Regulation of the \textit{INK4a/ARF} Locus by Histone Deacetylase Inhibitors*

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Despite the importance of the \textit{INK4a/ARF} locus in tumor suppression, its modulation by histone deacetylase inhibitors (HDACis) remains to be characterized. Here, we have shown that the levels of p19\textit{ARF} are decreased in human and murine fibroblasts upon exposure to relatively high concentrations of trichostatin A and sodium butyrate. Interestingly, the levels of p19\textit{ARF} are strongly up-regulated in murine cells even at low concentrations of HDACis. Using \textit{ARF}-deficient cells, we have demonstrated that p19\textit{ARF} plays an active role in HDACi-triggered cytostasis and the contribution of p19\textit{ARF} to this arrest is of higher magnitude than that of the well-established HDACi target p21\textit{Waf1/Cip1}. Moreover, chemically induced fibrosarcomas in \textit{ARF}-null mice are more resistant to the therapeutic effect of HDACis than similar tumors in wild type or p21\textit{Waf1/Cip1}-null mice. Together, our results have established the tumor suppressor ARF as a relevant target for HDACi chemotherapy.

Histone deacetylase inhibitors (HDACis) have emerged as a promising class of anti-neoplastic agents (reviewed in Refs. 1–3). Trichostatin A (TSA) and sodium butyrate (NaB) are prototypical of two different classes of chemical inhibitors of histone deacetylases (HDACs) that interfere with the activity of HDAC classes I and II; the remaining class of HDACs, class III, also known as the Sir2 family, is TSA resistant but nicotinamide sensitive (reviewed in Refs. 1, 2; see also Refs. 4, 5). The molecular events that mediate the biological effects of HDACis are incompletely understood. HDACs can be recruited to chromatin through interaction with a large variety of DNA-binding proteins, and upon recruitment deacetylate histones, generally resulting in heterochromatinization and transcriptional silencing (6). An additional layer of complexity derives from the fact that the substrates of HDACs are not restricted to histones but include transcriptional regulators, such as p53 or E2F-1, and may extend to general transcription factors, such as TFIIB or TFIIF (1, 7–15). Because of the complexity and variety of transcriptional processes that involve HDACs, it is anticipated that a multitude of mechanisms underlie the transcriptional effects of HDACs. In this regard, global gene expression analyses have shown that HDACs affect the expression levels of 2–20% of genes in the genome, of which about half are up-regulated and half down-regulated (Refs. 16, 17, and references therein).

Given the key role of cell cycle integrity in tumor suppression (reviewed in Refs. 18, 19) and cancer therapy (reviewed in Ref. 20), attention has focused on the ability of HDACis to alter the levels of cell cycle regulatory proteins (reviewed in Ref. 2). Numerous studies have provided evidence that HDACis induce cell cycle arrest mediated, at least in part, by a substantial increase in the expression of the \textit{CDKN1A} gene, which encodes the cyclin-dependent kinase inhibitor p21\textit{Waf1/Cip1} (Ref. 21 and references therein). Other cell cycle inhibitors that participate in the proliferative arrest elicited by HDACis are three of the four members of the \textit{INK4} family of inhibitors of the cyclin D-dependent kinases CDK4 and CDK6, namely, p16\textit{INK4a}, p18\textit{INK4c}, and p19\textit{INK4d} (22, 23). Moreover, positive regulators of proliferation, such as cyclins D1 and D2, c-Myc, or c-Src, are down-regulated by HDACis (24–29). Finally, p53 is activated both by inhibitors of HDAC class I/II and by inhibitors of the Sir2 family (12–15).

