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Role for Histone Deacetylase 1 in Human Tumor Cell Proliferation▽

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Posttranslational modifications of core histones are central to the regulation of gene expression. Histone deacetylases (HDACs) repress transcription by deacetylating histones, and class I HDACs have a crucial role in mouse, *Xenopus laevis*, zebra fish, and *Caenorhabditis elegans* development. The role of individual class I HDACs in tumor cell proliferation was investigated using RNA interference-mediated protein knockdown. We show here that in the absence of HDAC1 cells can arrest either at the G_1 phase of the cell cycle or at the G_2/M transition, resulting in the loss of mitotic cells, cell growth inhibition, and an increase in the percentage of apoptotic cells. On the contrary, HDAC2 knockdown showed no effect on cell proliferation unless we concurrently knocked down HDAC1. Using gene expression profiling analysis, we found that inactivation of HDAC1 affected the transcription of specific target genes involved in proliferation and apoptosis. Furthermore, HDAC2 downregulation did not cause significant changes compared to control cells, while inactivation of HDAC1, HDAC1 plus HDAC2, or HDAC3 resulted in more distinct clusters. Loss of these HDACs might impair cell cycle progression by affecting not only the transcription of specific target genes but also other biological processes. Our data support the idea that a drug targeting specific HDACs could be highly beneficial in the treatment of cancer.

In eukaryotic cells, the balance between acetylation and deacetylation is critical for gene transcription and for the functions of different cellular proteins. Histone acetylation is regulated by both histone acetyltransferases and histone deacetylases (HDACs), which are conserved from yeast (*Saccharomyces cerevisiae*) to humans. Mammalian HDACs have been grouped into three classes according to their homology to yeast proteins. The class I enzymes HDAC1 and HDAC2 are ubiquitously expressed and seem to be involved in more-general cellular processes. According to its sequence, HDAC3 also belongs to the class I family, but since it can interact with both class I and class II enzymes it could represent the functional link between the two families (11). Class II enzymes have tissue-specific functions, and class III enzymes are NAD dependent and are involved in the control of the life span of certain organisms (15, 16, 33). Recently, class IV, which includes HDAC11-related enzymes, has been added (12, 15). Because of the connection between transcriptional repression and HDAC recruitment, HDAC inhibitors may reverse silenced genes (reviewed in reference 30).

HDAC inhibitors are a new class of drugs with anticancer potential. They have been shown to induce apoptosis effectively in cancer cells and are currently in clinical trials for a variety of cancers (4, 40). One example includes the HDAC inhibitor suberoylanilide hydroxamic acid, which recently has been shown to induce polyploidy in human cancer cell lines (55). As we learn more about HDACs and the biological processes they regulate, a strong idea that the identities of the relevant target deacetylases need to be determined to aid in the development of HDAC inhibitors as anticancer agents is emerging. One clear example is the demonstration of HDAC2 induction upon loss of the adenomatosis polyposis coli tumor suppressor in colorectal tumorigenesis, providing a reason for treating certain carcinomas with HDAC inhibitors (58). Interestingly, it has been demonstrated previously that the HDAC inhibitor valproic acid selectively induces polyubiquitination and proteasomal degradation of HDAC2 (35). Lately, HDAC3 was found to be required for normal mitotic progression and maximal phosphorylation of histone 3 (H3) on Ser 10 (S10) in mitosis (38). Phosphorylation of H3 on S10 is a well-characterized and evolutionary conserved mitotic event (6, 17, 22, 23, 45).

Recent experiments with small interfering RNA (siRNA) implicated HDAC1 and HDAC3 in the regulation of proliferation and survival of cancer cells (13). In particular, the idea that HDAC1 can also be a potential target for therapeutic intervention in certain cancers stems from a series of studies. HDAC1 has been shown to inhibit estrogen receptor alpha protein expression and transcriptional activity, suggesting that it can modulate breast cancer progression (32). Overexpression of HDAC1 at mRNA and protein levels was reported for human gastric and prostate tumors (5, 18, 34, 44), whereas overexpression of HDAC3 is seen with colon tumors (54). Furthermore, published data indicate a role of mouse HDAC1 in the regulation of proliferation and development. For instance, the expression of HDAC1 is induced upon growth

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factor activation of mouse T cells and fibroblasts (3, 19) and HDAC1 levels were found to be elevated in highly proliferative tissues, embryonic stem (ES) cells, and several transformed cell lines (3, 37), suggesting a link between HDAC1 function and proliferation. In accordance with this idea, disruption of the HDAC1 gene resulted in reduced proliferation of mouse embryos and ES cells (37), whereas overexpression of HDAC1 led to impaired cell proliferation of murine fibroblasts (3). Taken together, these results suggest that class I HDACs are crucial in controlling the proliferation state of mammalian cells.

HDAC1 and HDAC2 often heterodimerize and are generally found in large multiprotein complexes: the Sin3, NuRD, and CoREST complexes (16). Numerous transcription factors, including regulators of cell cycle, differentiation, and development, have been shown to associate directly with HDAC1 and HDAC2 or with HDAC1/HDAC2 complexes, thereby mediating the repression of specific target genes (1, 7, 42). Furthermore, the loss of Sds3, a component of the Sin3/HDAC1/HDAC2 complex and a putative recruiting factor, led to cell death due to missegregation of chromosomes during mitosis (8). Therefore, both HDAC1- and HDAC1/HDAC2-mediated chromatin modifications seem to be important for cell cycle control and development. In light of this evidence, we conducted a study to analyze the effect of depleting class I deacetylases in human tumor cells by RNA interference (RNAi). We show here that by ablating either HDAC1 or HDAC3 protein expression there is an inhibition of tumor cell proliferation. Clearly, HDAC1 knockdown abolishes the ability of tumor cells to proceed through mitosis. On the contrary, HDAC2 knockdown shows no effect, unless we concurrently knock down both HDAC1 and HDAC2. In addition, we also conducted an analysis of the changes in gene expression in U2OS cells in response to knockdown of deacetylases and demonstrated that a large number of HDAC target genes are involved in proliferation and apoptosis.

The overall evidence therefore points to the idea that each individual class I HDAC plays a different role in regulating genes involved in various biological processes.

MATERIALS AND METHODS

Cell culture. U2OS and MCF7 cell lines were grown in Dulbecco's modified Eagle's medium (Cambrex) supplemented with 10% fetal bovine serum (Cambrex), antibiotics, and 2 mM L-glutamine (Cambrex). The MCF10A cell line was maintained in F12-Dulbecco's modified Eagle's medium (1:1) supplemented with 5% horse serum, 20 ng/ml epidermal growth factor, 0.5 μg/ml hydrocortisone, 50 ng/ml cholera toxin, 10 μg/ml insulin, and antibiotics. All cell lines were cultured in a humidified 37°C incubator with 5% CO_2.

Transfection of cells with siRNA, electrophoresis, and Western blotting. siRNAs were ordered from MWG BIOTECH and prepared according to the manufacturer's instructions (stock concentration of 20 μM in molecular-biology-grade water). Oligofectamine was used for all siRNA transfections (Invitrogen, San Diego, CA). The following siRNA sequences were used: 5′-CGUACGCG GAAUACUUCGATT-3′ for siRNA LUC (control), 5′-CAGCGACUGUUUG AGAACCTT-3′ and 5′-CUAAUGAGCUUCCAUACAATT-3′ for siRNA HDAC1, 5′-UCCGUAAUGUUGCUCGAUGTT-3′ for siRNA HDAC2, and 5′-GAUGCUGAACCAUGCACCUTT-3′ for siRNA HDAC3.

Oligofectamine reagent was diluted and used according to the manufacturer's instructions. The amount of siRNA used was optimized and fixed for each knockdown experiment. A second cycle of siRNA transfection was performed 24 h later. Cells were harvested 72 h after the first cycle of transfection and stored at −80°C until lysis in urea buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8).

