

Histone Deacetylase 2 Modulates p53 Transcriptional Activities through Regulation of p53-DNA Binding Activity

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Abstract

Histone deacetylase (HDAC) inhibitors are emerging as promising cancer therapeutics. HDAC inhibitors have been found to induce cellular activities that are strikingly similar to p53-mediated responses to genotoxic stress. For example, HDAC inhibitors induce cell cycle arrest, apoptosis, and cellular senescence. Because at least 11 HDACs are affected by the current HDAC inhibitors, the HDAC critical for tumor cell survival and proliferation remains unknown. Thus, we sought to characterize the distinct roles of HDACs in the p53 pathway. Through the use of stable MCF7 cell lines which inducibly express short hairpin RNA targeting HDAC2, we found that HDAC2 plays important roles in the p53 pathway. Specifically, we found that knockdown of HDAC2 inhibited cellular proliferation in a dose-dependent manner which was also partly p53-dependent. Furthermore, knockdown of HDAC2 induced cellular senescence. Importantly, we found that knockdown of HDAC2 enhanced p53-dependent trans-repression and trans-activation of a subset of target genes. We found that the enhancement was due to increased p53-DNA binding activity but not alterations in p53 stability or posttranslational modification(s). Thus, for the first time, our data suggest that HDAC inhibitors function through the p53 pathway, at least in part, by activating p53-DNA binding activity. [Cancer Res 2007;67(7):3145–52]

Introduction

The p53 tumor suppressor, a transcription factor, is well known to regulate target genes that mediate cell cycle arrest, apoptosis, senescence, DNA repair, and other responses to genotoxic stress (1, 2). The ability of p53 to regulate gene expression is under stringent control. The p53 protein is maintained at a low abundance in nonstressed cells by the E3 ubiquitin ligases Mdm2, Pirh2, and COP (3, 4). DNA damage-induced phosphorylation and acetylation of p53 promote the stabilization and transcriptional activities of p53 (5–7). Because p53 has the potential to induce many responses, we and others have investigated mechanisms of differential target gene regulation by p53. Through the use of histone deacetylase (HDAC) inhibitors, we have previously shown that HDACs play a role in differential target gene selection by p53 family proteins (8).

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

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HDACs play important roles in many diverse processes such as transcriptional regulation, protein-protein interaction, protein subcellular localization, and organismal aging (9, 10). HDACs, which deacetylate histones and non-histone proteins, are organized into four classes: class I (HDAC 1, 2, 3, 8), class II (HDAC 4, 5, 6, 7, 9, 10), class III (SIRT 1–7), and class IV (HDAC 11; refs. 9, 11). HDACs in classes I, II, and IV contain a conserved catalytic domain and are commonly inhibited by HDAC inhibitors such as trichostatin A and sodium butyrate (10). These inhibitors chelate the zinc cation within the enzyme active site (10). Class III HDACs, which contain a NAD-dependent catalytic domain, are insensitive to these agents (10). Recent evidence shows that HDAC inhibitors induce apoptosis and inhibit proliferation in tumor cells (12–16). Although HDAC inhibitors are in phase I/II clinical trials and are emerging as promising cancer chemotherapeutics (9), the HDAC(s) critical for tumor cell survival and proliferation remains unknown.

p53 has recently been found to play an important role in mediating the effects of HDAC inhibitors. Upon HDAC inhibition, p53 has been found to be stabilized and acetylated at lysines 320, 373, and 382 (15–17). Although several lines of evidence suggest that HDAC inhibitors activate the p53 pathway, the role of p53 during HDAC inhibition and mechanisms by which individual HDACs regulate p53 activity remain unclear.

In this study, we sought to characterize the function of individual HDACs on p53 activity. Through the use of stable MCF7 cell lines which inducibly express short hairpin RNA (shRNA) targeting distinct HDACs, we found that knockdown of HDAC2, but not HDAC1, induced G₁ arrest and inhibited cellular proliferation in a manner that partly depended on p53. Furthermore, knockdown of HDAC2 induced cellular senescence. Importantly, we found that knockdown of HDAC2 enhanced trans-repression and trans-activation of a subset of target genes by endogenous p53. Investigation into the mechanism revealed that knockdown of HDAC2 enhanced the ability of p53 to bind DNA *in vivo*. Thus, for the first time, we found that HDAC2 negatively regulates p53 activity by inhibiting p53-DNA binding.