The \textit{INK4a/ARF} locus is of critical importance in tumor suppression. Its relevance is reflected in the fact that this locus is inactivated in \textit{~}40% of human cancers, a frequency only comparable with that of p53 inactivation (18, 30, 31). The \textit{INK4a/ARF} locus encodes two tumor suppressors, p16\textit{INK4a} and p14\textit{ARF}/p19\textit{ARF} (p14 when referred to the human protein and p19 when referred to the murine protein), which share common exons 2 and 3 but differ in their first exons and their respective promoters. Protein p16\textit{INK4a} inhibits the activity of the CDK4,6/cyclin D kinases, thus contributing to the maintenance of the active, growth-suppressive form of the retinoblastoma family of proteins (31). On the other hand, the tumor suppressor ARF contributes to the stability of p53 by inhibiting the p53-degrading activity of MDM2 (31). A large amount of accumulated evidence indicates that the \textit{INK4a/ARF} locus is a sensor of oncogenic stress, its expression being up-regulated upon the detection of aberrant oncogenic signals (32–34). Given the importance of the \textit{INK4a/ARF} locus in tumor suppression, it is of obvious interest to understand the effects of HDACis on these two genes and their encoded proteins. Few studies have addressed the impact of HDACis on the expression of the \textit{INK4a/ARF} locus. Specifically, HDACis can cooperate with DNA methylation inhibitors to reactivate a silenced, aberrantly methylated p16\textit{INK4a} promoter (35). In a different context, HDACis can up-regulate p16\textit{INK4a} in rat synovial fibroblasts from arthritic joints, but not from normal ones (36). Finally, long-term exposure of human fibroblasts to low concentrations of HDACis affects their subsequent proliferative potential, shortening their replicative lifespan and eventually entering into premature senescence, the latter being mediated by p16\textit{INK4a} (37). Here, we have used primary human and murine fibroblasts to study the effect of short-term treatments with HDACis on the expression of p16\textit{INK4a} and ARF. We have also provided biochemical and genetic evidence for the causal implication of p19\textit{ARF} in HDAC-mediated inhibition of proliferation and tumorigenesis.

**EXPERIMENTAL PROCEDURES**

\textit{Cell Culture and Drug Treatment}—Normal human lung diploid fibroblasts (IMR90: ATCC CCL-186; WI38: ATCC CCL-75) and mouse
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embryo fibroblasts (MEFs) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma) and 1% penicillin G/streptomycin sulfate (Sigma). MEFs were derived from embryos obtained from the following colonies of mice: wild type, p53−/− (38), p21Clp1Waf1−/− (39), and p19ARF−/− (40). All the MEFs used in this work were minimally cultured (<3 passages). Mice were maintained at the Spanish National Center of Biotechnology, Madrid, in a genetic background highly enriched in C57BL6. MEFs were prepared from individual embryos at day 13.5 of gestation and passaged as described previously (41). Stock solutions of TSA (Sigma) and lactacystin (Sigma) were prepared in Me2SO; NaB (Sigma) and nicotinamide (NAM) (Sigma) stocks were in phosphate-buffered saline (PBS). Aliquots of these drugs were stored at −20°C and added to the culture medium as 1000-fold concentrated stock solutions 16–24 h after cell plating.

Determination of Cell Proliferation and Apoptosis—Cell proliferation was quantitated either by measuring [3H]thymidine incorporation or by counting cell numbers, as indicated in the respective figure legends. For [3H]thymidine incorporation assays, 2 × 104 cells were plated in triplicate in 96-well microtiter plates, and DNA synthesis was monitored by incubating the cells in a medium containing 5 μCi/ml of [methyl-3H]thymidine (Amersham Biosciences). After 24 h, cells were trypsinized and harvested using cell harvester LKB 1295–001. Incorporation of radioactivity into DNA was measured with a liquid scintillation counter, Betaplate 1205. Alternatively, cell proliferation was quantified by counting cells in a Neubauer chamber at the indicated time points after plating of 2 × 104 cells in 12-well plates. For apoptosis measurements, cells were lysed, stained with propidium iodide using the DNA-Prep reagents (Coulter Corp.), and analyzed in an Epics XL cytometer (Coulter Corp.). Relative proportions of cells with sub G0/G1 DNA contents (apoptotic cells) and cells in G1, S, or G2 phase of the cell cycle were determined using the ModFit software package.

RNA and Protein Expression Analysis—For Northern blot analysis, total RNA was extracted using TRizol (Invitrogen) following the manufacturer’s instructions. Total RNA (10 μg) was electrophoresed in denaturing agarose gels, transferred to Hybond-N+ membranes (Amersham Biosciences), and probed with random priming labeled probes against exon 1 of p16INK4a or exon 1β of ARF (42). After exposure, membranes were stripped and rehybridized with a probe derived from the γ-actin gene to estimate the amount of RNA loaded in each lane.