After Western blotting, primary antibodies anti-HDAC1 (monoclonal mouse antibody; Upstate), anti-HDAC3 (ab 7030; Abcam), and anti-caspase-3 (H-277; Santa Cruz Biotechnology) were incubated with membrane overnight at 4°C; anti-HDAC2 (ab 7029; Abcam), anti-p21 (a kind gift from K. Helin's lab), anti-acetylated H3/H4 (in-house antibody), antitubulin (DM1A; Sigma), and anti-H3 (ab 1791; Abcam) were incubated for 2 h at room temperature. Proteins were visualized by chemiluminescence with ECL Western blotting detection reagents (Amersham Biosciences).

Growth curve. To determine the effects of siRNA knockdown on cell proliferation, 24 h after transfection, cells were collected by trypsinization, counted by use of a hemacytometer with trypan blue dye, and plated at 3,000 viable cells/well for U2OS and MCF7 or 2,000 viable cells/well for MCF10A into a 96-well tissue culture dish in a final volume of 200 μl. Every 24 h, a 96-well tissue culture dish was stained with crystal violet solution and left to dry. Then, crystal violet incorporated in the cellular membrane was solubilized with a solution of 10% acetic acid–phosphate-buffered saline (PBS) and the absorbance at 595 nm was measured with an MRX microplate reader (Dynatech). We calculated the increase (*n*-fold) of the absorbance as the ratio of absorbance at time *i*/absorbance at time zero (where time *i* is 48, 72, or 96 h after siRNA transfection and time zero is 48 h after transfection). Each experimental point is the average of three independent experiments, with the respective standard deviation.

Colony formation assay. Twenty-four hours after transfection, cells were collected by trypsinization, counted by use of a hemacytometer with trypan blue dye, and plated at 3,000 viable cells/well for U2OS and MCF7 or 2,000 viable cells/well for MCF10A into a six-well tissue culture dish. Six days after plating, cells were stained with crystal violet solution (1% crystal violet, 35% ethanol) and colonies were counted. We calculated the relative CFU as follows: number of colonies of each sample/number of colonies of the control. The average relative CFU of three plates was calculated, with the respective standard deviation.

Fluorescence-activated cell sorter (FACS) analysis of cell cycle. To detect HDAC1, phosphorylated S10 at H3 (H3-P), and DNA or HDAC1, cyclin A, and DNA, 72 h after the first cycle of transfection, both attached cells and supernatant were collected and resuspended at 10^6 cells/ml in 1% formaldehyde for 15 min. After PBS washing, the pellet was resuspended in ice-cold 70% ethanol and stored at 4°C. For immunodetection, cells were washed twice in PBS and permeabilized in 0.1% Triton X-100 in PBS for 10 min. After being blocked in 5% normal goat serum for 20 min, cells were incubated with anti-HDAC1 mouse monoclonal antibody (Upstate), 1:250, and anti-H3-P rabbit polyclonal antibody (Upstate) or anti-cyclin A (Pharmingen), 1:100, in PBS plus 1% bovine serum albumin for 2 h at room temperature. Cells were rinsed in PBS and incubated for 1 h with Cy5-conjugated goat anti-mouse (1:50; Jackson Immunoresearch) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibodies. Cells were washed again and resuspended in 1 ml of a solution containing 2.5 μg/ml propidium iodide (PI) (Sigma) in PBS and 250 μg/ml RNase in PBS and incubated overnight at 4°C before acquisition.

Samples were acquired on a FACSCalibur (Becton Dickinson) flow cytometer. At least 10,000 events were acquired. Analysis was performed using CellQuest 3.3 (Becton Dickinson).

FACS analysis for activation of caspase-3. For active caspase-3 analysis, 96 h after the first cycle of transfection, both attached cells and supernatant were collected, fixed in 1% formaldehyde in PBS and then in 70% cold ethanol, and permeabilized in 0.1% Triton X-100 for 10 min. Prior to incubation with anti-caspase-3 polyclonal antibody (9661L; Cell Signaling), cells were incubated for 30 min with 10% goat serum in PBS. After being washed, cells were incubated with anti-rabbit FITC antibody (Sigma) for 1 h at room temperature (light protected). The labeled cells were stained overnight at 4°C in 2.5 μg/ml PI and RNase 250 μg/ml in PBS. The cellular suspension was passed through a FACSCalibur (Becton Dickinson) flow cytometer. At least 10,000 events were acquired, and analysis was performed using CellQuest 3.3 (Becton Dickinson) and ModFit LT 3.1 software.

Cell synchronization. Twenty-four hours after the first cycle of siRNA transfection (see above), cells were synchronized in the G_1/S transition by treatment with 2 mM thymidine for 14 h, released for 8 h, and then treated again with thymidine for 14 h. After two washes with PBS, cells were cultured in fresh medium or in medium with 0.1 μg/ml nocodazole for different times (as indicated in each experiment) and harvested. Cells were synchronized in prometaphase by treatment with 0.1 μg/ml nocodazole for 16 h and then released from the drug-induced cell cycle block by being washed three times with PBS.

Time-lapse microscopy. Time-lapse microscopy was performed by use of a Cell R imaging station for live-cell microscopy (Olympus) mounted on an IX81 inverted microscope (Olympus) equipped with an incubation chamber (Evotec). The system includes a Hamamatsu ORCA-ER charge-coupled-device camera