Materials and Methods

Plasmids and reagents. HAP53/pcDNA3, p21A/pGL2, and pBabe-U6-p53-siRNA were as described (8, 18). To generate tetracycline-inducible *PoIII*-driven expression of shRNA targeting HDAC1 or HDAC2, oligos were designed, annealed, and cloned into pTER vector. For HDAC1 shRNA, oligos were HDAC1-si-CR-F, 5'-GAT CCC CGC AGA TGC AGA GAT TCA ACT TCA AGA GAG TTG AAT CTC TGC ATC TGC TTT TTG GAA A-3' and HDAC1-si-CR-R, 5'-AGC TTT TCC AAA AAG CAG ATG CAG AGA TTC AAC TCT CTT GAA GTT GAA TCT CTG CAT CTG CGG G-3' with HDAC1 targeting sequence in boldface. For HDAC2 shRNA, oligos were HDAC2-CR-644-F, 5'-GAT CCC CAA GCA TCA GGA TTC TGT TAT TCA AGA GAT AAC AGA ATC CTG ATG CTT TTT TTG GAA A-3' and HDAC2-CR-644-R, 5'-AGC TTT TCC AAA AAA AGC ATC AGG ATT CTG TTA TCT CTT GAA TAA CAG AAT CCT GAT GCT TGG G-3' with HDAC2 targeting sequence in boldface. Murine HDAC2 was subcloned from pME18s into pcDNA3 using

EcoRI/XhoI. Reagents were tetracycline in ethanol at 2 $\mu\text{g}/\text{mL}$, doxycycline (Sigma, St. Louis, MO) in H_2O at 1 $\mu\text{g}/\text{mL}$, nutlin-3 (Cayman Chemical Company, Ann Arbor, MI) in ethanol, *cis*-diamineplatinum(II) dichloride (Sigma) in H_2O , and cycloheximide (Sigma) in ethanol at 10 $\mu\text{g}/\text{mL}$. Annealed RNA oligos (21 bp; HDAC2 siRNA, AAG CAU CAG GAU UCU GUU A and scrambled HDAC2 siRNA, GGC CGA UUG UCA AAU AAU U) were purchased from Dharmacon RNA Technologies (Layayette, CO).

Cell culture and transfection. MCF7, HCT116 p53^{-/-}, and MCF7-p53KD-3 cell lines were maintained as described (8, 18–20). Stable cell lines were generated as described (20). Individual clones were screened to identify MCF7 clone with stable integration of pcDNA6/TR (Invitrogen, Grand Island, NY), named MCF7-TR7. Individual clones were screened to identify tetracycline-inducible knockdown of HDAC1 or HDAC2 by Western blot analysis, named MCF7-si-HDAC1-7, MCF7-si-HDAC2-10, MCF7-si-HDAC2-B18, and MCF7-si-HDAC2-B38. To generate MCF7 cell lines with tetracycline-inducible knockdown of HDAC2 and constitutive knockdown of p53, pBabe-U6-p53-siRNA plasmid was transfected into MCF7-si-HDAC2-10 cells. Individual colonies were screened to identify clones with inducible knockdown of HDAC2 and constitutive knockdown of p53 by Western blot analysis, named MCF7-si-HDAC2-stable si-p53-13. For transient knockdown of HDAC2, MCF7, or MCF7-p53KD-3 (with stable p53-knockdown) cells were transfected with 50 nmol/L of oligos using siLentFect (Bio-Rad, Richmond, CA) for 3 days.

Western blot analysis. Western blot analysis was done as described (20) using anti-p21 (C19; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Ac-p53 (AcK373/382; Upstate, Lake Placid, NY), anti-actin (Sigma), anti-HDAC1 (E210; Upstate), anti-HDAC2 (3F3; Upstate), anti-c-Myc (9E10.2), and anti-p53 (DO-1, PAb1801, PAb240, and PAb421) antibodies.

Growth rate, colony formation assay, and DNA histogram analysis. Growth rate was assayed as described (20). Briefly, 3×10^4 cells were seeded with or without tetracycline in triplicate. Attached cells were counted at the indicated times. For colony formation assay, 500 cells were seeded with or without tetracycline in triplicate. Cells were fixed in methanol/glacial acetic acid (7:1), washed in H_2O , and stained with crystal violet (0.2 g/L). DNA histogram analysis was done as described (20). Briefly, 1×10^5 cells were seeded with or without tetracycline in triplicate, harvested, fixed in 100% ethanol, stained with propidium iodide, and analyzed by fluorescence-activated cell sorting.

