Published OnlineFirst July 21, 2009; DOI: 10.1158/0008-5472.CAN-08-2796

Loss of Histone Deacetylase 4 Causes Segregation Defects during Mitosis of p53-Deficient Human Tumor Cells

Bruno Cadot, Mirko Brunetti, Sabina Coppari, Silvia Fedeli, Emanuele de Rinaldis, Claudio Dello Russo, Paola Gallinari, Raffaele De Francesco, Christian Steinkühler, and Gessica Filocamo

Department of Oncology, IRBM-Merck Research Laboratories, Rome, Italy

Abstract

We investigated the role of histone deacetylase 4 (HDAC4) using RNA interference (RNAi) and knockout cells to specifically address its role in cell cycle progression in tumor and normal cells. Ablation of HDAC4 led to growth inhibition in human tumor cells but not to detectable effects in normal human dermal fibroblasts (NHDF) or myelopoietic progenitors. HDAC4−/+ or HDAC4−/− murine embryonic fibroblasts showed no detectable growth defects. On the other hand, HDAC4 RNAi in HeLa cells produced mitotic arrest followed by caspase-dependent apoptosis. Mitotically arrested cells showed chromosome segregation defects. Even though the growth of both p53–wild-type and p53-null tumor cells were affected by HDAC4 ablation, segregation defects were observed only in p53-null cells. HDAC4 associates with the PP2A-B56 regulatory subunit, which is known to be involved in chromosome segregation, and RNAi of either the structural subunit A or the regulatory subunit B56 of PP2A also caused chromosome segregation defects. We conclude that HDAC4 is required for cell cycle progression of tumor cells by multiple mechanisms, one of which seems to be specific to p53-deficient cells through chromosome segregation defects. On the contrary, HDAC4 is not required for the progression of NHDF. We therefore suggest that systemic selective interference with the expression or function of HDAC4 is expected to have a significant therapeutic window, in particular, for p53-deficient tumors. [Cancer Res 2009;69(15):6074–82]

Introduction

Histone deacetylases (HDAC) are involved in the control of histone acetylation status and form a group of 18 proteins divided into two major families: the zinc-dependent hydrolases, arranged into class I, II, and IV, and the evolutionarily distinct, NAD-dependent, sirtuin-like class III proteins (1–3). HDACs are deregulated or aberrantly expressed in several forms of human cancer (4), and the HDAC inhibitor vorinostat was recently approved for the treatment of cutaneous T-cell lymphoma (5). Vorinostat and other HDAC inhibitors exert their function by inhibiting several zinc-dependent HDACs. This is in line with the emerging biology of several HDAC subtypes which suggests that they play important roles in cancer. For example, each of the members of the class I enzymes (HDAC1, HDAC2, HDAC3, and HDAC8) was shown to be functionally involved in tumor growth and survival, either by aberrant recruitment, expression, or control of crucial growth and prosurvival pathways in the context of the transformed phenotype (4–6).

Class IIa HDACs (HDAC4, HDAC5, HDAC7, and HDAC9) are involved in the control of gene expression through their recruitment by transcription factors, notably of the Mef and Runx families, or by transcriptional corepressor complexes such as CtBP (7–9). They also associate with heterochromatin via direct binding to HP1 and thus participate in higher-order structures, possibly contributing to the propagation of repressive chromatin states (7). Stimulus-dependent nucleocytoplasmic shuttling emerged as a major control mechanism of the biological functions of class IIa HDACs. They are targets of several kinases and some phosphorylated forms are confined to the cytosol by interaction with 14-3-3 proteins (10, 11). HDAC4 was found to be frequently mutated in human cancers (12), to associate with oncoproteins (13), to stabilize hypoxia-inducible factor 1α (14), and to be a downstream effector of p53-mediated senescence (15). In tumors, oncogenic Ras (16), DNA damage (17), and caspase cleavage (18, 19) were also shown to promote nuclear localization of HDAC4. Once imported, HDAC4 may exert different nuclear functions that affect differentiation, cell hypertrophy, and cell cycle progression. In cancer cells, HDAC4 was shown to associate with 53BP1 (20) and to be recruited to DNA-damage foci, in which it seems to play a pivotal role in foci resolution. In addition, HDAC4 may localize to and repress G_2-M promoters in a p53-dependent way (17).

In an attempt to define the contributions of individual HDAC subtypes to tumor proliferation and survival, we performed a small interfering RNA (siRNA) screen on several human tumor cell lines. Here, we report that siRNAs directed against HDAC4 turned out to be particularly effective in inducing cell killing and showed an excellent window with respect to normal cells. We propose that HDAC4 has specialized functions in the context of cells with activated DNA-damage pathways that render these cells particularly sensitive to the loss of HDAC4 expression.

Materials and Methods

Cell culture. HeLa, Saos-2, HCT-116, and A549 cells were from American Type Culture Collection and were grown according to standard protocols. Normal human dermal fibroblasts (NHDF; Cambrex) were grown according to the suggestions of the manufacturer.

Human umbilical cord blood mononuclear cells (Cambrex) were plated in Iscove's modified Dulbecco's medium supplemented with 30% fetal

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Current address for B. Cadot: UMRS 787 Inserm-Groupe Myologie, Faculté de Médecine-Pitié-Salpétrière, 105 boulevard de l'Hôpital, 75634 Paris Cedex 13, France.

Requests for reprints: Gessica Filocamo, Department of Oncology, IRBM, via Pontina Km. 30,600, 00040 Pomezia, Italy. Phone: 39-06-9109-3619; Fax: 39-06-9109-3654; E-mail: gessica_filocamo@merck.com.

doi:10.1158/0008-5472.CAN-08-2796

Published OnlineFirst July 21, 2009; DOI: 10.1158/0008-5472.CAN-08-2796

bovine serum, 2 mmol/L glutamine, and 1% bovine serum albumin. Murine embryonic fibroblasts (MEF) were prepared from E13.5 embryos and cultured in DMEM supplemented with 10% fetal bovine serum, 2 mmol/L of L-glutamine (Life Technologies), 100 units/mL of penicillin, and 100 μg/mL of streptomycin (Life Technologies).

Transfections. Adherent cells at 60% confluency were transfected for 4 h with a final concentration of 50 nmol/L siRNA by using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. Cells were trypsinized and re-plated at different concentrations for the different assays. For cell cycle, HeLa and Saos-2 cells were transfected by electroporation following the protocols of the manufacturer (Amaxa) using siRNA at a 10 μmol/L concentration. Myelopoietic progenitors were electroporated (two pulses at 560 V, 5 ms, square-wave) with 10 μmol/L siRNAs.

Phenotype rescue. HDAC4-wt inducible clone was plated 12 h before transfection in the presence or in the absence of 125 ng/mL of doxycycline. Three millions cells were electroporated with 10 μmol/L siRNAs (Amaxa, solution V, program O-17) and plated in complete medium with or without doxycycline.

Cell cycle analysis. Cells and supernatant were collected, centrifuged, washed, and resuspended in PBS, 0.1% Triton X-100, 40 mmol/L of EDTA, and 40 μg/mL of propidium iodide. Cell cycle was monitored using a FACSCalibur (BD) instrument and analyzed with CellQuest Software. Cell synchronization was performed by adding thymidine at a 2.5 mmol/L concentration for 24 h, or nocodazole at a 100 ng/mL concentration for 18 h. Caspase inhibitor Q-VD-OPh (R&D Systems) was used at a 100 μmol/L final concentration.

