an activator of cell cycle blocking and pro-apoptotic genes, exerts its effects by inhibiting the transcription of specific genes (11, 64, and references therein). This repressive capacity is exerted through two mechanisms: (i) involving direct sequence-specific binding of activated p53 to *bona fide* target sites (65) and (ii) repression of promoters via other DNA-binding proteins that normally function as activators (11). Genes coding for cell cycle regulators specifically activated during G₂/M are specifically inhibited, and this phenomenon contributes to the DNA damage-induced block in G₂ (3, 7, 8, 12). p53 is associated with unstressed conditions through NF-Y binding to multiple CCAAT boxes, becoming rapidly acetylated following DNA damage. HDACs are then differentially recruited, suggesting differential temporal roles in repression. These data suggested a role of NF-Y-p53 complexes, through CCAAT boxes, in HDAC recruitment, which is consistent with interactions documented with both p53 (66) and NF-Y (67). In untransformed NIH3T3 cells, we detailed little difference in p53 association before and after adriamycin addition. However, in HCT116 cells, there is a clear increase in p53 binding upon stress. The reason for this is unclear, but it is tempting to speculate that the far more pronounced degree of G₂/M arrest in NIH3T3 (compare Figs. 1A and 3A) is indeed related to this type of tight promoter control, which is more delayed in HCT116. It will be interesting to extend these observations to other transformed and non-transformed cells. We established that p53 acetylation is important for HDAC4 recruitment. This stems from two types of data. (i) Unlike NIH3T3 and HCT116, HCT116-E6 cells show no recruitment capacity of HDAC4 in ChIP assays (Fig. 5). Note that NF-Y association is not altered, further enforcing the notion that it is required, but not sufficient, for HDAC recruitment. Also, HDAC5 binding is increased on many promoters. (ii) ChIP assays with the p53-9KR indicate that little recruitment of HDAC4 is observed after DNA damage on the transfected Cyclin B2, whereas it is loaded on the endogenous gene where wild-type p53 is presumably dominant. This behavior is not due to a lack of recruitment of the mutant p53 (Fig. 6). We conclude that lysine residues at the C-terminal are necessary for HDAC4 recruitment. They are known to be acetylated by PCAF (Lys-320) by p300/CREB-binding protein (Lys-373 and Lys-382), and methylated by Set9 (Lys-372) (53, 68–70). It is unclear what the relationship is between such modifications and whether they synergize in activity. We found that p300 activates, whereas pCAF represses Cyclin B2 (11, 71). It will now be important to establish the role of the Set9 methylase in G₂/M promoter function. Mechanistically, these modifications could confer conformational changes, or the presence of specific acetyl and/or methyl groups could lead to improved affinity to co-repressors. In general, p53 would act as an adaptor molecule. In keeping with this, although co-expressing wild-type p53 and HDAC4 yields an additive negative effect on Cyclin B2, no effect is observed with the p53-9KR mutant (Fig. 7). Finally, inhibition of HDACs by trichostatin A leads to reversal of the repression of Cyclin B2, CDC25C, and to a lesser extent, topoisomerase II α (Fig. 8C), proving that repression is actively requiring HDACs. In keeping with this, the acetylation levels of histone H3-H4 tails in the Cyclin B2 promoter decrease rapidly, confirming the functionality of the enzymatic activity of HDACs (11). Our data on the HDAC4 inactivation, however, suggests that HDAC4 alone is not responsible for this function and could be helped or surrogated by additional mechanisms. There are three possible explanations for this finding. (i) In incomplete inactivation of HDAC4, the residual activity, which was low but clearly visible by Western blot (Fig. 9B), might be sufficient to repress gene transcription. (ii) Regarding the requirement for Class I HDACs, it has been shown, in fact, that Class I HDACs are important for Class II HDAC function (72,

73, and references therein). Note that the lack of positivity of HDAC1 in some promoters at later hours does not necessarily imply a release from the promoter. The HDAC1 epitope(s) might be masked, becoming inaccessible to the antibody used. Thus HDAC1 might still be required for “late” repressive events. (iii) Redundancy with other Class II HDACs, such as HDAC5, is indeed found here on many promoters following damage.

Adriamycin, TSA, and Cell Cycle Control—It has been reported that HDAC inhibitors, TSA and SAHA, increase the sensitivity to several anti-cancer drugs (60); interestingly, however, this effect is observed only when the inhibitors are incubated before the addition of DNA-damaging drugs, as if a general loosening of chromatin structure through histone hyperacetylation would predispose chromatin to better targeting. The findings presented here are consistent with this view and present a rationale for this effect. When given together with adriamycin, TSA is capable of reversing the transcriptional block of key G₂/M regulators (Fig. 8). Most importantly, treatment of cells with the non-apoptotic doses of adriamycin used here led to an increase in the population of cells in G₂/M, both in NIH3T3 and in HCT116 (Figs. 1A and 3A), whereas an addition of TSA partially reverted this block (Fig. 8A). Inactivation of p53 reduced the G₂/M effect (Fig. 3A), consistent with previous findings (12). It is possible that cells blocked for a prolonged period of time in G₂/M might be more prone to enter an apoptotic pathway; thus, rather than use an HDAC inhibitor, which if anything, counterbalances the block, one should associate drugs that enhance the G₂/M block or devise a protocol in which cells are presensitized by HDAC inhibitors to lower doses of DNA-damaging agents, as suggested by Kim *et al.* (60). Reagents specifically targeting Class I or Class II HDACs should be important in pointing to their relative contribution in cell cycle block and could be used to fine tune protocols for a better, and less toxic, sensitization to DNA-damaging agents.

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