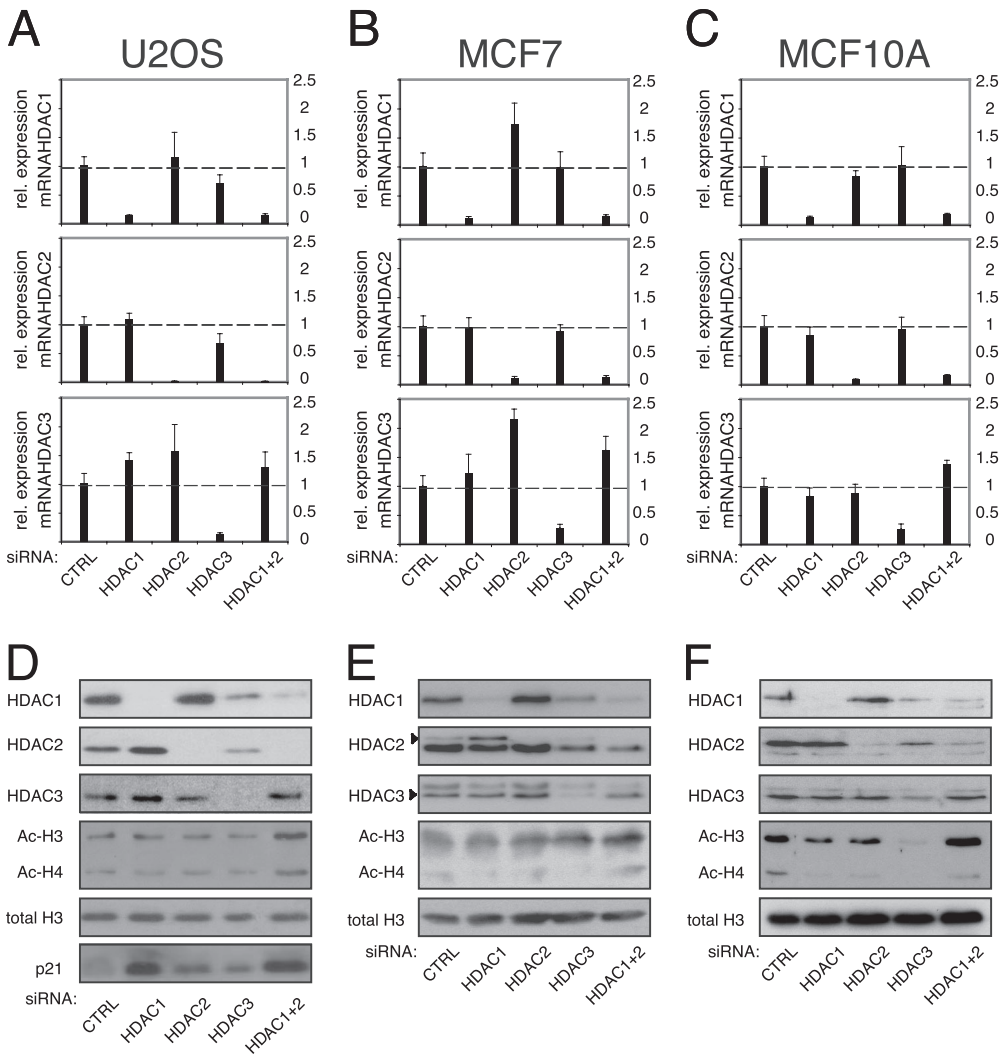

FIG. 1. siRNA-mediated knockdown of HDAC1, HDAC2, and HDAC3 in U2OS, MCF7, and MCF10A cells. The loss of both HDAC1 and HDAC2 produced a hyperacetylation of histones H3 and H4. (A to C) Relative (rel.) expression levels of the indicated mRNAs were determined by real-time PCR, as described in Materials and Methods. Averages of three independent experiments are shown, and standard deviations are indicated by the error bars. (D to F) Western blots of U2OS whole-cell extracts after transfection of siRNA HDAC1, HDAC2, HDAC3, and HDAC1 plus HDAC2. Cells were transfected and processed as described in Materials and Methods, and 30 μg was loaded per lane. As a control (CTRL), we used transfection of antiluciferase siRNA.

for image acquisition. A movie montage was performed using ImageJ image analysis software (W. Rasband, National Institutes of Health, Bethesda, MD).

Statistical immunofluorescence. Forty-eight hours after siRNA transfection, cells were plated on four-chamber slides pretreated with 15 μg/ml poly(D)lysine (Sigma) and treated with 0.1 μg/ml nocodazole for 16 h to enrich the mitotic population. Slides were fixed in 4% paraformaldehyde–1× PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] for 10 min, permeabilized with 0.1% Triton X-100 and 0.2% bovine serum albumin in PBS, and processed using standard protocols. The primary antibodies used were anti-HDAC1 monoclonal (Upstate) at 1:250 and anti-H3-P rabbit polyclonal (Upstate) at 1:100, and the conjugated secondary antibodies were Cy3-conjugated goat anti-mouse (Jackson Laboratories) and FITC-conjugated goat anti-rabbit (Sigma). DAPI (4′,6′-diamino-2-phenylindole) (Sigma) was used for nuclear counterstaining. Fluorescence microscopy images were acquired using a BX61 (Olympus) motorized fluorescence microscope. Acquired images were visualized using a browser developed with the ImageJ macro language. After acquisition of DAPI, Cy3, and FITC channels, DAPI staining was employed to identify cell nuclei and to build a mask for successive calculations. The mean nuclear levels of DAPI, HDAC1, and H3-P were stored in the memory to allow later statistical analysis. HDAC1 fluorescence distribution was evaluated in control- and HDAC1-interfered cells in order to establish an arbitrary threshold used to discriminate HDAC1-positive (gate II) and HDAC1-negative (gate I) cells. H3-P fluorescence distribution was evaluated in gate I and gate II to fix a value above which cells were H3-P positive and below which they were negative. We evaluated for both gate I and gate II the percentages of double positive cells.

Extraction of RNA and reverse transcription-PCR. For validation of the microarray results, we employed independent RNA preparations of the samples described above. Total RNA was isolated by using a QIAGEN RNeasy Protect mini kit as described by the manufacturer. cDNA was generated by reverse transcription-PCR with PE Applied Biosystem TaqMan reverse transcription reagents. Relative levels of specific mRNA were determined with a 5′ nuclease assay (TaqMan) chemistry system. All PCRs were performed with an ABI 7900HT sequence detection system. The GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene was used as a control gene for normalization.

Microarray analysis. Biotin-labeled cRNA targets were obtained from 5 μg of total RNA derived from samples as described above. cDNA synthesis was performed with a Gibco SuperScript custom cDNA synthesis kit, and biotin-labeled antisense RNA was transcribed using an in vitro transcription system (Ambion, Inc., Austin, TX) including Bio-11-UTP and Bio-11-CTP in the reaction (NEN Life Sciences, Boston, MA). GeneChip hybridization, washing, staining, and

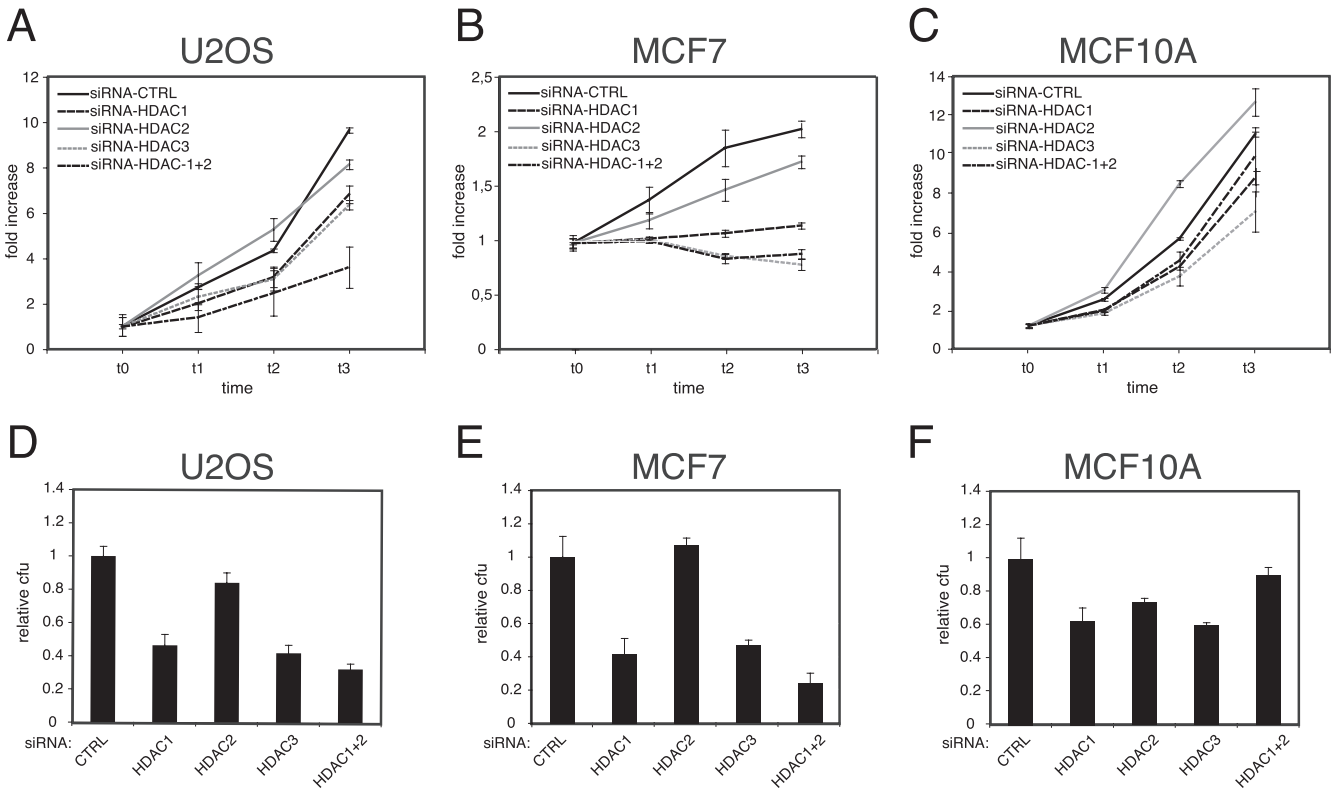

FIG. 2. Absence of either HDAC1 or HDAC3 causes a defective proliferation. (A to C) Growth curves of U2OS, MCF7, and MCF10A transfected with HDAC siRNAs. The increase of absorbance at 595 nm is directly correlated with cell proliferation, as described in Materials and Methods. Each curve represents the average of three independent experiments, and the standard deviation is indicated by the error bars. (D to F) CFU assay of U2OS, MCF7, and MCF10A cells transfected with HDAC siRNAs. The ratio of the CFU number of each sample/CFU number of the control was calculated. Each column represents the average of three independent experiments, with the respective standard deviations. CTRL, control.

scanning were performed according to Affymetrix protocols (Santa Clara, CA). Two copies of the HG-U133Plus GeneChip were hybridized with each target. Full details of microarray methods are described in the supplementary data posted at http://bio.ifom-ieo-campus.it/supplementary/MCB49407/.