Senescence-associated β -galactosidase assay. MCF7-si-HDAC2-10 cells (1×10^3) were seeded in triplicate with or without tetracycline. Cells were fixed in 2% formaldehyde/0.2% glutaraldehyde in PBS, washed in PBS, and stained overnight with 40 mmol/L of citric acid/sodium phosphate (pH 6.0), 150 mmol/L of NaCl, 2 mmol/L of MgCl_2 , 5 mmol/L of potassium ferrocyanide, 5 mmol/L of potassium ferricyanide, 1 mg/mL of X-gal. The percentage of SA- β -galactosidase-positive colonies were scored.

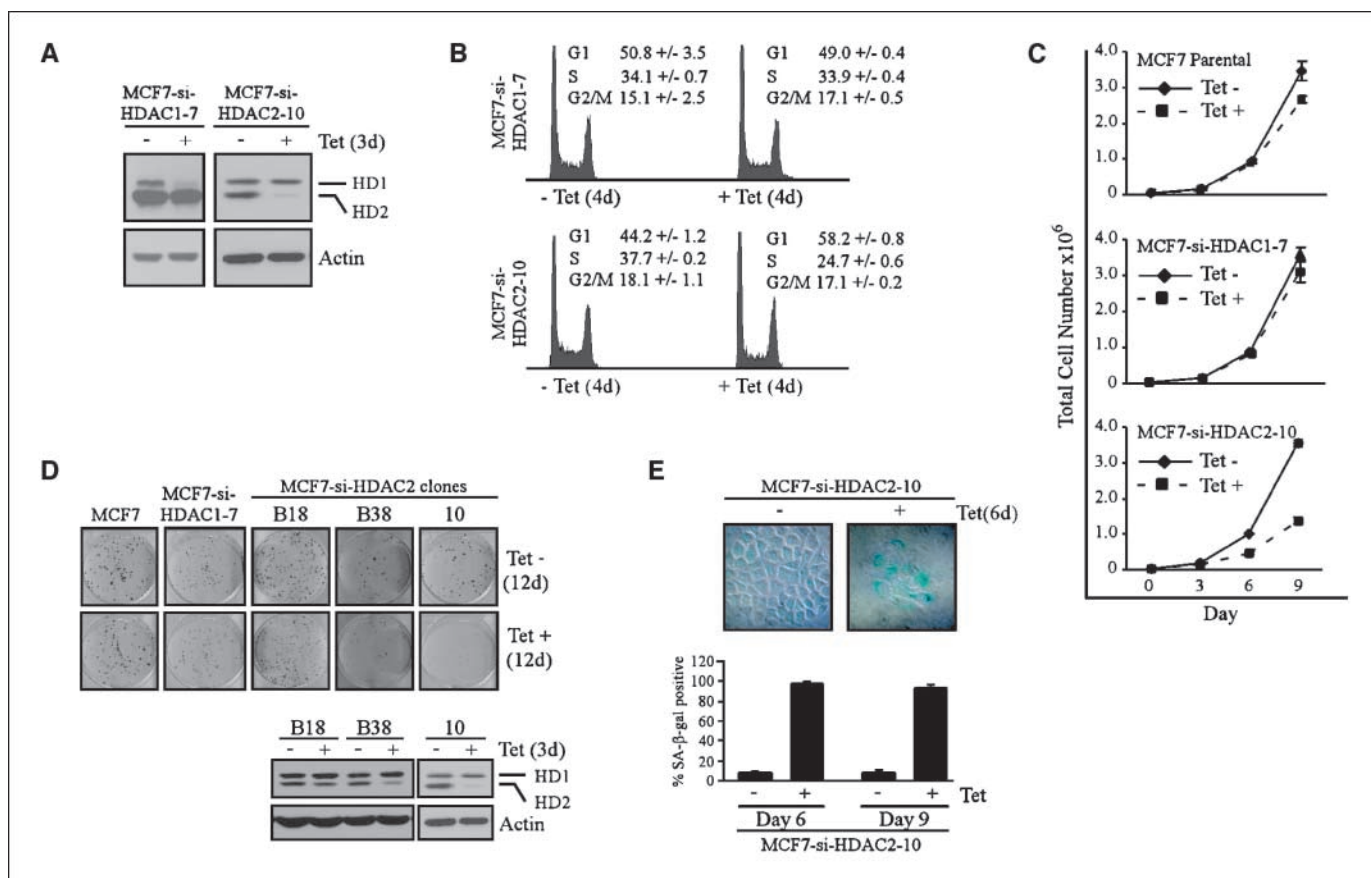


Figure 1. Knockdown of HDAC2, but not HDAC1, induces G₁ cell cycle arrest, inhibits cellular proliferation, and induces cellular senescence. **A**, the abundance of HDAC1 and HDAC2 is reduced in stable MCF7 cell lines upon tetracycline-inducible expression of shRNA targeting HDAC1 and HDAC2, respectively. The levels of HDAC1, HDAC2, and actin were assayed by Western blot analysis with antibodies against HDAC1, HDAC2, and actin, respectively, in MCF7-si-HDAC1-7 and MCF7-si-HDAC2-10 cells grown with or without tetracycline for 3 d. **B**, knockdown of HDAC2, but not HDAC1, induces G₁ arrest. The percentages of MCF7-si-HDAC1-7 and MCF7-si-HDAC2-10 cells in G₁, S, and G₂-M phases grown with or without tetracycline for 4 d were determined by DNA histogram analysis. **C**, knockdown of HDAC2 inhibits cell proliferation. Growth rates for parental MCF7, MCF7-si-HDAC1-7, and MCF7-si-HDAC2-10 cells grown with or without tetracycline were measured by Coulter cell counter. Bars, SD. **D**, colony formation