RNA extraction and TaqMan. Total RNA was extracted at 24 h posttransfection using the RNeasy kit (Qiagen), according to the instructions of the manufacturer. Quantitative reverse transcription-PCR (qRT-PCR) of HDAC4 mRNA was performed in triplicate by using the One-step RT-PCR Master Mix (Applied Biosystems) with the following set of primers and probe: primer sense, 5′-GAGGTTGAGCGTGAGCAAGAT-3′, 400 nmol/L; primer antisense, 5′-TAGCGGTGGAGGGACATGTAC-3′, 400 nmol/L; and probe (FAM-TAMRA), 5′-TCATCGTGGACTGGGACGTG-CAC-3′, 200 nmol/L. Normalization was done on the same amount of template by amplification of human glyceraldehyde-3-phosphate dehydrogenase with the corresponding predeveloped TaqMan Assay Reagents (Applied Biosystems), in triplicate. Detection was performed with an ABI Prism 7900HT Sequence Detection System.

Immunofluorescence analysis and antibodies. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100, then incubated with primary antibodies followed by secondary antibody (Alexa) diluted 1:3,000. Coverslips were mounted with ProLong anti-fade (Molecular Probes) containing 4′,6-diamidino-2-phenylindole (DAPI). Images were acquired using a Leica microscope coupled to Metamorph software. HDAC4 and phosphorylated H3 antibodies were from Cell Signaling, α-tubulin antibody was from Sigma, and Eg5 and PARP antibodies were from Novus Biologicals. Cyclin A and B1 antibodies were from Santa Cruz Biotechnology; terminal nucleotidyl transferase–mediated nick end labeling (TUNEL) assay was performed with the APO-Direct apoptosis kit (Phoenix Flow Systems).

Chromosome spreading. Cells were trypsinized, centrifuged, incubated in 0.075 mol/L KCl for 10 min at 37°C, and fixed thrice with methanol/acetic acid (3:1). A drop of this mixture was placed on a microscope slide and let dry before covering it with antifade containing DAPI.

Western blot and immunoprecipitation. Protein extracts in 1% SDS were sheared with QIAshredder columns (Qiagen), electrophoresed on 8% SDS/polyacrylamide gels, and blotted onto a nitrocellulose membrane. All primary antibodies were incubated overnight, and after incubation with the peroxidase-conjugated secondary antibody (Pierce), detection was carried out using the SuperSignal West Pico chemiluminescent substrate (Pierce). Antibodies used were HDAC4 (Cell Signaling), PARP (Novus Biologicals), cyclin A and cyclin B1 (Santa Cruz Biotechnology), 53BP1 (Novus Biologicals), γ-H2AX (Upstate), PP2A-A (Upstate), PP2A-C (Upstate), PP2A-B56 (Sigma), and anti-FLAG (Sigma). HDAC4-FLAG immunoprecipitation was carried out as previously described (21).

Results

HDAC4 siRNAs are antiproliferative in tumor cells. Several HDAC4-specific siRNAs were tested for their ability to inhibit cell proliferation in a clonogenic assay on HeLa cells. With two exceptions, all siRNAs produced a ≥50% decrease in colony formation with effects comparable to an siRNA directed against the mitotic kinesin Eg5 (Fig. 1*A*). Three of the siRNAs were chosen for further characterization, and transfected into three human tumor cell lines (HCT-116, HeLa, and A549). Residual HDAC4 protein expression (Fig. 1*B, c*) correlated with growth inhibition (Fig. 1*B, a*) and inversely correlated with the induction of TUNEL-positive cells (Fig. 1*B, b*). No significant effect was observed on the expression of other HDACs (see below).

To assess the specificity of the antiproliferative effects, we designed a phenotype-rescue experiment. An HCT-116 clone, stably expressing HDAC4 devoid of its natural 3′-untranslated region (UTR), was generated under the control of a doxycycline-inducible promoter (HCT-116_HDAC4; Fig. 1*C, a*) in a cell clone stably expressing the tetracycline promoter transactivator and suppressor. Subsequently, siRNAs directed against the 3′-UTR of the wild-type HDAC4 mRNA were generated and shown to both decrease endogenous HDAC4 and lead to proliferation arrest in parental HCT-116 cells as well as in the HCT-116_HDAC4 cell clone in the absence of doxycycline (Fig. 1*C, black columns* in *b* and *c*). However, the addition of doxycycline completely rescued the antiproliferative effect (Fig. 1*C, gray columns* in *c*). qRT-PCR showed efficient silencing of the endogenous but not of the inducible HDAC4, indicating that antiproliferative effects were specifically due to HDAC4 knockdown and not to off-target effects (Fig. 1*C, gray columns* in *b*).

Next, we determined gene expression changes induced by different siRNAs directed against HDAC4 (Supplementary Fig. S1). RNA was extracted 24 and 32 hours posttransfection of the siRNAs in HCT-116 cells, and analyzed by microarray. Under these conditions, a common, high-stringency, target-specific gene expression signature of 14 and 23 genes at 24 and 32 hours, respectively, out of ~18,000 human genes probed on the chip used (Supplementary Fig. S1; and Materials and Methods) was derived by hierarchical agglomerative clustering, and revealed the regulation of genes involved in cell-cell interactions, cell shape, apoptosis, and cell cycle in line with the early phenotypic effects of HDAC4 knockdown (see below). To validate microarray results, we performed qRT-PCR on a representative subset of these genes and confirmed the observed change in expression (Supplementary Fig. S1*C*). We concluded that HDAC4 knockdown specifically leads to proliferation defects in human tumor cell lines.

Loss of HDAC4 is well tolerated in normal cells. We next asked the question about the therapeutic index associated with loss of HDAC4. The much more tissue-restricted expression pattern of HDAC4 versus HDAC1 and HDAC3, as representatives of class I, found in several human tissues argued that its loss may be better tolerated (Fig. 2*A*).

Parallel transfection of NHDF and tumor HCT-116 cells with an HDAC4 siRNA led, in both cell types, to a decrease of ~80% in HDAC4 mRNA (Fig. 2*B, b*). Whereas no morphologic changes could be detected in fibroblasts for up to 72 hours posttransfection, HCT-116 cells lost cell-cell contacts, partly detached, and rounded up, in line with the changes in the expression levels of several genes functionally involved in these processes as found by microarray (Fig. 2*B, a*) and indicative of cell death.

To assess whether HDAC4 knockdown would cause myelotoxic effects, we performed an *in vitro* assay based on colony formation

Published OnlineFirst July 21, 2009; DOI: 10.1158/0008-5472.CAN-08-2796

of human cord blood myelopoietic progenitors (22, 23) that were transfected with HDAC4 siRNAs (Fig. 2*C*). As an internal positive control, we used an Eg5-siRNA. No significant decrease in colony formation was produced by any of the HDAC4 siRNAs tested, whereas Eg5-siRNA gave a significant decrease in colony number. Expression of HDAC4 and Eg5 was affected to a similar extent (data not shown).

Growth of normal MEFs from HDAC4 knockout mice is not affected by HDAC4 loss. To get more insight into the role of HDAC4 in the cell cycle progression of normal cells, we used MEFs from HDAC4-knockout (KO) mice. Growth curves of MEFs from wild-type, HDAC4−/+, and HDAC4−/− animals were superimposable, indicating that the proliferation of these cells was not impaired even in the complete absence of HDAC4 (Supplementary Fig. S2*A*).