For protein analysis by Western blot, whole cell extracts were prepared lysing cells in a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 4 μg/ml protease inhibitors mixture (aprotinin, leupeptin, and pepstatin). Following a 10-min incubation on ice, cellular debris was removed by centrifugation. Protein concentrations were measured using the Bio-Rad DC protein assay kit. Equal amounts of protein (30 μg) were separated on 15% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes (Bio-Rad). Membranes were blocked in PBS containing 0.2% Tween 20 and 5% skim milk. For immunodetection, the following primary antibodies were used: for the murine p16(INK4a), M-156 from Santa Cruz; for human p16(INK4a), DCS-50 from Oncogene Research Products; for murine p19ARF, we used either R562 (Figs. 3–5) or ab105569 (Figs. 6 and 7) from AbCam; and for β-actin, AC-15 from Sigma. Rabbit polyclonal Ac-H4 antibodies were from Upstate; mouse monoclonal HP1γ antibodies were from Chemicon. Following incubation with horseradish peroxidase-coupled secondary antibodies (Dako), signals were detected by chemiluminescence using ECL (Amersham Biosciences).

For immunofluorescence, cells were fixed with 4% paraformaldehyde for 15 min and extracted with PBS supplemented with 0.2% Triton X-100 and 1% fetal calf serum for 5 min at 4°C. Subsequent to blocking for 12 h with PBS and 1% fetal calf serum, cells were incubated with a p19ARF rabbit polyclonal antibody (R562; AbCam) at a 1:100 dilution or with a HP1γ mouse monoclonal antibody (Chemicon) at a 1:1000 dilution. Secondary antibodies were goat anti-rabbit IgG Cy3 (Jackson). Nuclear DNA was stained with a 4% PBS buffered paraformaldehyde solution containing 10 μg/ml 4’6-diamidino-2-phenylindole (Sigma).

Promoter Constructs and Reporter Assays—Human p16INK4a (hp16INK4a) promoter constructs cloned into the pgL2-basic luciferase reporter vector (Promega) and containing, respectively, 3017, 869, and 798 nucleotides upstream the ATG start codon were a generous gift from Dr. Gordon Peters (Cancer Research UK, London). Additional hp16INK4a promoter constructs (−611, −510, −378, and −284) were obtained by limited digestion of the linearized full-length promoter (−3017) with exonuclease III (New England Biolabs) according to the instructions of the manufacturer. The human p14ARF reporter (3.4 kb upstream the p14ARF ATG start codon cloned into the pgL3-basic luciferase reporter vector from Promega) as well as the murine p16INK4a (1.2 kb upstream the ATG) and p19ARF (1.2 kb upstream the ATG) reporters (cloned into pgL2-basic) were kind gifts from Dr. Gordon Peters (Cancer Research UK, London).

For reporter assays, HEK-293T cells were transfected with FuGENE transfection reagent (Roche Applied Science) according to the recommendations of the manufacturer. Cells were incubated for 12 h in the presence of the DNA/FuGENE mixture. The transfection medium was subsequently removed and replaced by fresh medium containing the indicated concentrations of TSA and NaB. After 24 h, cells were washed with PBS and resuspended in 5X reporter lysis buffer (Promega). Cell debris was removed by centrifugation, and supernatants were assayed immediately for luciferase activity in a Lumat LB 9509 luminometer (Berthold Technologies). Luciferase activities of the reporter constructs were normalized to luciferase activity of the empty vector constructs transfected under identical conditions and processed in parallel. Under basal conditions, i.e. in the absence of TSA and NaB, relative luciferase activities of the murine p19ARF and p16INK4a promoters were 116 ± 31 (n = 14) and 205 ± 40 (n = 8) arbitrary units, respectively, as compared with the empty luciferase reporter vector (pGGL2 basic, which is considered the reference, i.e. 1 unit of reporter activity). The human p14ARF (−3.4 kb) and p16INK4a (−3.0 kb) reporter constructs displayed a relative luciferase activity of 16 ± 7 (n = 11); normalized to the parent pGGL3-basic luciferase reporter vector) and 336 ± 78 (n = 15; normalized to the parent pGGL2-basic luciferase reporter vector) arbitrary units, respectively.