assay for parental MCF7, MCF7-si-HDAC1-7, and MCF7-si-HDAC2 clones B18, B38, and 10. Cells were grown with or without tetracycline for 12 d, fixed, and stained with crystal violet. The levels of HDAC1, HDAC2, and actin were assayed by Western blot analysis in MCF7-si-HDAC2 clones B18, B38, and 10 grown with or without tetracycline for 3 d. **E**, knockdown of HDAC2 induces cellular senescence. MCF7-si-HDAC2-10 cells were grown with or without tetracycline, fixed, and stained for senescence-associated β -galactosidase activity at the indicated times. Phase contrast microscopy of representative colonies (*top*) and quantitation of SA- β -galactosidase-positive colonies (*bottom*). Bars, SE.

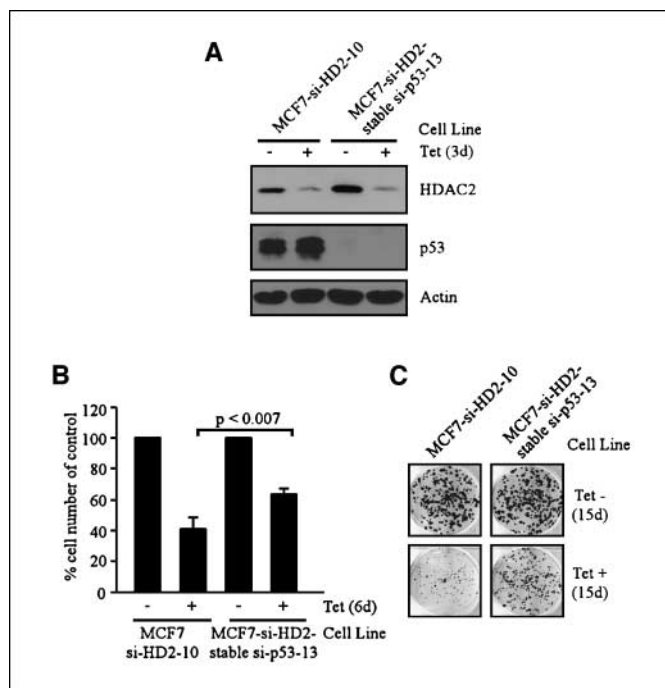


Figure 2. Knockdown of HDAC2 inhibits cellular proliferation in p53-dependent and -independent manners. *A*, MCF7-si-HDAC2-10 and MCF7-si-HDAC2-stable si-p53-13 cells were grown with or without tetracycline for 3 d. The level of HDAC2, p53, and actin was assayed by Western blot analysis. *B*, stable knockdown of p53 partly rescues growth arrest induced upon knockdown of HDAC2. MCF7-si-HDAC2-10 and MCF7-si-HDAC2-stable si-p53-13 cells were grown with or without tetracycline for 6 d, collected, and counted by Coulter cell counter. Columns, average of three independent experiments each done in triplicate; bars, SD. *C*, colony formation assay for MCF7-si-HDAC2-10 and MCF7-si-HDAC2-stable si-p53-13 cells. Cells were grown with or without tetracycline for 15 d, fixed, and stained with crystal violet.