HDAC4 has a well-documented role in DNA repair in transformed cells by associating with 53BP1 followed by recruitment to DNA-damage foci (20). HDAC4 depletion in HeLa cells impairs ionizing radiation–induced DNA-damage repair, leading to cell death. Gamma-irradiation of wild-type, HDAC4 heterozygous, or KO MEFs produced a prominent G_2-M arrest (Supplementary

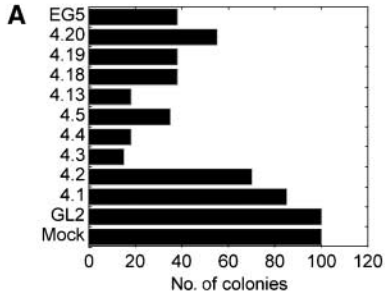
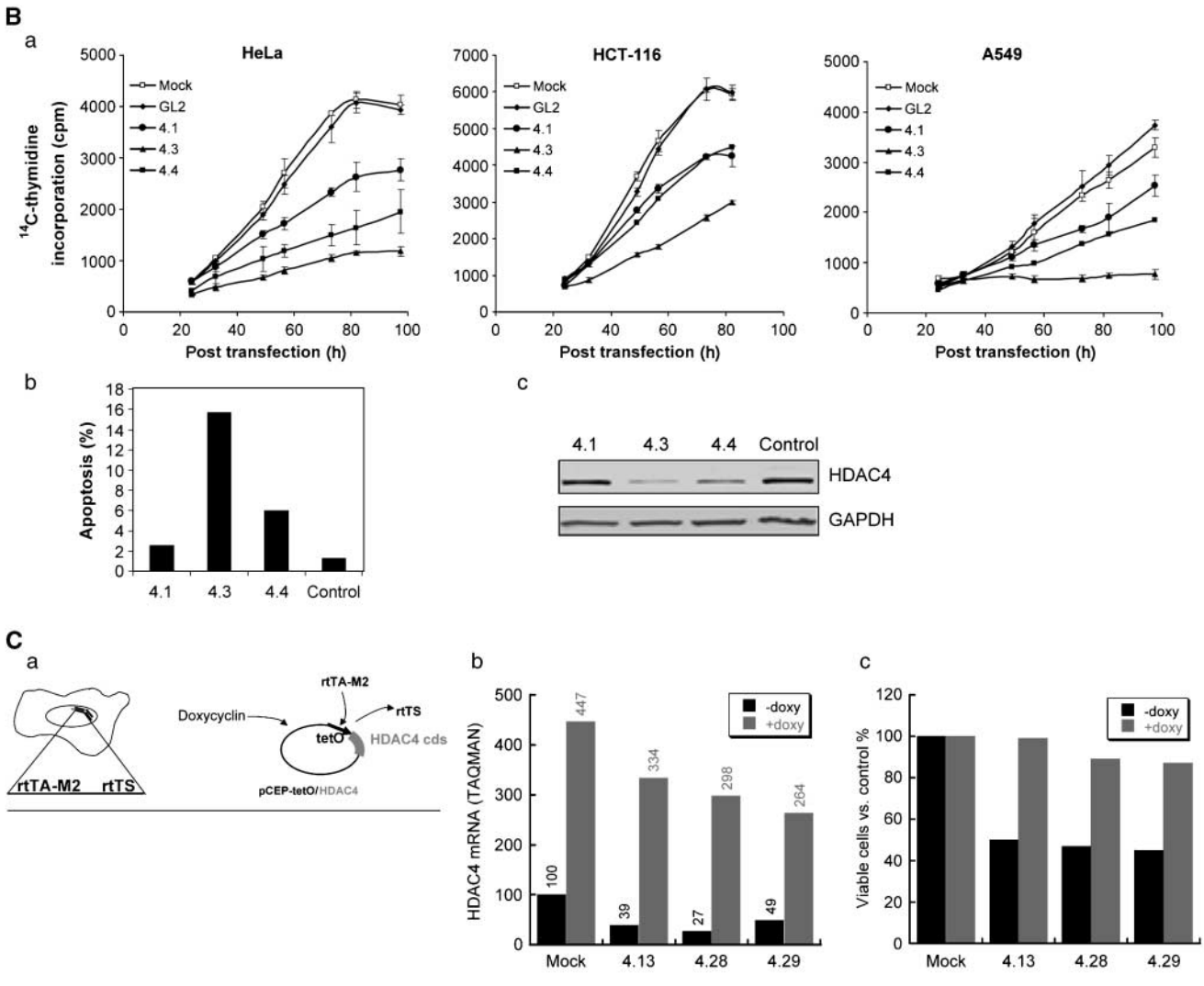


Figure 1. HDAC4 knockdown blocks the proliferation of tumor cells. *A,* colony formation assay of HDAC4 siRNA–treated HeLa cells; the decrease in colony number correlated with knockdown efficiency. *Eg5-siRNA,* positive control of growth inhibition; *GL2-siRNA,* negative control. *B, a,* ^{14}C-thymidine incorporation of tumor cells transfected with HDAC4 siRNAs. *b,* apoptosis measured after 72 h of silencing by TUNEL assay. *c,* HDAC4 knockdown monitored by Western blot of protein extracts at 48 h. *C, a,* phenotype rescue of HDAC4 knockdown was obtained in a stable, inducible cell clone stably expressing HDAC4 cds devoid of its natural 3′-UTR by using siRNAs mapping in HDAC4 3′-UTR. In uninduced conditions (−doxycycline), RNAi caused HDAC4 knockdown (*b, black columns*) and cell growth inhibition (*c, black columns*), whereas expression of exogenous HDAC4 (+doxycycline) resulted in no more knockdown of HDAC4 (*b, gray columns*) and no proliferation inhibition (*c, gray columns*).