RESULTS

siRNA-mediated knockdown of HDAC1 to HDAC3. We established the methodology for HDAC class I knockdown in various cell lines: U2OS (an osteosarcoma cell line), MCF7 (a mammary carcinoma cell line), and MCF10A (an epithelial mammary gland cell line). As shown in Fig. 1A to F, the expression levels of these HDACs were efficiently knocked down by the respective siRNAs. It is interesting to note that the levels of endogenous HDAC1 and HDAC2 in all three cell lines increase in opposition to one other (Fig. 1D to F), i.e., upon knockdown of one the other one increases at the protein level. On the other hand, we observed a decrease of both HDAC1 and HDAC2 upon HDAC3 knockdown, but only at the protein level. We still do not know the reason for this result, but preliminary data indicate that it is proteasome dependent. Nevertheless, reduced expression of HDAC1 and HDAC2 could also be a consequence of the reduced cellular viability after HDAC3 knockdown (Fig. 2).

The absence of HDAC1 induces p21 expression only in the U2OS cell line, and the absence of both HDAC1 and HDAC2 increases histone acetylation. One important proliferation-related function of HDAC1 is to prevent the inappropriate expression of the cyclin-dependent kinase (CDK) inhibitors p21 and p27 (37). In accordance with this model, the promoters of the genes for p21 and p27 are hyperacetylated in HDAC1 null cells (37). In addition, HDAC1 can be recruited to the p21 gene by the transcription factor SP1 (36) and Tbx2 (51) and p21 is one of the genes upregulated after HDAC inhibitor treatment (29, 30, 47, 48). Therefore, we analyzed endogenous p21 protein levels after RNAi treatment. Figure 1D indicates that p21 levels increase in the absence of either HDAC1 alone or HDAC1 plus HDAC2 in U2OS cells, in accordance with HDAC1 null embryo results (37) and with the effect of HDAC inhibitors (47). Conversely, in both MCF7 and MCF10A, we did not observe any changes in p21 levels after HDAC1 or HDAC1 and HDAC2 knockdown (data not shown). Interestingly, only in the absence of both HDAC1 and HDAC2 did Western blot analysis with modification-specific antibodies reveal increased acetylation levels of a subset of histones H3 and H4 (Fig. 1D to F) in all three cell lines. This result could indicate that these histones are substrates for both HDACs and that only the concurrent double knockdown of HDAC1 plus HDAC2 leads to their hyperacetylation. We also noted that in our system specific knockdown of any HDAC did not affect basal levels of p53 or its acetylation (data not shown), reflecting the idea that multiple deacetylases are involved in regulating p53 acetylation (28, 39, 52).

siRNA-mediated knockdown of HDAC1 and HDAC3 diminishes cellular viability and proliferation. It is known that HDAC inhibitors lead to p21 induction. They also induce cell cycle arrest in either G_1 or G_2/M and apoptosis, killing tumor cells (4, 29, 48). The pathway leading to tumor cell death is still unknown, but it is very unlikely that a single molecular pathway will be identified in all cell types for all HDAC inhibitors. We decided to investigate cellular viability and proliferation of U2OS, MCF7, and MCF10A cells after HDAC knockdown. Clearly, the absence of HDAC1, HDAC3, and HDAC1 plus HDAC2 generated a remarkable defect in proliferation in both U2OS and MCF7 cell lines (Fig. 2A and B) but had an almost unnoticeable effect in the MCF10A cell line (Fig. 2C). The lack of HDAC2 had a much less pronounced effect on all cells (Fig. 2A to C). This is inconsistent with published data but is probably due to the different cell lines used (24). We then analyzed the effect of the absence of all three HDACs on the life spans of all three cell lines. In correlation with the results in Fig. 2A to C, cells lacking HDAC1, HDAC3, or the combination HDAC1 plus HDAC2 had a diminished capacity to form colonies when plated at a low density (Fig. 2D to F). These data suggest that both HDAC1 (either alone or in combination with HDAC2) and HDAC3 have important roles in the proliferation of tumor cells, in agreement with published data indicating a relevant role of murine HDAC1 for the regulation of proliferation and development (37). An HDAC3-AKAP95/HA95-Aurora B pathway regulating mitosis has been demonstrated recently, explaining the mitotic defect observed after HDAC3 siRNA (38). It is interesting to note that in the cell line MCF10A, the untransformed counterpart of MCF7, the absence of all three HDACs had less effect on cellular proliferation.

HDAC1 knockdown cells have a marked reduction of H3 S10 phosphorylation. H3-P is a mitotic marker. To explore whether the defect in proliferation of HDAC1 knockdown cells was due to a mitotic defect, we applied single-cell statistical analysis to both control-interfered and HDAC1-interfered cells by using an anti-HDAC1 antibody and an anti-H3-P antibody (see Materials and Methods). We analyzed all three cell lines for mitotic cells in the presence and absence of HDAC1 (Fig. 3). Figure 3A, B, and C show histograms reporting the distributions of HDAC1 levels in control-interfered populations and HDAC1-interfered populations, as described in Materials and Methods. As shown in the upper panels, we were able to discriminate between HDAC1-positive cells (gate II) and HDAC1-negative cells (gate I). The middle panels represent the distributions of H3-P levels in the two gates in order to define the basal level of H3-P-positive cells. In the lower panels, the correlation between HDAC1 and H3-P levels in the two gates is represented. Interestingly, knockdown of HDAC1 led to a clear reduction in the number of H3-P-positive cells, suggesting that HDAC1 could be important for proper cell cycle progression. Again, the effect was not as drastic in the MCF10A cell line (Fig. 3C).

HDAC1 knockdown cells can either arrest in G_1 or die during G_2/M transition. We next tested whether HDAC1 knockdown affects cell cycle progression. U2OS cells were synchronized by a double thymidine block at the G_1/S transition, and cell cycle progression upon removal of thymidine was monitored by FACS analysis of PI-stained DNA (Fig. 4A, top). The bottom panel of Fig. 4A represents a biparametric analysis, done using anti-H3-P and PI staining levels in U2OS cells. This analysis shows an increase in G_1 cells in the absence of HDAC1 at all time points after thymidine release, accompanied by a lower percentage of mitotic cells.

Next, we released U2OS cells from the double thymidine block with nocodazole-containing culture medium to enrich the mitotic population. Biparametric analysis using anti-cyclin A, a G_2 marker, and PI staining (Fig. 4B) showed that by 24 h control cells were blocked in mitosis as expected but that HDAC1 knockdown cells were unable to remain in mitosis. In fact, over time, there was a larger amount of G_1/S HDAC1-negative cells, accompanied by a significant decrease of mitotic cells and increase of sub-G_1 cells.

We next analyzed the proportion of sub-G_1 cells after release from the nocodazole block. As shown in Fig. 4C, we observed massive apoptosis in HDAC1-negative U2OS cells. Taken together, these data demonstrate that the proliferative defect of HDAC1 knockdown cells is due to both partial G_1 arrest and cell death either at the G_2/M transition or at the very beginning of mitosis. Alternatively, HDAC1-deficient cells could become insensitive to nocodazole and grow to be G_1 tetraploids. However, when we followed living cells by time-lapse microscopy, we observed the inability of HDAC1-negative cells to condense and enter mitosis, suggesting that these cells die during G_2/M transition (Fig. 4D). Our data confirm and extend published findings showing that HDAC inhibitors induce G_1 and/or G_2/M arrest and/or apoptosis (4).