RNA isolation and Northern blot analysis. Total RNA was isolated using Trizol reagents (Invitrogen). Northern blot analysis was done as described (8). The DKK1, GAPDH, Mdm2, p21, and FDXR probes were prepared as described (8, 20–22), whereas the 338-bp c-Myc cDNA probe was amplified using primers as described (23) and cloned into pGEM-Teasy.

Luciferase assay. To determine the effect of HDAC2 knockdown on p53 activity, MCF7-si-HDAC2 cells were grown with or without tetracycline for 4 days and cotransfected in triplicate with p21A/pGL2, internal control Renilla luciferase vector pRL/CMV, and either empty pcDNA3 or pcDNA3 expressing HA-tagged wild-type p53 for 24 h. To determine the effect of HDAC2 on p53 activity, HCT116 p53^{-/-} and MCF7 cells were cotransfected in triplicate with p21A/pGL2, pRL/CMV, and an equal concentration of pcDNA3, p53, and HDAC2 expression vectors as indicated for 24 h. The dual luciferase reporter assay (Promega, Madison, WI) was done. Fold increase in luciferase activity is shown. SE was determined.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assay was done as described (8). Briefly, MCF7-si-HDAC2-10 cells were grown with or without tetracycline for 3 days and treated with 5 μ M of nutlin or 50 μ M of cisplatin as indicated. Chromatin was cross-linked and sonicated. DNA was quantitated and equal amounts of chromatin were immunoprecipitated with anti-mSin3A (K20; Santa Cruz), anti-Ach3 (Upstate), anti-p53 DO-1, or a mix of anti-p53 (DO-1, PAb1801, PAb240, and PAb421) antibodies. Control antibodies were nonimmune rabbit IgG (Sigma), mouse IgG_{2a} (UPC-10; Sigma), and antimouse (Sigma) antibodies. Immunoprecipitated DNA fragments were purified over a Qiagen (Chatsworth, CA) column and analyzed by PCR. Primers that amplify the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter and the 5' p53-responsive elements within the p21 and Mdm2 promoters were as described (8, 24, 25). Primers that amplify a 208-bp fragment

spanning the p53-responsive element within the human c-Myc gene were the 5'-end primer Myc-1765-5' (TGA GGG ACC AAG GAT GAG AAG AAT G) and 3'-end primer Myc-1104-3' (TGA AAG TGC ACT GTA TGT AAC CCG C). Primers that amplify a 189-bp fragment spanning the +1 site of the DKK1 gene were the 5'-end primer DK1-2996F (CAG TCA GGA CTC TGG GAC CGC AGG G) and the 3'-end primer DK1-3185R (GCC GCT ACC ATC GCG ACA AAG ACC C).

Affymetrix gene chip analysis. Total RNAs from MCF7-si-HDAC2-10 cells grown with or without tetracycline for 4 days were isolated, labeled, and hybridized to an Affymetrix gene chip (U133 plus 2.0).

Results

Knockdown of HDAC2, but not HDAC1, inhibits cellular proliferation and induces cellular senescence. To characterize the role of individual HDACs on p53 activity, we generated stable MCF7 cell lines which inducibly express shRNA targeting HDAC1 or HDAC2 in the tetracycline-inducible system named MCF7-si-HDAC1 clone 7 and MCF7-si-HDAC2 clone 10, respectively. Western blot analysis showed that HDAC1 and HDAC2 protein levels were significantly decreased upon expression of shRNA targeting HDAC1 and HDAC2, respectively (Fig. 1*A*). Interestingly, we found that knockdown of HDAC2, but not HDAC1, resulted in a G₁ arrest (Fig. 1*B*). Consistent with the arrest in G₁, cellular proliferation (Fig. 1*C*) and colony formation (Fig. 1*D*) were inhibited upon knockdown of HDAC2. We also found that HDAC2 affected colony formation in a dose-dependent manner because more effective knockdown of HDAC2 resulted in more marked growth inhibition (Fig. 1*D*). In contrast, knockdown of HDAC1 or tetracycline had no effect on cellular proliferation or colony formation (Fig. 1*C* and *D*). Furthermore, we found that knockdown of HDAC2 resulted in cellular senescence (Fig. 1*E*). Thus, HDAC2 is required for cellular proliferation and is critical for the inhibition of cellular senescence.