Published OnlineFirst July 21, 2009; DOI: 10.1158/0008-5472.CAN-08-2796

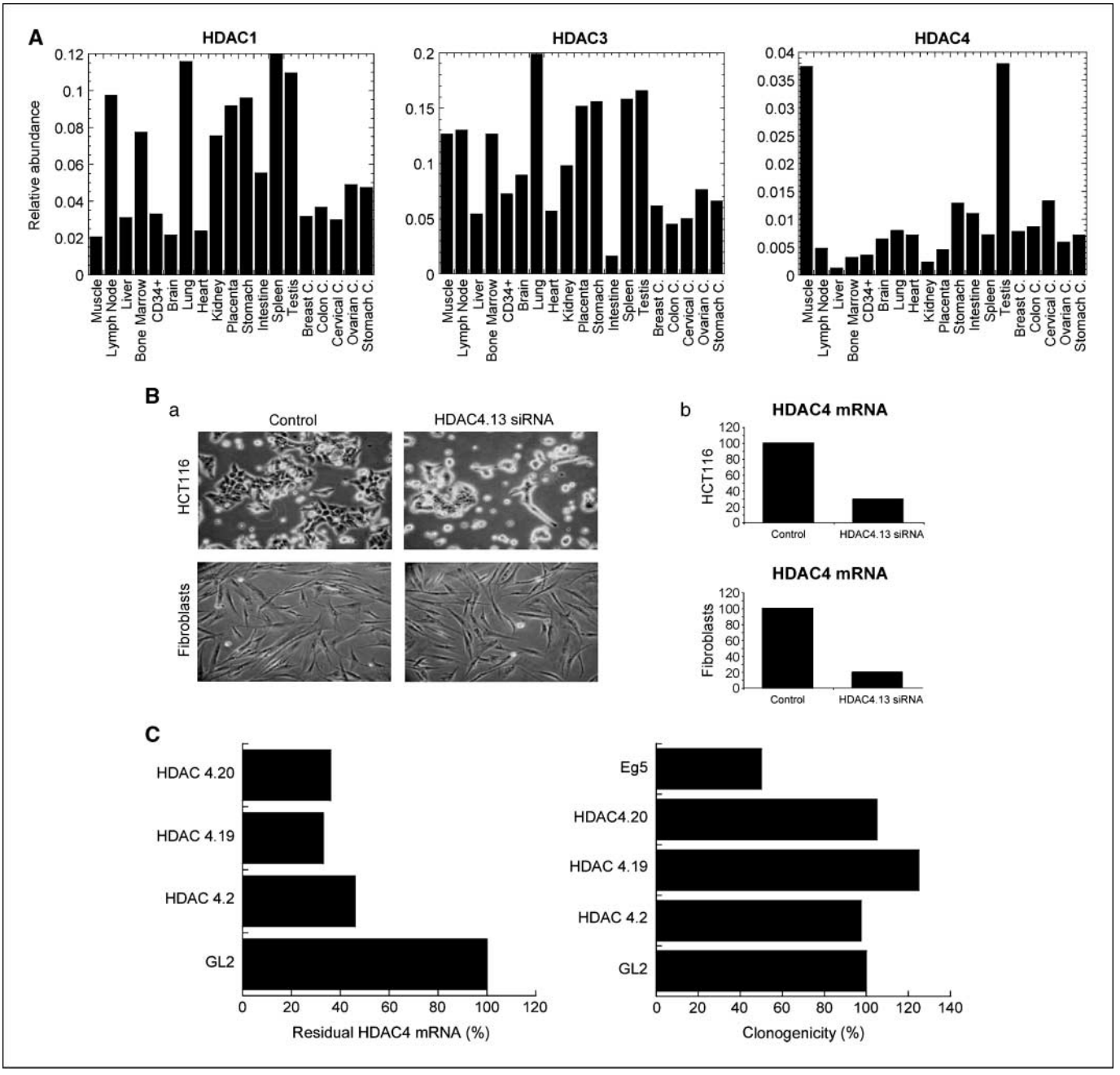


Figure 2. Loss of HDAC4 is well tolerated in normal cells. *A,* expression levels of HDAC4 in human normal tissues show that HDAC4 has a much more tissue-restricted expression, specifically, in muscle and testis, as compared with the ubiquitously expressed HDAC1 and HDAC3. *B,* parallel transfection of HCT-116 cells and NHDF with HDAC4.13 siRNA led to a cell death phenotype at 72 h posttransfection only in tumor cells, as shown by cell detachment and membrane blebbing (*a*), despite similar knockdown efficiencies measured at 24 h (*b*). *C,* human myelopoietic progenitors transfected with HDAC4 siRNAs; despite down-regulation of HDAC4 expression, no decrease in clonogenicity was observed. GL2 siRNA served as a negative control, and Eg5 siRNA as a positive control.

Fig. S2*B*). However, the kinetics of cell cycle re-entry were identical for all genotypes, and no increased apoptosis could be observed in the KO cells, indicating that in these normal cells, DNA-damage repair could efficiently occur in the complete absence of HDAC4 (Supplementary Fig. S2*B* and *C*).

To further investigate the role of HDAC4 in KO cells, we analyzed the transcriptional profile of wild-type, HDAC4+/−, and KO cells cultured *in vitro* for two passages after explantation from embryos, by microarray analysis (see Materials and Methods). As a result, 17 genes were identified to be differentially expressed in at least one genotype versus the others. Among these, the *prm1* gene, encoding protamine-1, emerged as having the most statistically significant level of perturbation upon HDAC4 impairment ($P < 10^{-6}$). It was up-regulated in the HDAC4+/− group and even more in the HDAC4−/− group, as compared with the wild-type counterpart, exhibiting an inverse correlation to HDAC4 expression. This observation was also confirmed by qRT-PCR analysis (Supplementary Fig. S2*D*).

Published OnlineFirst July 21, 2009; DOI: 10.1158/0008-5472.CAN-08-2796

In view of the high expression of HDAC4 in testis where histones are extensively substituted by protamines during spermiogenesis (24), this finding suggests that HDAC4 might have a specific role in this process. We concluded that the biology of HDAC4 is likely to differ in normal and transformed cells and that HDAC4 depletion seems to be well tolerated in normal human and murine cells.

Loss of HDAC4 leads to cell cycle arrest and apoptosis in HeLa cells. We next addressed the mechanism by which HDAC4 depletion leads to proliferation defects in tumor cells. Transfection of HeLa cells with HDAC4 siRNA led to cell cycle arrest in G_2-M 48 hours posttransfection, and to a significant sub-G_1 fraction at 72 hours (Fig. 3*A, top*) with induction of PARP cleavage (Fig. 3*B, a*). Addition of a pan-caspase inhibitor prevented the disappearance of a G_2-M peak and the appearance of a sub-G_1 fraction, indicating that HDAC4 depletion leads to caspase-dependent apoptosis (Fig. 3*A, bottom*).

HDAC4-interfered cells showed a decreased amount of 53BP1 and an increased amount of γ-H2AX proteins (Fig. 3*B, b*), in line with published data (20), and with the notion that inhibition of HDAC4 nuclear translocation inhibits the resolution of γ-H2AX foci (25).

We next synchronized HeLa cells either in G_1 or in G_2 phase using a double thymidine or a nocodazole blockage, respectively. Mock or siRNA-transfected cells released from the G_1 blockage could both proceed through S phase, but a substantial fraction of HDAC4 knockdown cells arrested in G_2-M (Fig. 3*C, a, left*). Similarly, upon the release of the nocodazole blockage (Fig. 3*C, a, right*), a large amount of HDAC4-interfered cells did not further proceed through the cycle. These data confirmed the observation that HDAC4 depletion leads to a G_2-M blockage in HeLa cells.

In HDAC4-depleted cells, only cyclin B1 and not cyclin A levels increased as compared with mock-transfected cells, arguing for a blockage in mitosis rather than in G_2 (Fig. 3*C, b*). This was confirmed by the immunofluorescence of cells double-stained with anti–α-tubulin and DAPI, in which we detected a substantial part of siRNA-transfected cells arrested in mitosis at 48 hours (Fig. 3*D, a*). Moreover, analysis of the mitotic index by phosphorylated H3 staining (Fig. 3*D, b, c, d*) further supported that HDAC4 RNA interference (RNAi) caused a blockage of cells in mitosis.