Induction of apoptosis in HDAC1-deficient U2OS cells. The observation that there were differences in the numbers of apoptotic cells after HDAC1 knockdown prompted us to confirm apoptotic cell death by examining caspase-3 activation, both by FACS analysis (Fig. 5A) and by Western blot analysis (Fig. 5B) (see Materials and Methods). Our results indicate that caspase-3 activation is involved in apoptosis after HDAC1 knockdown.

Comparison of gene expression profiles after depletion of HDAC1, HDAC2, HDAC3, and HDAC1 plus HDAC2. We then analyzed the gene expression profiles of U2OS cells upon loss of HDAC1, HDAC2, HDAC3, or HDAC1 plus HDAC2 by using HG-U133 Plus Affymetrix oligonucleotide chips, which explore the expression of approximately 54,000 human transcripts. Results were analyzed using GCOS and further elaborated with GenePicker and GeneSpring software (see the supplementary data posted at http://bio.ifom-ieo-campus.it/supplementary/MCB49407/) (2, 10). A detailed description of microarray methods, data analysis and elaboration, and comparative results can be found in the supplementary data at the URL mentioned above.

Ablation of HDAC1, HDAC2, HDAC3, or HDAC1 plus HDAC2 resulted in the altered expression of 987, 283, 1,317, or 601 genes, respectively (see supplementary Table 1 posted at http://bio.ifom-ieo-campus.it/supplementary/MCB49407/). There is a large degree of overlap between the lists of genes regulated by siRNA of HDAC1 and those regulated by siRNA of HDAC1 plus HDAC2. Ablation of HDAC2 results in a mild phenotype and, compared to the other conditions, is associated with the deregulated expression of few genes, 86% of which are also deregulated under one of the two above-mentioned conditions. Only 31% of genes deregulated by HDAC3 siRNA are

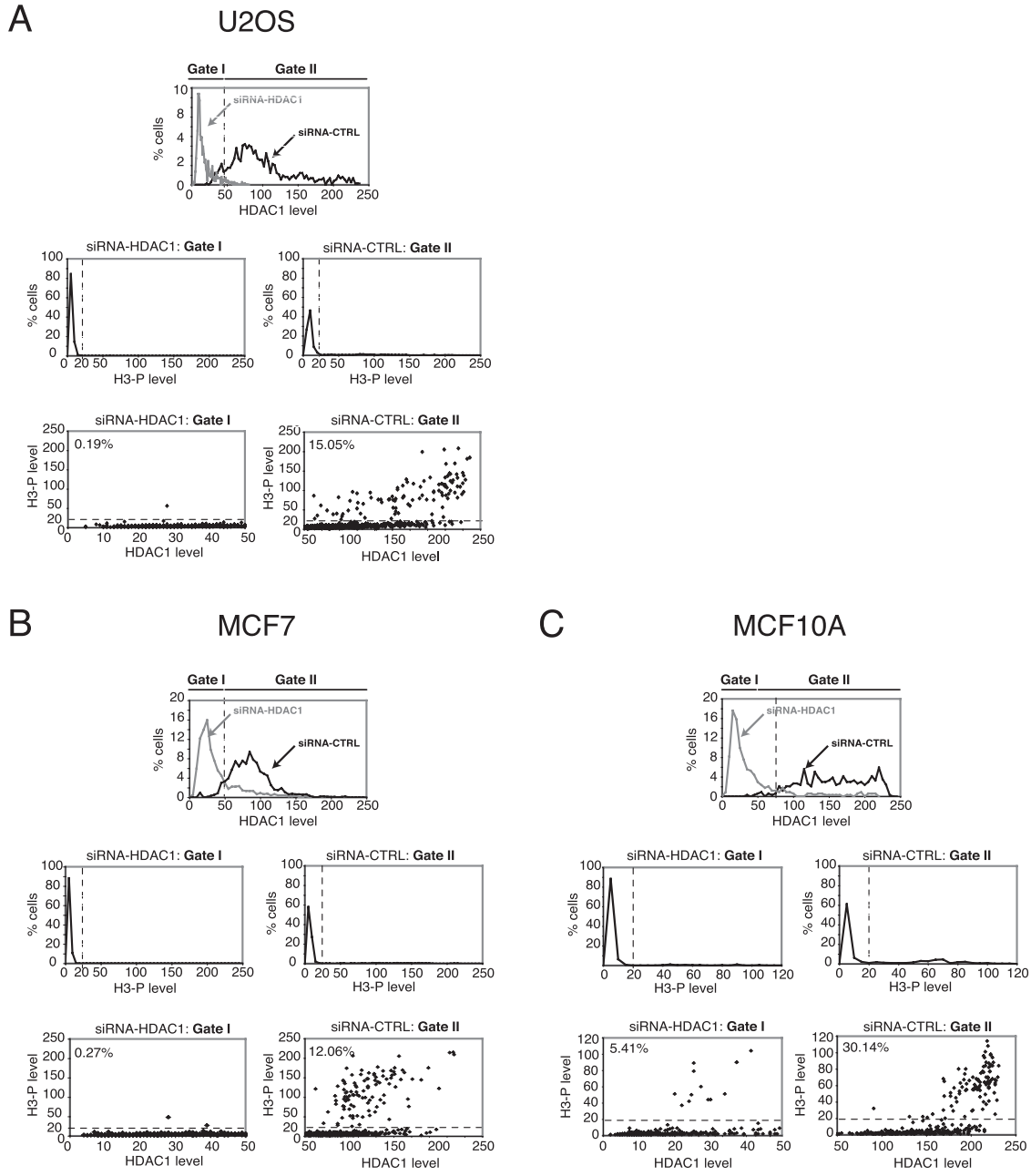

FIG. 3. Absence of the mitotic marker H3-P in HDAC1-deficient cells. U2OS, MCF-7, and MCF10A cells were analyzed for the presence of H3-P. (Top) Distribution of the HDAC1 signal over the siRNA-control (CTRL)-transfected and siRNA-HDAC1-transfected populations. The dashed line represents the arbitrary threshold used to distinguish HDAC1-negative (gate I) from HDAC1-positive (gate II) cells. (Middle) Distribution of H3-P signals in gate I and gate II. The dashed line represents the arbitrary threshold used to distinguish between positive and negative H3-P cells. (Bottom) Scatter plot of HDAC1 signal versus H3-P signal. The percentages inside the plot indicate the percentages of double positive cells.

in common with those regulated by siRNA of HDAC1 or HDAC1 plus HDAC2 (see the supplementary data and supplementary Table 1 posted at the URL mentioned above). Hierarchical clustering using any of the lists of regulated genes confirms there is a higher degree of similarity in the gene expression profiles resulting from ablation of HDAC1, HDAC2, or HDAC1 plus HDAC2 than in those resulting from ablation of HDAC3 (Fig. 6).