Because p53 is well known to inhibit cellular proliferation (1), we sought to determine if p53 was responsible for the growth arrest induced upon knockdown of HDAC2. Thus, we generated cell lines which inducibly express shRNA targeting HDAC2 and constitutively

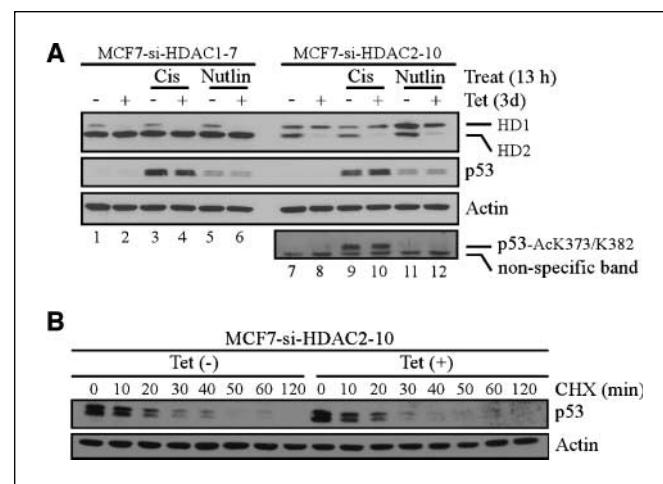


Figure 3. Knockdown of HDAC1 or HDAC2 does not affect p53 stability. *A*, MCF7-si-HDAC1-7 and MCF7-si-HDAC2-10 cells were grown with or without tetracycline for 3 d and treated with 50 μ M of cisplatin or 5 μ M of nutlin for 13 h. The level of HDAC1, HDAC2, p53, p53-AcK373/382, and actin was assayed by Western blot analysis. *B*, knockdown of HDAC2 does not prolong the half-life of p53. MCF7-si-HDAC2-10 cells were grown with or without tetracycline for 3 d and treated with cycloheximide (CHX) for the indicated times. The level of p53 and actin was assayed by Western blot analysis.