Loss of HDAC4 impairs segregation defects. To investigate the mechanism of mitotic blockage, we prepared chromosome spreads from HDAC4 siRNA–transfected HeLa cells. We found that whereas in control cells ~5% to 6% of mitotic cells had separated chromosomes, in the HDAC4 siRNA–transfected cells up to 52% of the karyotypes showed abnormal chromatid segregation with "partial" sister chromatid separation occurring in the presence of unseparated chromosomes; 13% of mitotic cells instead showed a "completely separated" phenotype (Fig. 4*A*). This pattern was found with four different HDAC4-specific siRNAs (data not shown) and the extent of mitotic anomalies correlated with the HDAC4 knockdown efficiency. Also, infection of HeLa cells with an shRNA-expressing lentivirus, targeting HDAC4, but not with a control virus, reduced their growth rate and led to mitotic arrest with segregation defects (Supplementary Fig. S3*A*). Segregation defects upon HDAC4 knockdown were also observed in Saos cells (p53 null) but not in HCT-116 or in A549 cells (p53 positive). Therefore, we reasoned that perhaps p53 loss might be necessary to observe segregation defects in cells with HDAC4 loss. To verify this hypothesis, we transfected HCT-116-E6 cells, harboring the papilloma E6 protein that blunts p53 expression, with an HDAC4 siRNA. Interestingly, HCT-116-E6 cells presented the same phenotype observed in HDAC4-depleted HeLa cells, indicating that this mitotic defect requires the absence of p53 to be detectable (Table 1). Moreover, we used A549 cells expressing a lentiviral vector bearing a p53-specific shRNA. Also in this case, we found that HDAC4 knockdown led to segregation defects only upon p53 ablation (Supplementary Fig. S3*B*). None of the p53-defective cell lines used in this study showed segregation defects in the presence of HDAC4, suggesting that this mitotic phenotype results from the simultaneous down-regulation of both gene products.

We recently discovered that HDAC4 associates with PP2A, which dephosphorylates HDAC4, thus promoting its nuclear import (21, 26). PP2A is a multi-subunit phosphatase and is involved in both the resolution of DNA repair foci (27) and the protection of cohesion (28, 29). We therefore asked the question of whether the impairment of PP2A nuclear import, as a consequence of loss of HDAC4 expression, might account for some of the phenotypic effects of HDAC4 siRNAs.

To determine if HDAC4 and PP2A also associate in HeLa cells, we immunoprecipitated ectopically expressed HDAC4-FLAG and determined the presence of PP2A subunits in the immunoprecipitate. Western blot analysis of anti-FLAG immunoprecipitates identified PP2A sub-A, PP2A sub-C, and PP2A sub-B56-α (Fig. 4*D*). Moreover, the inverse immunoprecipitation of PP2A (subunit C) confirmed that HDAC4 could be found complexed with PP2A.

We next asked the question of whether PP2A depletion led to similar mitotic phenotypes as HDAC4 RNAi. Indeed, we found that silencing of PP2A sub-A led to decreased cell proliferation, G_2-M arrest, increased levels of γ-H2AX, followed by PARP cleavage and apoptosis (Supplementary Fig. S4*B*).

In order to get insights into a possible link between HDAC4 and PP2A, we further investigated the mitotic phenotype of PP2A sub-A RNAi in HCT-116 wild type and HCT-116-E6 cells, as well as in NHDF (Fig. 4*B, a* and *b*). RNAi of PP2A sub-A phenocopied crucial aspects of the mitotic defect pattern observed upon HDAC4 ablation. Whereas no anomalies could be detected in PP2A sub-A–deficient NHDF, loss of PP2A sub-A lead to segregation defects with isolated chromatids and intact chromosomes in the same spread (partial phenotype) only in p53-deficient HCT-116-E6 cells but not in their wild-type counterparts. In all cases, we made sure that the knockdown efficiencies were comparable (Supplementary Fig. S4*A*).

To further dissect the role of PP2A in the mitotic phenotype observed upon HDAC4 depletion in p53-deficient cells, we determined the phenotypic consequences of selectively depleting the PP2A-B56 regulatory subunit, which was specifically found to be involved in chromatid cohesion (28–30). Indeed, RNAi of PP2A-B56 in HeLa cells also lead to mitotic arrest and chromatid segregation defects in ~24% of mitotic cells (Fig. 4*C*).

Despite these striking similarities, we noticed, however, that the phenotypes resulting from HDAC4 or PP2A ablation also had distinguishing features. In fact, RNAi of PP2A sub-A led to mitotic patterns with completely separated chromatids ("separated" phenotype) in HCT-116 wild-type cells (Fig. 4*B, b*) in which RNAi of HDAC4 never gave mitotic defects. These differences suggest that the roles of these two proteins only partially overlap.

Discussion

The most important known roles of class IIa HDACs relate to their involvement in the control of gene expression during muscle differentiation, regulation of cellular hypertrophy, clonal cell expansion, or vascular integrity (7, 25, 31–33). HDAC4-KO mice show pronounced chondrocyte hypertrophy and die of