We next performed a functional classification of genes deregulated upon knockdown of HDAC1 or HDAC1 plus HDAC2, according to Gene Ontology Biological Process categories (for a complete description, see the supplementary data posted at http://bio.ifom-ieo-campus.it/supplementary/MCB49407/). To investigate the molecular basis of HDAC1-mediated growth arrest, we focused our attention on two functional categories: apoptosis and proliferation. We found 36 probe sets (corresponding to 28 nonredundant genes) that were classified as relevant to apoptosis and 100 probe sets

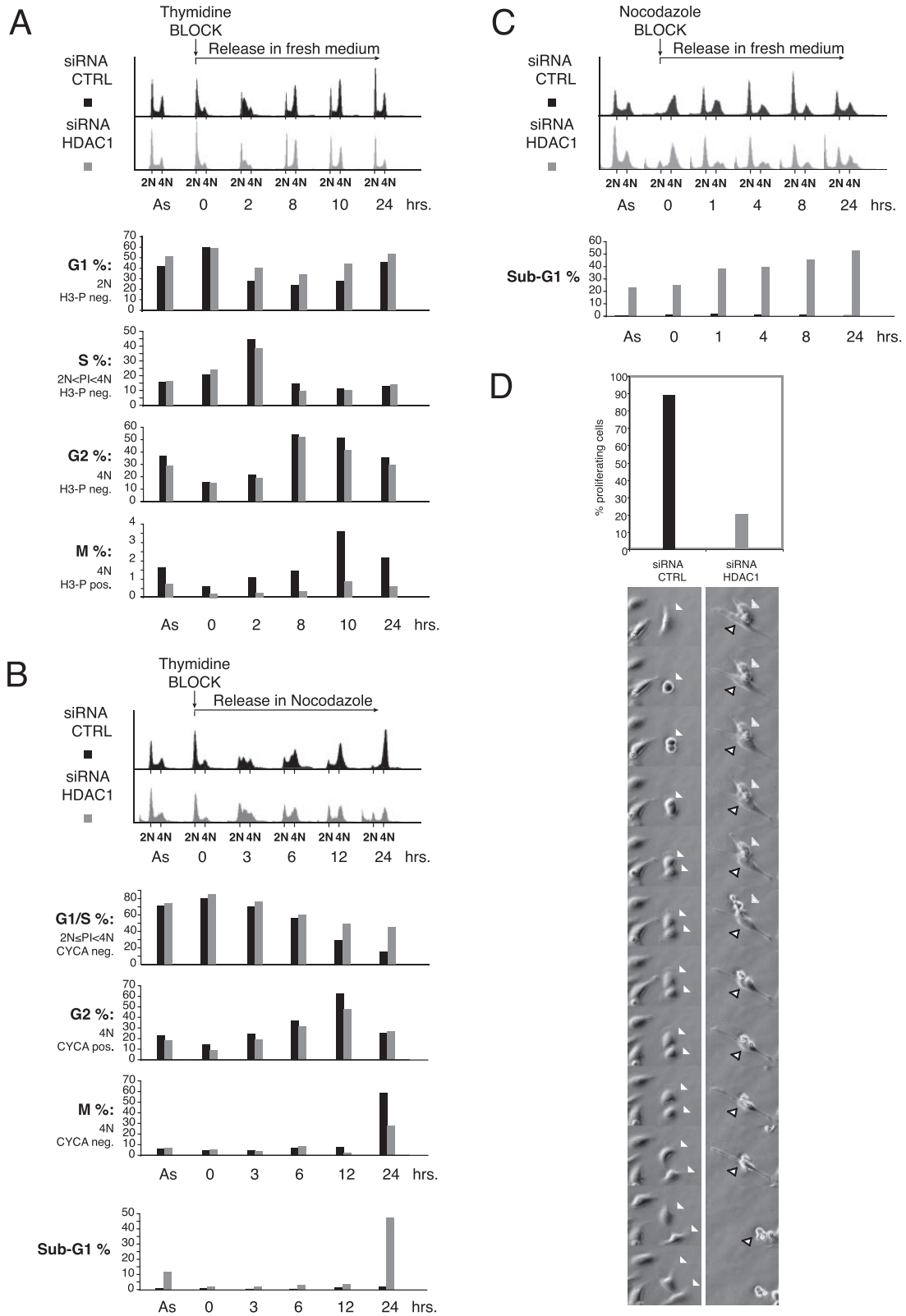

FIG. 4. HDAC1-deficient cells arrest in G_1 or die during G_2/M transition. (A) Asynchronous (As) siRNA-control (CTRL)-transfected (black) and siRNA-HDAC1-transfected (gray) U2OS cells were blocked in G_1/S transition by use of thymidine and then released in fresh medium. (Top) DNA content was analyzed by FACS analysis of PI-stained cells at different time points after the release in fresh medium. (Bottom) Biparametric FACS analysis using anti-H3-P and PI staining was performed to calculate the percentages of cells in G_1, S, G_2, and M phases at each time point. (B) U2OS control- and HDAC1-interfered cells were blocked in G_1/S transition by use of thymidine and then released in medium containing nocodazole. (Top) DNA content was analyzed by FACS analysis of PI-stained cells at different time points. (Middle) Biparametric FACS analysis

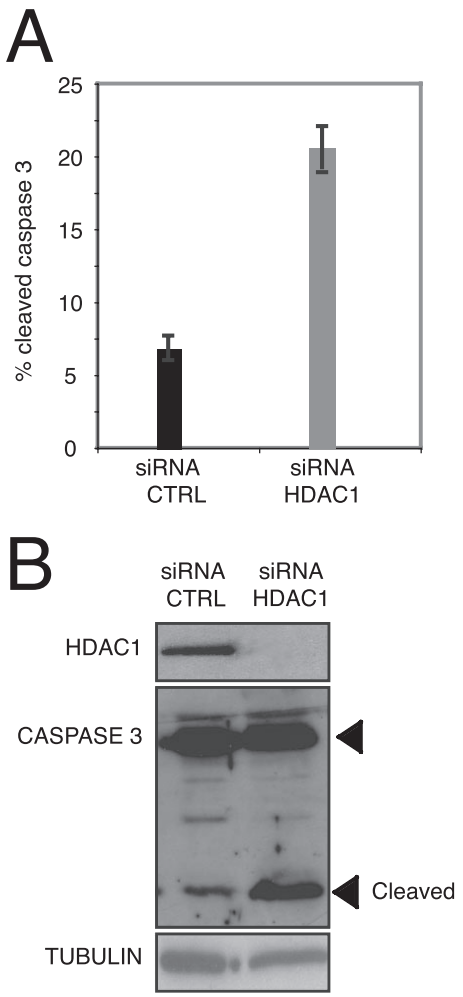

FIG. 5. HDAC1 knockdown cells undergo apoptosis. (A) Effect of siRNA HDAC1 on caspase-3 activation by FACS analysis. The bars represent the percentages of cleaved caspase-3-positive cells in control (CTRL) and siRNA-HDAC1-transfected U2OS cells after 96 h. The values correspond to the averages of three independent experiments, and the error bars indicate the associated standard deviations. (B) Western blots of U2OS whole-cell extracts after transfection of control or siRNA HDAC1. Cells were collected 120 h after transfection, and 50 μg of lysates was loaded per lane. As a control, we used an injection of antiluciferase siRNA. The anti-caspase-3 antibody used was able to recognize both full-length inactive and cleaved active forms.

(corresponding to 72 nonredundant genes) relevant to proliferation. Three genes were present in both categories (F2R, interleukin-1A [IL1A], and SPP1 genes). Figure 7 shows a graphical representation of the behavior of these genes in U2OS cells lacking HDAC1 or HDAC1 plus HDAC2 com-