Chromatin Immunoprecipitation Assays—Chromatin immunoprecipitation assays were performed according to standard procedures. Briefly, subconfluent MEF cultures (5 × 104 cells) were cross-linked with 1% formaldehyde for 15 min at room temperature and the cross-linking stopped with 0.13 M glycine. Subsequently, cells were washed with cold PBS, scrapped, lysed (1% SDS), and sonicated to obtain DNA fragments of 500–1000 base pairs. DNA fragments were immunoprecipitated with antibodies against Ac-H4 (Upstate Biotechnologies) or Sp1 (Santa Cruz) coupled to protein A-agarose beads previously blocked with salmon sperm DNA. The immunoprecipitated DNA was extracted and subjected to PCR amplification with primers directed against the proximal promoter of p16INK4a, amplifying a fragment of the promoter spanning from −796 to −407 upstream the p16INK4a ATG start codon (5’-CAG ATT GCC CTC CGA TGA CTT C-3’; 5’-TGG ACC CGC AGA AAG T-3’) and p19ARF, amplifying a frag-
ment of the promoter spanning −529 to −303 upstream the p19ARF ATG start codon (−5′-GCC TCG CCG ATC TTC TCT CTA TTT TCT-3′; −5′-CCC ATC GCG GTG ACA GC-3′). Chromatin immunoprecipitations were quantified by real-time quantitative RT-PCR using DNA Master SYBR Green I mix (Applied Biosystems, Foster City, CA) and an ABI PRISM 7700 (Applied Biosystems). For each sample, p16INK4a and p19ARF-specific PCR products amplified from Ac-H4 and Sp1 immunoprecipitates were quantified as the cycle threshold difference (ΔCt) to their corresponding input samples (diluted 1:10). The ΔCt values for HDACi-treated samples were then subtracted from the ΔCt value of the corresponding non-treated control (∆∆Ct). Fold expression changes were calculated as 2^∆∆Ct. All reactions were carried out in sextuplicate per assay, and at least three independent assays were performed per value obtained.

In Vivo Carcinogenesis Assays—For 3-methylcholanthrene (3MC)-induced carcinogenesis, mice of 4 months of age received a single intramuscular injection at one of the rear legs of 40 μl of 3MC (Sigma) dissolved in sesame oil (Sigma) at a concentration of 25 μg/μl (Sigma). Mice were observed on a daily basis until tumors became palpable (usually when the diameter of the leg, which normally is 3–5 mm, was increased to 6–8 mm). Once the tumors reached the above-indicated palpable size, mice were subjected to chemotherapy by intraperitoneal injection of 200 μl of NaB solution (100 mg/ml in PBS) with periodicity of 3 times a week. Treatment with NaB was continued until tumors reached 12 mm (i.e. when the diameter of the leg was increased to 15–17 mm).

Data Evaluation—Data are presented as mean values ± S.E. with the number of experiments (n) in parenthesis. Unless otherwise indicated, statistical significance (p values) was calculated using the Student’s t-test. Asterisks (*, **, and *** ) indicate statistical significance (p < 0.05, p < 0.01, and p < 0.001, respectively). Where indicated, experimental data were fitted to concentration response curves by the Hill equation using the KaleidaGraph 3.6 graphing and data analysis package from Synergy software.

RESULTS

Effects of HDACis on Histone Acetylation and Heterochromatin—The effects of the HDACis TSA and NaB on histone acetylation and heterochromatin have been characterized for the most part in immortal cancer cell lines, which invariably have deregulated expression of the INK4a/ARF locus. For this reason, we have focused on the analysis of normal cells, and therefore we have first validated some of the known effects of TSA and NaB on primary fibroblasts. We incubated primary MEFs with both drugs at concentrations within their usual working range, and we verified their activity by measuring the levels of acetylated histone H4 (Ac-H4). In agreement with previous reports (43), Ac-H4 levels accumulated significantly upon treatment with TSA or NaB (Fig. 1a). Histone acetylation participates, together with histone methylation, in the recruitment of a family of proteins known as heterochromatin protein 1 (HP1), and consequently inhibition of histone acetylation by HDACis results in dissociation of HP1 from chromatin and in loss of heterochromatin (43–46). In agreement with this, we observed that TSA and NaB significantly decreased the number of HP1-positive MEFs (Fig. 1b). These results confirm that in primary fibroblasts both TSA and NaB are functional as manifested by the presence of hallmarks of HDAC inhibition.