Published OnlineFirst July 21, 2009; DOI: 10.1158/0008-5472.CAN-08-2796

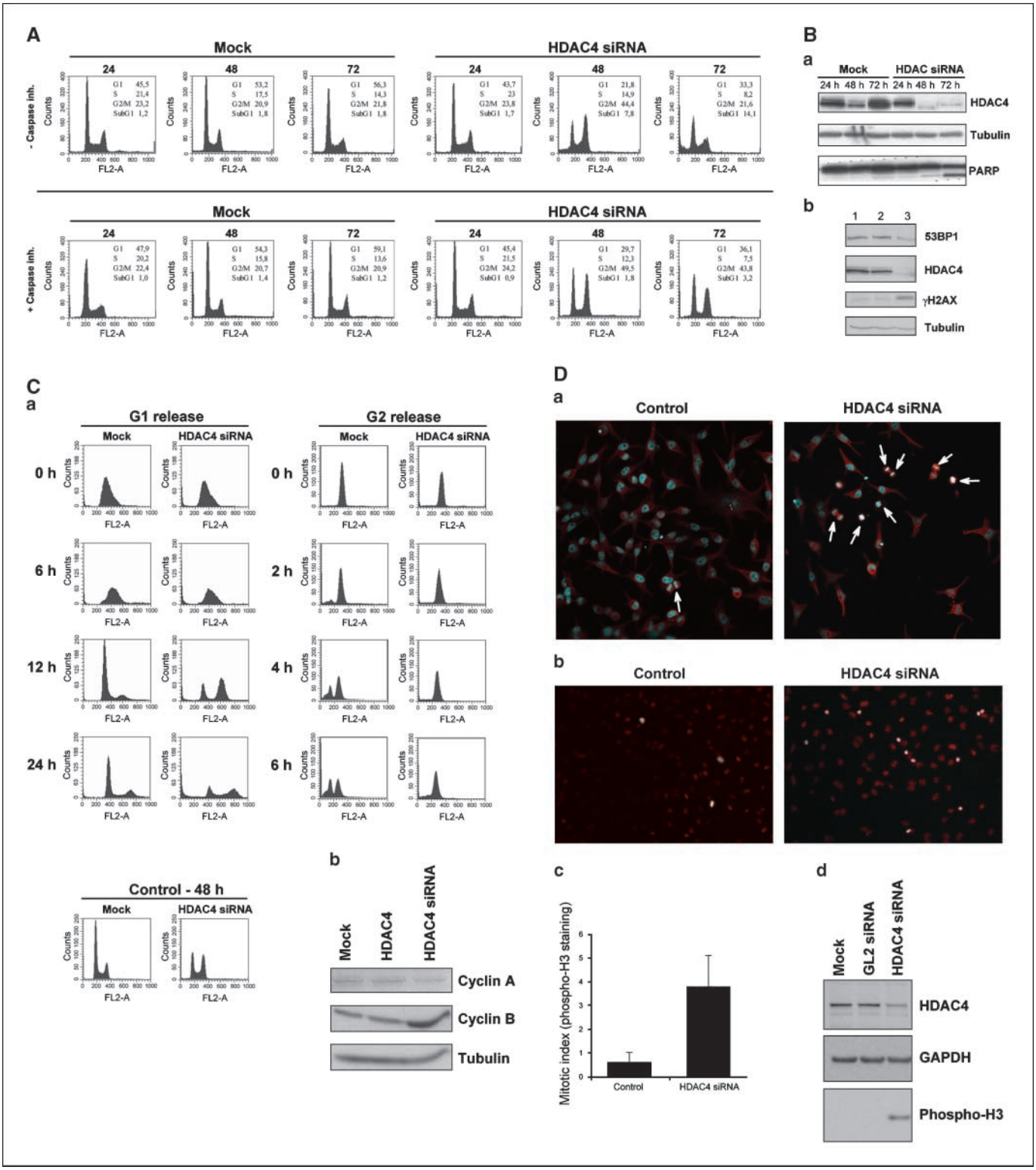


Figure 3. Loss of HDAC4 leads to cell cycle arrest and apoptosis of HeLa cells. *A,* cell cycle of mock or HDAC4 siRNA–transfected cells was analyzed by fluorescence-activated cell sorting at 24, 48, and 72 h posttransfection in the presence or absence of a pan-caspase inhibitor (percentages of cells in G_1, S, G_2 phases as well as the sub-G_1 population). *B,* HDAC4 knockdown induced PARP cleavage (*a*) and induction of γH2AX (*b; lane 1,* mock; *lane 2,* GL2-siRNA; *lane 3,* HDAC4 siRNA). *C, a,* thymidine blockage in G_1 (*left*) or a nocodazole blockage in G_2-M (*right*) were induced 24 h after HDAC4.13 siRNA transfection; 24 h later, re-entry into the cell cycle was monitored at different time points, showing that HDAC4-interfered cells could not exit a G_2-M arrest but could proceed from G_1 to G_2-M normally. *Bottom histograms,* control transfection of HDAC4 siRNA run in parallel, and analyzed 48 h posttransfection. *b,* Western blot analysis of cyclin A and B1 48 h posttransfection showed an increase in cyclin B1 level in HDAC4 siRNA–transfected cells arguing for an arrest in mitosis. *D, a,* immunofluorescence of HeLa cells 48 h posttransfection with DAPI and an anti–α-tubulin antibody showed a significant increase of mitotic cells in HDAC4-interfered cells compared with controls (*white arrows*). *b,* the same sample as in *a* was stained with DAPI and an anti–phosphorylated H3 histone to determine mitotic index, which was quantified with an InCell instrument (*c*), and confirmed by Western blot analysis (*d*).

Published OnlineFirst July 21, 2009; DOI: 10.1158/0008-5472.CAN-08-2796

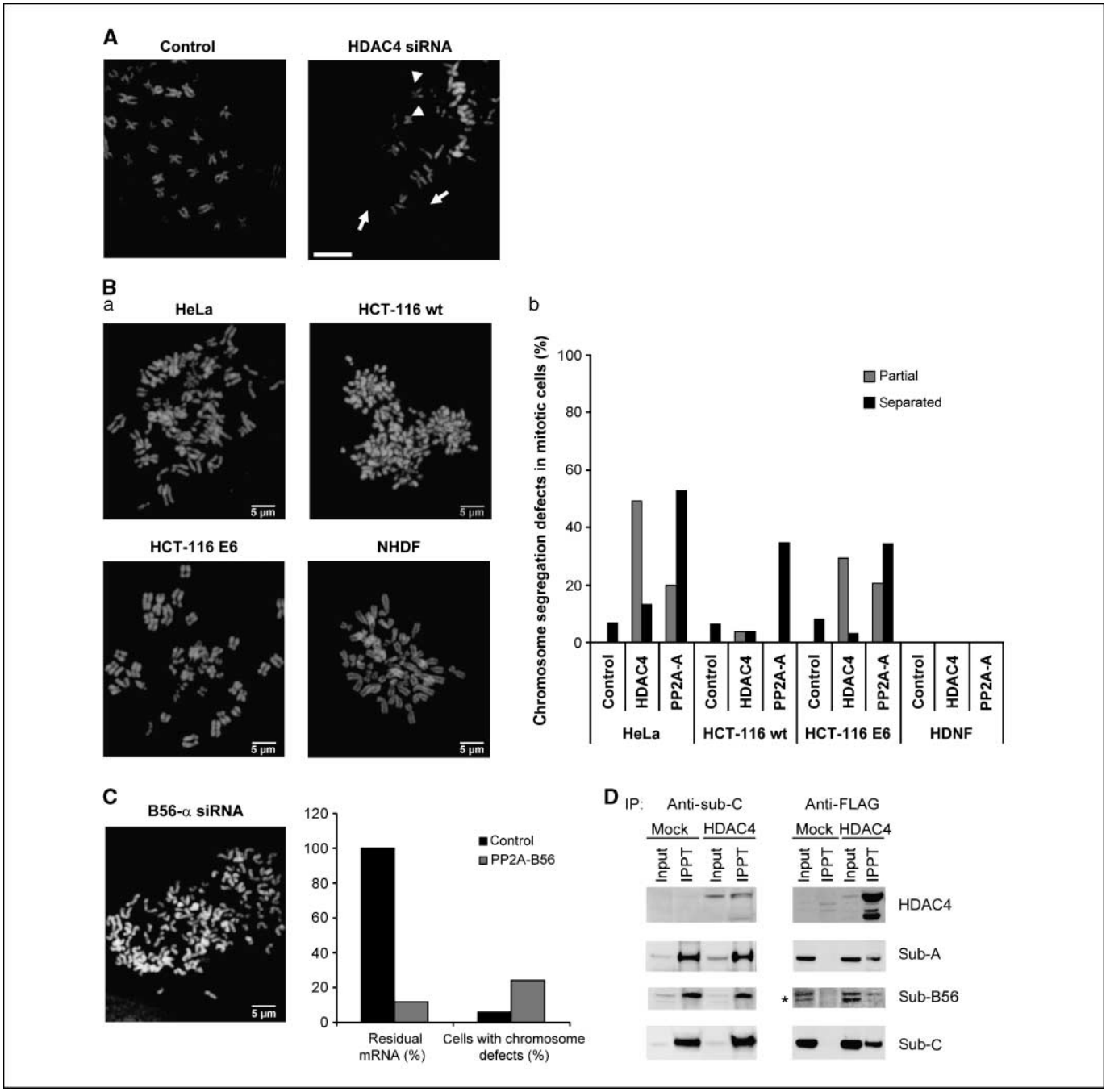


Figure 4. *A,* chromosome spreads of HeLa cells 48 h posttransfection with HDAC4 siRNA. The abnormal partial phenotype is characterized by the presence in the same karyotype of normal X-shaped chromosomes (*arrowheads*) mixed with free chromatids (*arrows*). *B, a,* chromosome spreads of cells upon PP2A-A RNAi show representative examples of the partial defect (*HCT-116-E6*) or the "complete separation" defect (*HCT-116 wt*) or no effect (normal fibroblasts). *b,* percentages of chromosome segregation defects in the different cell lines tested ($n > 50$). *C,* chromosome spreads of HeLa cells 48 h posttransfection with PP2A-B56 siRNA show segregation defects. Knockdown efficiency of PP2A-B56 siRNA was determined by qRT-PCR vs. control cells; in these conditions, chromosome defects were observed in 24% of mitotic cells ($n > 50$). *D,* immunoprecipitates of mock-transfected or HDAC4-FLAG–transfected HeLa cells, with an anti-FLAG or anti-PP2A subunit C antibody, were analyzed by Western blot with the indicated antibodies. *Input,* 0.01% of whole cell extracts.