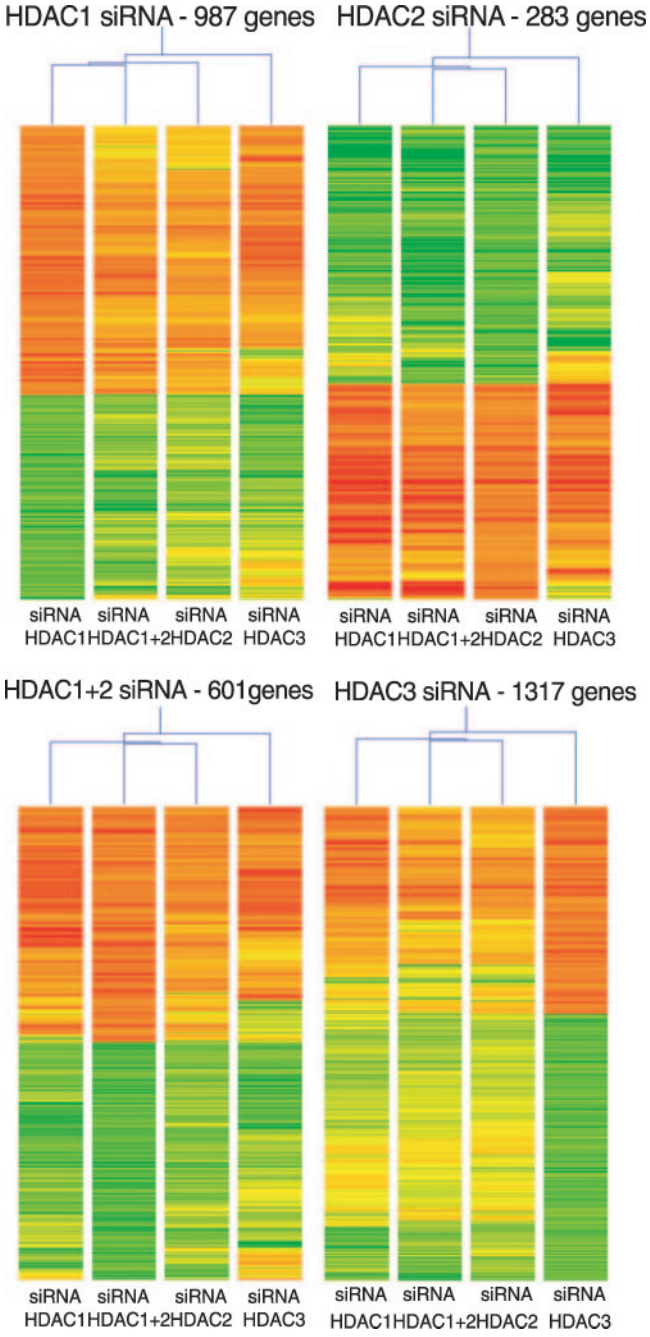

FIG. 6. Gene expression profiles. Supervised hierarchical clustering (according to Pearson's correlation) was performed using comparative expression values of the gene lists indicated above each dendrogram (for gene lists, see supplementary Table 1 posted at http://bio.ifom-ieo-campus.it/supplementary/MCB49407/). In all cases, the expression value for the control cells was used as the baseline (value of 1).

using anti-cyclin A (CYCA) and PI staining was performed to calculate the percentages of cells in G_1/S, G_2, and M phases at each time point. (Bottom) DNA content was analyzed by FACS analysis to evaluate the percentages of sub-G_1 cells. (C) U2OS control- and HDAC1-interfered cells were blocked in G_2/M transition by use of nocodazole and then released in fresh medium. (Top) DNA content was analyzed by FACS analysis on PI-stained cells at different time points. (Bottom) DNA content was analyzed by FACS analysis to evaluate the percentages of sub-G_1 cells. (D) (Top) Percentages of cells in the control- and HDAC1-interfered population able to undergo mitosis at least once in 30 h. (Bottom) Images of time-lapse experiments with U2OS siRNA-CTRL-transfected and siRNA-HDAC1-transfected cells. Control cells experience normal mitosis (white arrowheads), while HDAC1 knockdown cells cannot enter in mitosis and they die (yellow and black/white arrowheads).

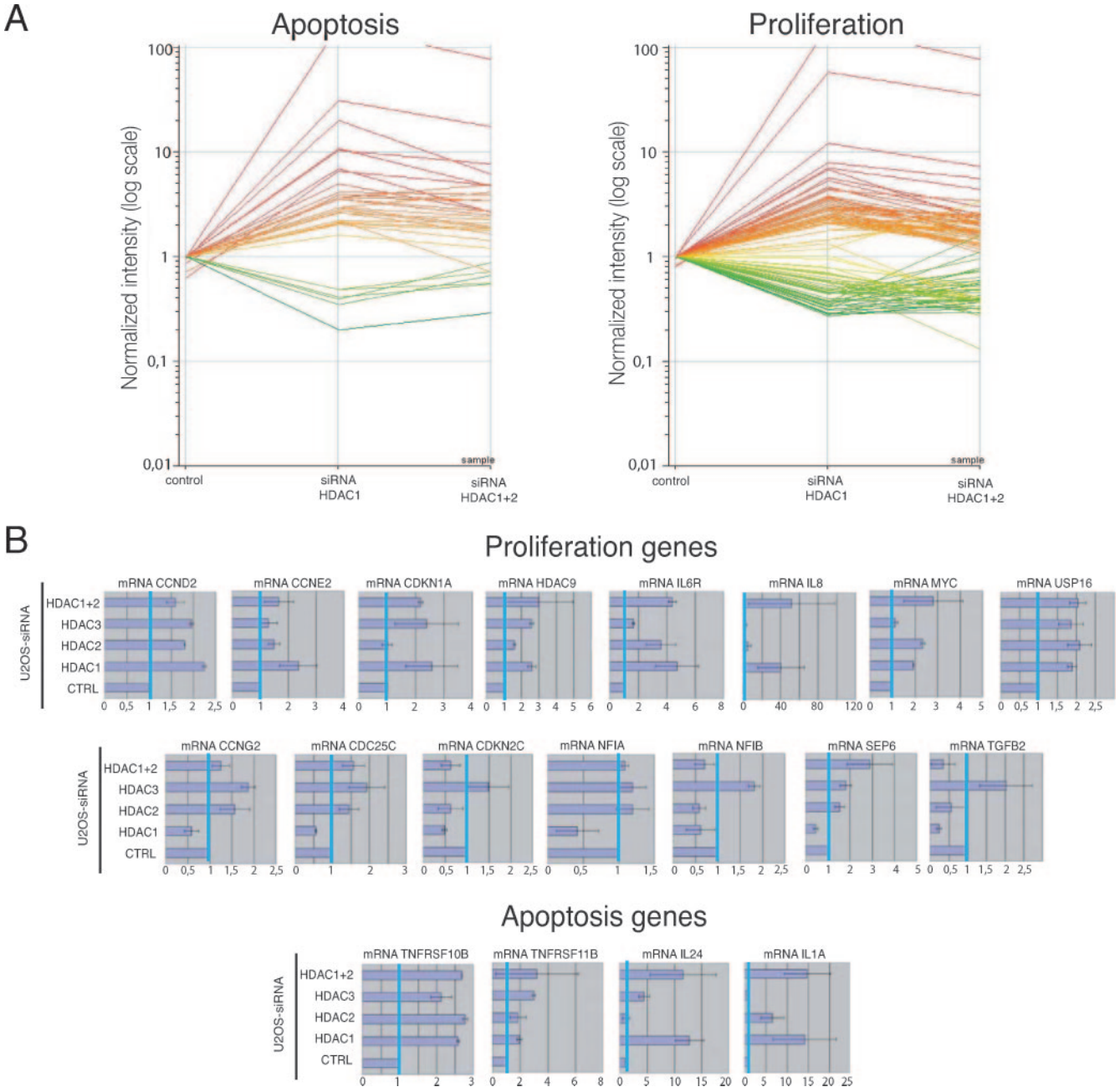

FIG. 7. Gene expression profiles and quantitative real-time PCR. (A) Graphic representation of the expression patterns of genes functionally involved in apoptosis and proliferation regulated by ablation of HDAC1 or HDAC1 plus HDAC2 in U2OS cells. (B) Validation of microarray data by TaqMan quantitative real-time PCR. The error bars indicate the standard deviations from three independent experiments. CTRL, control.

pared to the behavior in vector-transfected U2OS cells (control). Clearly, more genes involved in apoptosis were induced than repressed by RNAi of HDAC1 and/or HDAC1 plus HDAC2. Multiple proapoptotic genes, including FAS, BCL2-like 1, and members of the tumor necrosis factor (TNF) family receptors and ligands (TNFRSF10B, TNFRSF11B, and TNF RSF12A), were activated. Conversely, approximately equal numbers of genes involved in cell proliferation were up- and downregulated. The complete gene lists can be found in supplementary Tables 1 and 2 posted at http://bio.ifom-ieo-campus.it/supplementary/MCB49407/. To verify changes in gene expression detected by our microarray analysis, by using independent RNA preparations of the described experimental conditions, we performed quantitative real-time PCR analysis on 19 genes whose expression was altered and which regulate proliferation and apoptosis (cyclin D2 [CCND2], CCNE2, CDKN1A [p21], HDAC9, IL6R, IL-9, MYC, ubiquitin-specific peptidase 16 [USP16], CCNG2, cell division cycle 25C [CDC25C], CDKN2C, nuclear factor 1A [NF1A], NFIB, septin 6 [SEP6], transforming growth factor β2 [TGFB2], TNFRSF10B,

TNFRSF11B, IL-24, and IL1A genes). As shown in Fig. 7, there was a high correlation between the microarray and the real -time data for all 19 genes.