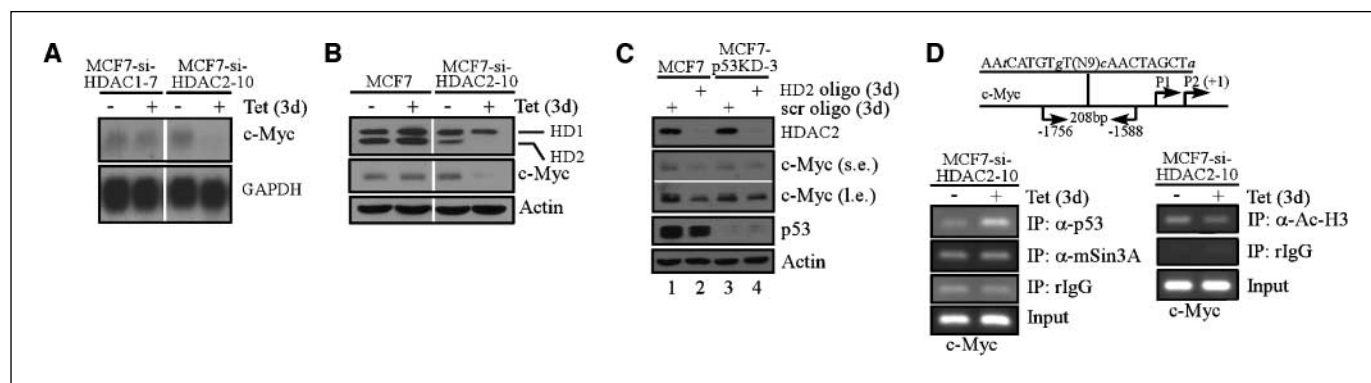


Figure 4. Knockdown of HDAC2 represses *c-Myc* in a p53-dependent manner. *A*, knockdown of HDAC2, but not HDAC1, represses *c-Myc* mRNA. A Northern blot was prepared using total RNAs isolated from MCF7-si-HDAC1-7 and MCF7-si-HDAC2-10 cells grown with or without tetracycline for 3 d. The blot was sequentially probed with cDNAs derived from *c-Myc* and *GAPDH* genes. *B*, knockdown of HDAC2 represses *c-Myc* protein. The level of HDAC1, HDAC2, *c-Myc*, and actin was assayed by Western blot analysis in MCF7 parental and MCF7-si-HDAC2-10 cells grown with or without tetracycline for 3 d. *C*, repression of *c-Myc* upon knockdown of HDAC2 is partially p53-dependent. Parental MCF7 cells and MCF7 cells in which p53 was stably knocked-down (MCF7-p53KD-3) were transiently transfected with scrambled or HDAC2 siRNA oligos for 3 d. The level of HDAC2, *c-Myc*, p53, and actin was assayed by Western blot analysis. *s.e.*, short exposure; *l.e.*, long exposure. *D*, knockdown of HDAC2 enhances p53-DNA binding to the p53-responsive element within the *c-Myc* promoter. *Top*, schematic representation of the human *c-Myc* promoter and location of transcriptional start sites, p53-responsive element, and primers used for ChIP assay. Lowercase italicized nucleotides identify mismatches from p53 consensus binding site. *Bottom left*, ChIP assay was done using MCF7-si-HDAC2-10 cells grown with or without tetracycline for 3 d. Chromatin was immunoprecipitated with p53, mSin3A, and control rabbit IgG antibodies. The fragment containing the p53-responsive element in the *c-Myc* promoter was amplified by PCR. *Bottom right*, ChIP assay was done using Ac-histone H3 and control rabbit IgG antibodies.

express shRNA targeting p53. Western blot analysis showed that p53 and HDAC2 protein levels were constitutively and inducibly down-regulated, respectively, in a representative cell line named MCF7-si-HDAC2-stable si-p53 clone 13 (Fig. 2A). Consistent with the role of p53 in growth arrest, we found that the proliferative defect induced upon knockdown of HDAC2 was partly rescued by knockdown of p53 as measured by cellular proliferation (Fig. 2B) and colony formation (Fig. 2C) assays. Thus, knockdown of HDAC2 inhibited proliferation in both p53-dependent and -independent manners. In sum, the inhibition of proliferation and the induction of cellular senescence upon knockdown of HDAC2 suggest that HDAC2 plays an important role in the promotion of tumorigenesis, in part through the inhibition of p53 activity, and that HDAC2 is a critical target of HDAC inhibitors for cancer therapy.

HDAC2 does not affect p53 stability. Because we have found that knockdown of HDAC2 inhibited proliferation in a manner that partly depended on p53, we sought to determine the mechanism by which HDAC2 inhibited p53 activity. Because HDAC inhibitors have been found to induce the stabilization of p53 (15–17), we wanted to determine if knockdown of HDAC1 or HDAC2 affected p53 stability. We found that knockdown of HDAC1 or HDAC2 did not affect the stabilization of p53 in cells treated with the chemotherapeutic agent cisplatin or the Mdm2-inhibitor nutlin (Fig. 3A; also see Fig. 5B). Although a small increase in the abundance of p53 was detected upon knockdown of HDAC2 together with cisplatin treatment, this increase was not consistently observed and was not detected upon knockdown of HDAC2 together with nutlin treatment (Fig. 3A, compare lanes 9 and 10 with lanes 11 and 12, respectively). Furthermore, knockdown of HDAC2 did not enhance cisplatin-induced acetylation of p53 as measured by Western blot analysis of whole cell lysates or p53 immunoprecipitates with anti-acetyl-p53 (K373/K382) antibody or anti-acetyl lysine antibody, respectively (Fig. 3A; Supplementary Fig. S1). This is consistent with evidence that p53 can be deacetylated by HDAC1 and HDAC3, but not by HDAC2 (26–28). In line with this, knockdown of HDAC2 did not prolong the half-life of p53 (Fig. 3B). Taken together, these data suggest that HDAC2

alone does not greatly affect p53 stabilization in response to cisplatin or nutlin.