aberrant ossification (9). In addition to these developmental functions, HDAC4 has several hallmarks of a tumor suppressor gene: it was identified as a mediator of p53 and $p19^{ARF}$-dependent proliferation arrest and senescence (15), it was found to be frequently mutated in human cancers (12), and to be required for the repair of ionizing radiation–induced DNA damage (20). Intriguingly, activated Ras acts on HDAC4, even though indirectly, by promoting its nuclear localization (16). HDAC4 is also a caspase substrate and one of the caspase cleavage fragments is imported into the nucleus and induces cell death (18, 19). In addition, we recently identified PP2A, another well-known tumor suppressor gene, to associate with HDAC4 and to promote its nuclear localization (21, 26). The apparent tumor suppressor role of HDAC4 is seemingly at odds with our

Published OnlineFirst July 21, 2009; DOI: 10.1158/0008-5472.CAN-08-2796

Table 1. Dependence of chromosome segregation defects on p53 status

Cell line	p53 status	Chromosome segregation defects
HeLa	Null	Yes
SAOS	Null	Yes
A549 p53+	Wild-type	No
A549 p53−	Null	Yes
HCT-116	Wild-type	No
HCT-116-E6	Null	Yes

findings of a rather selective requirement for HDAC4 expression in transformed cells. This latter conclusion is based on experiments looking at the ablation of HDAC4 expression via RNAi, making use of siRNAs or shRNAs, or on results obtained with primary cells from mice with targeted disruptions of the *hdac4* gene. In HeLa cells, loss of HDAC4 resulted in a DNA-damage response, mitotic arrest, chromatid segregation defects, and caspase-dependent apoptosis. We note that our data are in agreement with those reported by Kao and colleagues (20), who first described the role of HDAC4 in DNA-damage repair. It is tempting to speculate that at least some of these effects are mediated by PP2A. In fact, in our hands, loss of either the PP2A sub-A or PP2A sub-B56 recapitulated the phenotypic features observed upon HDAC4 RNAi to a significant extent, in line with some of the known functions of this phosphatase. Indeed, PP2A is known to be involved in the resolution of DNA-damage foci by dephosphorylating γ-H2AX and, in association with shugoshin, in the protection of cohesin (28–30). HDAC4 could therefore act on PP2A trafficking, targeting this phosphatase to both DNA-damage foci, perhaps via its interaction with 53BP1, and to cohesin.

The roles of HDAC4 in maintaining genome integrity and in allowing progression through mitosis are apparently not required to the same extent in normal cells. This is perhaps best exemplified by the lack of proliferation defects in HDAC4-KO MEFs, the normal development of most tissues in the KO animals and the lack of correlation between HDAC4 expression levels in human tissues and their proliferative index. Notably, loss of HDAC4 is well tolerated in human myelopoietic progenitors that readily respond to the ablation of other genes involved in mitotic progression. Again, this finding is in line with the lack of hematologic defects in HDAC4-KO animals. Interestingly, and differently from observations in HeLa cells, HDAC4-KO MEFs are proficient in ionizing radiation–induced DNA-damage response, and are indistinguishable from their wild-type counterparts (Supplementary Fig. S2).

Our data suggest that HDAC4 is not required for normal "maintenance" cell cycle progression in untransformed cells, in which it may instead function as a tumor suppressor upon activation of p53 or other stimuli, thus mediating cell cycle arrest and exit. In contrast, HDAC4 seems to become indispensable in transformed cells with a constitutively activated DNA-damage repair machinery and blunted apoptotic and senescence responses. Under these conditions, its recruitment to DNA-damage foci, the stabilization of 53BP1, the association with PP2A, and the resulting possible effects on DNA-damage foci resolution and cohesin protection may all contribute to an eventless cell cycle progression in the presence of replicative stress.

The predominant mechanism(s) by which HDAC4 contributes to cell cycle progression in transformed cells may differ in a context-dependent way. Thus, loss of HDAC4 was antiproliferative in both p53+ and p53− cells, but cohesion defects were noticeable only in the latter. Interestingly, loss of PP2A sub-A also led to mitotic segregation defects in p53 wild-type cells, suggesting that the roles of the two proteins overlap only partially.

Recently, small molecule HDAC inhibitors were described to affect chromatid cohesion (34–36). In addition, HDAC3 was also found to be required for centromeric H3K4 deacetylation and sister chromatid cohesion (37), suggesting a broader role for HDACs during mitotic progression. It is interesting to note that HDAC3 and HDAC4 may associate and can be found in the same multiprotein complexes, leading to the question of whether the phenotypes we have observed in this work may actually involve additional HDAC subtypes.

Even though not yet understood in detail, the emerging role of HDAC4 in human malignancies is an attractive one in terms of therapeutic potentials. A key question is whether deacetylase activity is involved in the processes described in this article and if small molecule active site binders are able to recapitulate the RNAi phenotype. In this respect, a recent publication suggests that the hydroxamic acid inhibitor LBH589 affects DNA-damage response and simultaneously influences subcellular localization of HDAC4 (38). This opens the intriguing possibility that HDAC4 inhibition may contribute to the antitumor activity of the present compounds. The answer to this question will have to await the availability of highly selective small molecule inhibitors that could also validate the hypothesis that selective targeting of this HDAC subtype may lead to novel compounds with an improved therapeutic index.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 8/8/08; revised 4/22/09; accepted 5/3/09; published OnlineFirst 7/21/09.

Grant support: Italian Ministry of Education, University and Scientific Research.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The HDAC4-KO mice were received from Eric Olson, UT Southwestern. We thank Gabriella Biasiol for qRT-PCR of tissue RNAs, and Manuela Emili for graphics.

This article is dedicated to the memory of our beloved colleague and husband Giovanni Migliaccio.