Effect of HDACis on p16INK4a Protein Levels—We found that in MEFs TSA (5 μM) reduced p16INK4a protein levels in a time-dependent manner, resulting in almost complete elimination of p16INK4a after 48 h of treatment (Fig. 2a, left panel). This inhibitory effect of TSA was concentration dependent, with 0.5 μM TSA inhibiting protein levels of p16INK4a to ~50% at 24 h (Fig. 2a, right panel). Similar results were obtained using the HDAC inhibitor NaB, which reduced p16INK4a protein levels in a time-dependent manner (Fig. 2b, left panel) and was half-maximally active at a concentration of 5 mM (Fig. 2b, right panel). Histone deacetylases of class I/II are inhibited by TSA, whereas the remaining class III, also known as the Sir2 family, is insensitive to TSA but can be effectively blocked by NAM (1, 4, 5). Fig. 2c shows that NAM did not interfere with p16INK4a expression, suggesting that HDACs of class I/II, but not class III, are involved in regulating the levels of p16INK4a.

We asked whether the effect of HDAC inhibition on p16INK4a was restricted to primary murine fibroblasts or could be also observed in their human counterparts. Fig. 2d shows that TSA and NaB effectively suppressed p16INK4a expression both in WJ38 and IMR90 diploid human fibroblasts, pointing to a common mechanism of regulation of p16INK4a expression by HDACs in murine and human cells. Finally, in ARF-deficient MEFs (40), p16INK4a expression was repressed by HDACs in the same manner as in wild type MEFs (data not shown). Together, these observations demonstrate that inhibition of class I/II HDACs produces a significant reduction in the levels of p16INK4a.

Effect of HDACis on p19ARF Protein Levels—In contrast to the above-described down-regulation of p16INK4a by HDACis, we found that both TSA (Fig. 3a) and NaB (Fig. 3b) strongly induced the accumulation of p19ARF in a time- and concentration-dependent manner, and this induction was further confirmed in intact cells by immunofluorescence (Fig. 3c). p19ARF was remarkably sensitive to HDACs, showing a robust up-regulation at relatively low concentrations such as 25 mM TSA or 0.5 mM NaB (see Fig. 7b) that on the other hand were ineffective on p16INK4a (Fig. 7b). Finally, inhibition of the Sir2 family of HDACs by NAM did not modify p19ARF levels (Fig. 3d). These data are consistent with the involvement of class I/II HDACs in the up-regulation of p19ARF.

Inhibitors of class I/II HDACs are known to activate both p53 and p21 (see the Introduction), which, in turn, are downstream effectors of p19ARF.
We wondered whether the activation of p53 and/or p21 by HDACis could mediate indirectly the activation of p19ARF. As shown in Fig. 3e, MEFs deficient in p53 or in p21 were sensitive to the stimulatory effect of TSA and NaB on p19ARF protein levels. As previously reported, the basal levels of p19ARF were higher in p53-null cells than in wild type cells (see Fig. 3e), a reflection of the negative regulation of p19ARF exerted by p53 (47). The transcription factors E2F-1 and E2F-2 are positive regulators of ARF expression (48), whereas E2F-3 is a negative regulator of ARF (49). To explore the involvement of E2F members on the up-regulation of ARF by HDACis, we treated MEFs deficient in either E2F-1 or E2F-2 (see Ref. 50) with HDACis, and we observed the same response as in wild type cells (data not shown), thus concluding that neither of these regulators is essential on its own for HDACi up-regulation of p19ARF. Additionally, we explored by chromatin immunoprecipitation whether HDACis affected the binding of E2F-1 or E2F-3 to the murine ARF promoter. We observed specific binding of these two regulators to the ARF promoter, but the binding was not affected by treatment with HDACis (data not shown). Finally, examination of p14ARF in normal human diploid fibroblasts was not possible because the levels of p14ARF were below detection level (51), even in the presence of HDACis (data not shown). Collectively, these observations indicate that
inhibition of class I/II HDACs results in increased levels of p19ARF through a mechanism that is independent of the p53/p21 pathway.