We also found some consistency with a recent study identifying several genes within both apoptosis and cell proliferation pathways regulated by two different HDAC inhibitors over a 16-h culture treatment (46). Although with siRNA experiments it is difficult to discern between early and late events, it is interesting to note that this study identified that, for example, the cell division cycle gene CDC25C and genes encoding TNF family receptors and ligands were modulated by both HDAC inhibitors (46). Altogether, these findings support the idea that HDAC inhibition affects the expression of genes within pathways that regulate tumor cell growth and survival.

DISCUSSION

HDAC inhibitors induce cell cycle arrest, apoptosis, or differentiation in tumor cells, and a number of them have entered early-phase clinical trials. Based on the phenotype of HDAC1-deficient embryos and ES cells, HDAC1 was implicated in proliferation control (37). In this study, we show that HDAC1 has an important role in the proliferation of human tumor cells and that it influences their capacity to undergo mitosis. Although our study also shows that the absence of HDAC3 has a drastic effect on cell proliferation, we directed our attention mostly to the HDAC1 knockdown phenotype. Clearly, our microarray data indicate that HDAC3 favors more than a few uniquely expressed genes, compared to HDAC1 and HDAC1 plus HDAC2 (Fig. 6) (also see supplementary Table 1 posted at http://bio.ifom-ieo-campus.it/supplementary/MCB49407/), suggesting different and specific pathways of action. Recent work has clearly shown that HDAC3 is part of a novel HDAC pathway regulating mitosis by Aurora B kinase activity modulation (38). The aim of this study was to define in detail the role of HDAC1 in tumor cells and, in parallel, to provide molecular insight into HDAC1 functions by conducting a global gene expression study in the U2OS cell line. It is known that the effects of HDAC inhibitors are cell line specific and can be HDAC inhibitor selective (14), providing ground for detailed studies of which HDAC is responsible for which phenotype in the cell line of interest. It has also been demonstrated that the expression profiles induced by HDAC inhibitors are cell line dependent and that, since the expression patterns of HDACs vary from cell line to cell line, we can anticipate different effects upon knockout of any particular HDAC (4, 14). In U2OS and MCF7 cells, the absence of HDAC1 had a remarkable effect on both apoptosis and proliferation and this phenotype was exacerbated by the simultaneous lack of HDAC2. Interestingly, MCF10A, the untransformed counterpart of the MCF7 cells, was less sensitive to the lack of HDAC1. In fact, the effects on both proliferation and cell cycle were not as evident (Fig. 2 and 3). These results are in agreement with the idea that HDAC inhibitors have antiproliferative and proapoptotic properties mainly in transformed cells (4, 41). We also identified caspase-3 as a mediator of the apoptosis provoked by the absence of HDAC1. Caspase-3 is a proapoptotic enzyme and triggers apoptosis through both the intrinsic and the death ligand pathways (20, 21). It is therefore a very good apoptosis indicator. However, the most striking effect we observed was the lack of mitotic cells in the absence of HDAC1 (Fig. 3 and 4). This result implies that HDAC1 is essential for mitosis, a yet-undescribed observation. As expected by published data showing that HDAC6 is the major tubulin deacetylase and that HDAC1 has no specific tubulin deacetylase activity (25, 57), we did not observe changes in tubulin acetylation following siRNA-mediated knockdown of HDAC1 to HDAC3 (data not shown). All cells interfered against HDAC1 had a nuclear appearance typical of interphase or apoptosis. Taken together, these data suggest that human tumor cells lacking HDAC1 cannot enter mitosis and therefore undergo apoptosis through caspase-3 activation. Future work will further characterize the mechanism of mitotic arrest. Our data are, however, consistent with studies pointing to the importance of histone deacetylation in the formation of pericentric heterochromatin (9, 50) and showing that loss of a component of the Sin3/HDAC1/HDAC2 complex leads to cell death due to missegregation of chromosomes during mitosis (8). Interestingly, loss of HDAC1 in untransformed cells, such as ES cells, fibroblasts, and neurons, does not induce apoptosis (37; G. Lagger, R. Grausenburger, and C. Seiser, unpublished data), perhaps because of a possible checkpoint activation in these cells or compensation by HDAC2.

Based on our microarray results, the analysis of global gene expression profiles of U2OS cells in the absence of HDAC1, HDAC2, HDAC3, or HDAC1 plus HDAC2 yielded a large number of genes that present altered expression levels (see the supplementary data posted at http://bio.ifom-ieo-campus.it/supplementary/MCB49407/). HDAC1 and HDAC1 plus HDAC2 knockdown regulated a largely overlapping set of genes, including most of those regulated by HDAC2 knockdown. HDAC3 knockdown caused the deregulation of a vast number of genes. Interestingly, 50% of genes induced by HDAC3 knockdown were also induced by ablation of HDAC1 or HDAC1 plus HDAC2 expression, whereas only 21% of genes repressed by HDAC3 knockdown were in common with the other conditions. These results could be a consequence of the protein reduction of HDAC1 and HDAC2 we observed after HDAC3 knockdown (Fig. 1).

Again, we analyzed in greater detail the genes regulated by HDAC1. In accordance with the proposed role of HDAC1 as a transcriptional repressor, more HDAC1 target genes were upregulated than downregulated in the absence of HDAC1. Interestingly, this result evened out when both HDAC1 and HDAC2 were ablated. This is in line with several reports that have demonstrated the independent recruitment of HDAC1 and HDAC2 to target genes (26, 49).

Because of the phenotype observed in the absence of HDAC1 (and HDAC1 plus HDAC2), we validated genes involved in apoptosis and proliferation. Notably, a number of cyclins, cyclin-dependent kinases, and the cyclin-dependent kinase inhibitor p21, whose expression is tightly coordinated to regulate appropriate cell cycle progression, were induced after RNAi treatment against HDAC1. p21 has been regarded as an attractive therapeutic target in human cancer. Although a plethora of data regarding the role of this multifunctional protein in many cellular pathways has emerged, the precise mechanism by which p21 regulates cell cycle progression is still unknown, partly because p21 can act in both a positive and a negative fashion toward cell proliferation (53). Given that p21

expression is increased upon HDAC1 knockdown in U2OS cells but not in MCF7 cells, we would argue that the mitotic phenotype observed in these two tumor cell lines is not linked to p21. Our microarray data also showed repression of CDC25C and septin 6. In *Saccharomyces cerevisiae*, the septins are a family of cell division cycle regulatory proteins and have been associated with actin stress fibers in interphase cells, the cleavage furrow of dividing cells, and the bud neck of budding yeast (reviewed in reference 31). The human homologue of septin 6 has been found to be fused to MLL in a few cases of infant acute myeloid leukemia (43). Cdc25C phosphatase is a major cell cycle regulator in mammals. Its C-terminal catalytic phosphatase domain dephosphorylates Cdc2 to promote G_2/M transition (27), whereas the N-terminal regulatory domain contains multiple phosphorylation sites. Multisite phosphorylation of Cdc25C by different kinases recruits specific proteins, controlling the normal cellular mitotic progression (reviewed in reference 56). Clearly, both Septin6 and Cdc25C have important roles in mitosis. Finally, the activation of many interleukins after HDAC ablation could be because of a postapoptotic activation of an inflammation response.

In summary, we have defined a role for HDAC1 in the proliferation of human tumor cells and, most importantly, identified a mitotic defect in human tumoral cells after knockdown of HDAC1. These results confirm once again the importance of characterizing the biological function of HDAC1, also in light of its potential significance as a target for cancer therapy.

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