References

1. Roth SY, Denu JM, Allis CD. Histone acetyltransferases. Annu Rev Biochem 2001;70:81–120.
2. Marks PA, Miller T, Richon VM. Histone deacetylases. Curr Opin Pharmacol 2003;3:344–51.
3. Gallinari P, Di Marco S, Jones P, Pallaoro M, Steinkuhler C. HDACs, histone deacetylation and gene transcription: from molecular biology to cancer therapeutics. Cell Res 2007;17:195–211.
4. Minucci S, Pelicci PG. Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. Nat Rev Cancer 2006;6:38–51.
5. Olsen M, Madsen HO, Hjalgrim H, Gregers J, Rostgaard K, Schmiegelow K. Preleukemic TEL-AML1-positive clones at cell level of 10(-3) to 10(-4) do not persist into adulthood. J Pediatr Hematol Oncol 2006;28:734–40.
6. Rosato RR, Grant S. Histone deacetylase inhibitors: insights into mechanisms of lethality. Expert Opin Ther Targets 2005;9:809–24.
7. Verdin E, Dequiedt F, Kasler HG. Class II histone

Published OnlineFirst July 21, 2009; DOI: 10.1158/0008-5472.CAN-08-2796

deacetylases: versatile regulators. Trends Genet 2003;19:286–93.
8. Zhang CL, McKinsey TA, Lu JR, Olson EN. Association of COOH-terminal-binding protein (CtBP) and MEF2-interacting transcription repressor (MITR) contributes to transcriptional repression of the MEF2 transcription factor. J Biol Chem 2001;276:35–9.
9. Vega RB, Matsuda K, Oh J, et al. Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis. Cell 2004;119:555–66.
10. Grozinger CM, Schreiber SL. Regulation of histone deacetylase 4 and 5 and transcriptional activity by 14-3-3-dependent cellular localization. Proc Natl Acad Sci U S A 2000;97:7835–40.
11. McKinsey TA, Zhang CL, Olson EN. Activation of the myocyte enhancer factor-2 transcription factor by calcium/calmodulin-dependent protein kinase-stimulated binding of 14-3-3 to histone deacetylase 5. Proc Natl Acad Sci U S A 2000;97:14400–5.
12. Sjoblom T, Jones S, Wood LD, et al. The consensus coding sequences of human breast and colorectal cancers. Science 2006;314:268–74.
13. Chauchereau A, Mathieu M, de Saintignon J, et al. HDAC4 mediates transcriptional repression by the acute promyelocytic leukaemia-associated protein PLZF. Oncogene 2004;23:8777–84.
14. Qian DZ, Kachhap SK, Collis SJ, et al. Class II histone deacetylases are associated with VHL-independent regulation of hypoxia-inducible factor 1α. Cancer Res 2006;66:8814–21.
15. Berns K, Hijmans EM, Mullenders J, et al. A large-scale RNAi screen in human cells identifies new components of the p53 pathway. Nature 2004;428:431–7.
16. Zhou X, Richon VM, Wang AH, Yang XJ, Rifkind RA, Marks PA. Histone deacetylase 4 associates with extracellular signal-regulated kinases 1 and 2, and its cellular localization is regulated by oncogenic Ras. Proc Natl Acad Sci U S A 2000;97:14329–33.
17. Basile V, Mantovani R, Imbriano C. DNA damage promotes histone deacetylase 4 nuclear localization and repression of G2/M promoters, via p53 C-terminal lysines. J Biol Chem 2006;281:2347–57.
18. Paroni G, Mizzau M, Henderson C, Del Sal G, Schneider C, Brancolini C. Caspase-dependent regulation of histone deacetylase 4 nuclear-cytoplasmic shuttling promotes apoptosis. Mol Biol Cell 2004;15:2804–18.
19. Liu F, Dowling M, Yang XJ, Kao GD. Caspase-mediated specific cleavage of human histone deacetylase 4. J Biol Chem 2004;279:34537–46.
20. Kao GD, McKenna WG, Guenther MG, Muschel RJ, Lazar MA, Yen TJ. Histone deacetylase 4 interacts with 53BP1 to mediate the DNA damage response. J Cell Biol 2003;160:1017–27.
21. Paroni G, Cernotta N, Dello Russo C, et al. PP2A regulates HDAC4 nuclear import. Mol Biol Cell 2008;19:655–67.
22. Pessina A, Croera C, Bayo M, et al. A methylcellulose microculture assay for the *in vitro* assessment of drug toxicity on granulocyte/macrophage progenitors (CFU-GM). Altern Lab Anim 2004;32:17–23.
23. Pessina A, Malerba I, Gribaldo L. Hematotoxicity testing by cell clonogenic assay in drug development and preclinical trials. Curr Pharm Des 2005;11:1055–65.
24. Sassone-Corsi P. Unique chromatin remodeling and transcriptional regulation in spermatogenesis. Science 2002;296:2176–8.
25. Dequiedt F, Kasler H, Fischle W, et al. HDAC7, a thymus-specific class II histone deacetylase, regulates Nur77 transcription and TCR-mediated apoptosis. Immunity 2003;18:687–98.
26. Illi B, Dello Russo C, Colussi C, et al. Nitric oxide modulates chromatin folding in human endothelial cells via protein phosphatase 2A activation and class II histone deacetylases nuclear shuttling. Circ Res 2008;102:51–8.
27. Chowdhury D, Keogh MC, Ishii H, Peterson CL, Buratowski S, Lieberman J. γ-H2AX dephosphorylation by protein phosphatase 2A facilitates DNA double-strand break repair. Mol Cell 2005;20:801–9.
28. Kitajima TS, Sakuno T, Ishiguro K, et al. Shugoshin collaborates with protein phosphatase 2A to protect cohesin. Nature 2006;441:46–52.
29. Tang Z, Shu H, Qi W, Mahmood NA, Mumby MC, Yu H. PP2A is required for centromeric localization of Sgo1 and proper chromosome segregation. Dev Cell 2006;10:575–85.
30. Riedel CG, Katis VL, Katou Y, et al. Protein phosphatase 2A protects centromeric sister chromatid cohesion during meiosis I. Nature 2006;441:53–61.
31. Chang S, McKinsey TA, Zhang CL, Richardson JA, Hill JA, Olson EN. Histone deacetylases 5 and 9 govern responsiveness of the heart to a subset of stress signals and play redundant roles in heart development. Mol Cell Biol 2004;24:8467–76.
32. Chang S, Young BD, Li S, Qi X, Richardson JA, Olson EN. Histone deacetylase 7 maintains vascular integrity by repressing matrix metalloproteinase 10. Cell 2006;126:321–34.
33. Zhang CL, McKinsey TA, Chang S, Antos CL, Hill JA, Olson EN. Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy. Cell 2002;110:479–88.
34. Kimata Y, Matsuyama A, Nagao K, et al. Diminishing HDACs by drugs or mutations promotes normal or abnormal sister chromatid separation by affecting APC/C and adherin. J Cell Sci 2008;121:1107–18.
35. Magnaghi-Jaulin L, Eot-Houllier G, Fulcrand G, Jaulin C. Histone deacetylase inhibitors induce premature sister chromatid separation and override the mitotic spindle assembly checkpoint. Cancer Res 2007;67:6360–7.
36. Robbins AR, Jablonski SA, Yen TJ, et al. Inhibitors of histone deacetylases alter kinetochore assembly by disrupting pericentromeric heterochromatin. Cell Cycle 2005;4:717–26.
37. Eot-Houllier G, Fulcrand G, Watanabe Y, Magnaghi-Jaulin L, Jaulin C. Histone deacetylase 3 is required for centromeric H3K4 deacetylation and sister chromatid cohesion. Genes Dev 2008;22:2639–44.
38. Geng L, Cuneo KC, Fu A, Tu T, Atadja PW, Hallahan DE. Histone deacetylase (HDAC) inhibitor LBH589 increases duration of γ-H2AX foci and confines HDAC4 to the cytoplasm in irradiated non-small cell lung cancer. Cancer Res 2006;66:11298–304.

Cancer Research
The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

AACR American Association for Cancer Research

Loss of Histone Deacetylase 4 Causes Segregation Defects during Mitosis of p53-Deficient Human Tumor Cells

Bruno Cadot, Mirko Brunetti, Sabina Coppari, et al.

Cancer Res 2009;69:6074-6082. Published OnlineFirst July 21, 2009.