**Differential Impact of HDACis on INK4a and ARF Transcription**—Most of the present evidence implicates HDACs in the regulation of transcriptional processes (1). Therefore, we anticipated that the observed changes in p16INK4a and p19ARF expression were mainly due to changes in the transcriptional activity of their respective promoters. To examine the possible implication of protein stabilizing/destabilizing effects through the proteasome, we treated MEFs simultaneously with HDACis (TSA 5 μM and NaB 10 mM) and with the proteasome-specific inhibitor lactacystin (10 μM). As shown in Fig. 4a, neither the stimulatory nor the inhibitory effects of HDACis on p19ARF and p16INK4a protein levels, respectively, were affected by proteasome inhibition, thus excluding the implication of proteasome-mediated protein degradation. On the other hand, the analysis of p16INK4a and p19ARF mRNA levels indicated that TSA and NaB modify the levels of
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FIGURE 6. Effect of HDACis on cell proliferation and viability. a, determination of cell proliferation by measuring [3H]thymidine incorporation into MEFs cultured for the indicated times in the absence (control, closed circles) or presence of TSA (5 μM, open squares) and NaB (10 mM, closed triangles). The graph is representative of eight similar experiments. b, quantification by fluorescence-activated cell sorter analysis of the percentage of MEFs undergoing apoptosis (sub-G1 DNA contents) or at G1, S, or G2 phases of the cell cycle upon culture for 24 h in the absence (control) or presence of TSA (5 μM) and NaB (10 mM). Data are presented as mean values ± S.E. of five experiments. Asterisks (*, **, and *** indicate statistical significance (p < 0.05, p < 0.01, and p < 0.001, respectively) as compared with untreated controls. c, MEFs were cultured in the absence (control, closed circles) and presence of 5 μM TSA (left panel) or 10 mM NaB (right panel). As indicated by an asterisk, after 24 h the HDAC inhibitors were either washed out (closed triangles) or kept in the culture medium (open squares). Cell proliferation was quantified by counting cell numbers at the indicated time points. The graph is representative of two experiments. d, immunodetection of p16INK4a and p19ARF in MEFs either cultured in the absence (−) or presence (+) of 5 μM TSA (left panel) or 10 mM NaB (right panel). Cells were either cultured for a total time of 72 h in the presence of TSA or NaB, i.e. without washing out the drugs (−), or the HDAC inhibitors were removed during the last 24 or 48 h of the 72-h incubation. The blots shown are representative of at least two similar experiments.

these mRNAs in a manner that parallels the protein levels, i.e. down-regulation of p16INK4a mRNA and up-regulation of p19ARF mRNA (Fig. 4b).

To further characterize the transcriptional effect of HDACis on the INK4a and ARF promoters, we transiently transfected HEK-293T cells with ARF and p16INK4a promoter constructs placed upstream of a luciferase reporter. Fig. 4c, left panels, shows that TSA (5 μM) and NaB (10 mM) stimulated both the murine and human ARF promoters. Conversely, TSA and NaB inhibited the murine and human INK4a promoters (Fig. 4c, right panels). These results suggest that the opposing effects of HDACis on the transcription of the INK4a/ARF locus are intrinsically dictated by the corresponding proximal promoter sequences of INK4a and ARF.

In contrast to the wealth of data on the mechanisms of transcriptional activation by HDACis, the mechanisms involved in transcriptional down-regulation by HDACis are poorly understood. Motivated by this, we have mapped the promoter region involved in the repression of p16INK4a. A series of truncated human INK4a promoter constructs were assayed in HEK-293T cells in the absence or presence of TSA or NaB (Fig. 5a). HDACis suppressed INK4a promoter activity in all the constructs that contained at least 510 nucleotides upstream of the human p16INK4a ATG start codon. The inhibitory effect was abolished, and even reversed, upon further truncation of the promoter to 378 nucleotides or less. These data suggest that the region of 133 bp between positions −378 and −510 is important in mediating the inhibitory effect of HDACis on the human INK4a promoter. Of note, previous investigations have demonstrated that this region contains functional binding sites for Sp1 and Sp3 transcription factors (52, 53). Transcription factors Sp1 and Sp3 have been identified in numerous promoters as mediators of both HDACis-induced activation (see Ref. 22 and references therein) and of HDACis-induced repression (54). Interestingly, the murine INK4a promoter contains two predicted Sp1 sites at positions −162 and −344 upstream the ATG start codon (data not shown); therefore, in this regard the HDACi-induced repression of the human and mouse promoters could be explained by a common mechanism mediated by Sp1.

To further support the role of Sp1 in the regulation of the INK4a promoter by HDACis, we performed chromatin immunoprecipitation assays in intact MEFs. In line with the finding that HDACis induce global histone acetylation and loss of heterochromatinization (see Fig. 1), we observed that these drugs in fact promote histone H4 acetylation at the endogenous INK4a and ARF promoters (Fig. 5b). Chromatin immunoprecipitation of Sp1 revealed detectable Sp1 binding to the ARF promoter, but not to the INK4a promoter (Fig. 5b). Treatment with TSA or NaB resulted in a 4- to 8-fold increase in the binding of Sp1 to the INK4a promoter, whereas the occupancy of the ARF promoter was largely unaffected (Fig. 5b). These findings associate the repressive effect of HDACis on p16INK4a with the binding of Sp1 to the INK4a promoter.

Effect of HDACis on Cell Growth and Viability—Similar to what has been described previously for a variety of cell types, TSA (5 μM) and NaB (10 mM) induced a robust proliferative arrest in primary MEFs (Fig. 6a). After exposure to HDACis for 3 days, cells were completely arrested but were not positive for senescence-associated β-galactosidase (results not
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**FIGURE 7.** Implication of ARF in the cytostatic and anti-tumorigenic effects of HDACis. a, wild type MEFs (black symbols) and MEFs null for p21 (p21−/−, green), p53 (p53−/−, blue), or ARF (ARF−/−, red) were cultured for 4 days in the presence of increasing concentrations of TSA (left panel) or NaB (right panel). Cell proliferation was quantified by counting cell numbers and is given relative to untreated control cultures (mean values ± S.E. of two to eight experiments). Concentration response curves were fitted to the experimental data by the Hill equation. b, immunodetection of p19ARF, p16INK4a, p21, and β-actin in the indicated concentrations of TSA or NaB. The blots shown are representative of at least two experiments. c, left panel, wild type mice (black symbols, n = 10) and mice null for p21 (green, n = 8) or ARF (red, n = 6) were treated with a single intramuscular injection of MMC into the rear leg to induce fibrosarcoma formation. Animals were scored as positive for fibrosarcoma when tumors became palpable (“gained” diameter of the leg of 1–3 mm, see “Experimental Procedures”). Right panel, animals with palpable tumors were subjected to chemotherapy with NaB (filled circles) or left untreated (open circles) (see “Experimental Procedures”). Tumors were scored as full-blown fibrosarcoma when the diameter of the leg reached 15–17 mm (gained diameter of the leg of 12 mm). The median time from tumor detection to full-blown fibrosarcoma is indicated by a straight line.

shown). Based on a previous report, positive staining for senescence-associated β-galactosidase requires the continuous presence of the HDACi during a much longer time (equivalent to several culture passages (see Ref. 37). Importantly, TSA and NaB did not exert a significant cytotoxic effect as evidenced by the low amount (<6%) of apoptotic cells in cultures treated with HDACis for 24 h (Fig. 6b). The cell cycle profile of these cells revealed that the cytostatic effect of TSA and NaB is primarily due to a proliferation arrest in the G1 phase of the cell cycle (Fig. 6b).

We wished to know whether the anti-proliferative effect of HDAC inhibitors and their effects on p16INK4a and p19ARF were reversible. To address this issue, we cultured primary MEFs in the presence of TSA (5 μM) and NaB (10 mM) for 24 h and then washed out the drugs. As shown in Fig. 6c, MEFs previously arrested by TSA and NaB treatment started to proliferate upon washing out the inhibitors (Fig. 6c). Similarly, HDACi-triggered suppression of p16INK4a, as well as induction of p19ARF, were partially reversed upon removal of the HDACis (Fig. 6d). In summary, these data show that HDAC inhibitors exert a pronounced cytostatic effect that, in the case of primary normal cells, is not cytotoxic and can be reversible.

**Implication of p19ARF in the Cytostatic Effects of HDAC Inhibitors**—The p19ARF/p53/p21 tumor suppressor pathway plays a central role in the regulation of cell proliferation (reviewed in Refs. 31, 34). Remarkably, its three components are activated by HDAC inhibitors, specifically: transcription of p21 is strongly up-regulated by HDACis and it is considered a reference target for HDACi-mediated up-regulation (Ref. 21 and references therein); p53 is activated by direct protein acetylation and, accordingly, by inhibitors of HDACs (12–15); and, finally, as per our present study, p19ARF is transcriptionally up-regulated by HDACis. To evaluate the relative impact of each of the members of this pathway on the response to HDACis, we compared the behavior of MEFs deficient for p19ARF, p53, and p21, respectively. As shown in Fig. 7a, TSA (left panel) and NaB (right panel) inhibited the proliferation of MEFs in a concentration-dependent manner. Lack of ARF caused a marked increase in the IC50 values for TSA and NaB by a factor of 16- and 7-fold, respectively, relative to wild type MEFs (for IC50 values see TABLE ONE). Similarly, germ line deletion of p21 and p53 significantly impaired the potency of both drugs, although not as much as the absence of ARF.

The above concentration response curves further reveal that both TSA and NaB exert cytostatic effects at concentrations at least 10 times below the ones usually employed in cell culture assays. We therefore wished to confirm that relatively low concentrations of these drugs were in fact able to cause a detectable up-regulation of the p19ARF/p53/p21 pathway in our system. As shown in Fig. 7b, both TSA and NaB increased the expression of p19ARF (left panel) at concentrations as low as 25–50 nM TSA or 0.5 mM NaB, and this induction was accompanied by increases in p21 and p53 (right panel). Of note, at such low concentrations, these drugs had no detectable effect on the levels of p16INK4a (Fig. 7b). Together, these experiments provide both genetic (Fig. 7a) and biochemical (Fig. 7b) evidence for the causal implication of the p19ARF/p53/p21 tumor suppressor pathway in the cytostatic effects of TSA and NaB.

To further corroborate the relevance of p19ARF in the cytostatic response to HDACis in vivo, we studied the efficacy of NaB as a chemotherapeutic agent in a mouse model of chemically induced tumorigenesis. We compared wild type with ARF-deficient animals (40) and also included a positive control group of animals that were null for the
known NaB target p21. Fibrosarcoma formation was induced with a single intramuscular injection of 3MC. As shown in Fig. 7c, left panel, 100% of the wild type animals displayed tumors within 20 weeks following 3MC injection. Expectedly, ablation of the tumor suppressors p16INK4a and p14ARF/p19ARF has remained as a relevant mediator of the anti-tumorigenic action of HDAC inhibitors.

**DISCUSSION**

HDACs are known to be cytoplastic and cytotoxic for most cells, both normal and cancerous, and a number of mediators have been described in recent years (see the Introduction). Nonetheless, despite the importance of the INK4a/ARF locus in tumor suppression, the effect of HDACis on the expression of p16INK4a and p14ARF/p19ARF has remained largely unexplored. Here, we have shown that INK4a and ARF are oppositely regulated by HDACis in primary fibroblasts. It is important to note that there is at least a difference of one order of magnitude in the sensitivity of these two proteins to HDACis; specifically, p19ARF is efficiently up-regulated by low concentrations of HDACis (i.e. 25 mM TSA or 0.5 mM NaB), whereas p16INK4a is down-regulated only at rather high concentrations (i.e. 0.5 μM TSA or 5 mM NaB). The high concentrations of HDACis required to down-regulate the INK4a promoter could reflect the operation of indirect mechanisms, although this remains speculative. In the case of p19ARF, we have shown that this gene is up-regulated by HDACis playing an active role in the cytostatic effect of these drugs. It is interesting to note that HDACis activate independently the three members of the ARF/p53/p21 pathway, both by transcriptional activation as in the case of ARF and p21 and by protein stabilizing mechanisms as in the case of p53, thus implicating this pathway as a primary target of the cytostatic effect of HDACis.

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