HDAC2 inhibits p53-dependent trans-repression of *c-Myc*. Because knockdown of HDAC2 did not affect p53 stabilization or acetylation, we sought to determine if the expression of p53 target genes was affected upon knockdown of HDAC2. We found that p53-repressed genes, *c-Myc* and *Cyclin B1*, were down-regulated upon knockdown of HDAC2 (Fig. 4; Supplementary Fig. S2). We found that *c-Myc* mRNA was reduced upon knockdown of HDAC2, but not HDAC1 (Fig. 4A). The abundance of *c-Myc* protein was also reduced upon inducible as well as transient knockdown of HDAC2 (Fig. 4B and C). This is consistent with reports that *c-Myc* is repressed by HDAC inhibitors (29–31). Therefore, for the first time, we have identified that knockdown of HDAC2, but not HDAC1, results in the repression of *c-Myc* (Fig. 4A and B). Because p53 is known to directly repress *c-Myc* (32), we sought to determine the role of p53 in the repression of *c-Myc* upon knockdown of HDAC2. To do this, we transiently knocked-down HDAC2 in MCF7 cells and in MCF7 cells in which p53 was stably knocked-down. We found that the repression of *c-Myc* upon knockdown of HDAC2 was partly dependent on p53 (Fig. 4C, compare lanes 1 and 3 with lanes 2 and 4, respectively). To investigate the mechanism, we characterized the binding of p53 to the *c-Myc* promoter by ChIP analysis and found that in the unstressed condition, the extent of p53 bound to the p53-responsive element within the *c-Myc* promoter was markedly increased upon knockdown of HDAC2 (Fig. 4D). mSin3A, a component of the p53-repression complex, was also found to interact with the *c-Myc* promoter, but the interaction was not affected by HDAC2 knockdown (Fig. 4D). Consistent with the repressed state of the *c-Myc* promoter, the level of histone H3 acetylation was diminished upon knockdown of HDAC2 (Fig. 4D). Immunoprecipitation with rabbit-IgG served as a negative control in the ChIP assay (Fig. 4D). Thus, HDAC2 is required for the basal expression of *c-Myc* by inhibiting p53-dependent repression of *c-Myc*.

HDAC2 inhibits p53-dependent trans-activation of a subset of target genes. Because HDAC2 inhibited p53-dependent

trans-repression of *c-Myc*, we investigated whether HDAC2 could also affect p53-dependent trans-activation. First, we sought to determine if HDAC2 inhibited *IGFBP3* or *AQP3* induction by p53 because HDAC inhibitors restore the ability of endogenous p53 to induce *IGFBP3* and possibly *AQP3* in MCF7 cells (8). To test this, HDAC2 was inducibly knocked-down in cells treated with or without cisplatin or nutlin. We found that knockdown of HDAC2 was not sufficient to activate endogenous p53 to induce *IGFBP3* or *AQP3* in MCF7 cells (data not shown). Thus, a combination of HDAC2 and another HDAC may be involved in repressing endogenous p53 to induce *IGFBP3* and *AQP3*.

To identify other genes regulated by HDAC2, we did a microarray study. Although 2% of genes can be affected upon HDAC inhibition (33), only a small number of genes, such as Dickkopf-1 (*DKK1*; NM_012242.1), *DUSP5* (NM_004419.2), and claudin-1 (*CLDN1*; AF101051.1), were found to be affected ≥ 2 -fold upon knockdown of HDAC2. As an internal control, the expression of *HDAC2* was found to be decreased by 5.3-fold upon knockdown of HDAC2. Because we have previously found *DKK1* to be a p53 target gene (21), we chose *DKK1* for further analysis. Indeed, knockdown of HDAC2 led to a modest *DKK1* induction (Fig. 5A). Strikingly, we found that knockdown of HDAC2 together with cisplatin or nutlin treatment led to a robust *DKK1* induction, whereas treatment with cisplatin or nutlin alone led to a modest *DKK1* induction (Fig. 5A). The induction of the *p21*, *Mdm2*, and *FDXR* genes by p53 was also further enhanced upon knockdown of HDAC2 (Fig. 5A). Thus, HDAC2 regulates p53 transcriptional activity, at least for a subset of p53 target genes.

To further characterize the role of HDAC2 in the p53 pathway, we examined the effect of HDAC2 on *p21* induction by p53. Consistent with the level of *p21* mRNA, *p21* protein was induced to a greater extent upon knockdown of HDAC2, either inducibly in MCF7 cells or transiently in RKO colon carcinoma cells, together with nutlin treatment compared with nutlin treatment alone (Fig. 5B). We also found that knockdown of HDAC2 led to a modest activation of the *p21* promoter and that knockdown of HDAC2 further enhanced the activation of the *p21* promoter by p53 (Fig. 5C). Conversely, overexpression of HDAC2 repressed the ability of p53 to activate the *p21* promoter in HCT116-p53^{-/-} and MCF7 cells (Fig. 5D). Taken together, HDAC2 inhibits the induction of *p21* by p53.

HDAC2 inhibits p53-DNA binding activity. Because knockdown of HDAC2 did not alter the stability or acetylation of p53 (Fig. 3), enhanced p53 transcriptional activities upon knockdown of HDAC2 are not likely due to p53 abundance. Because knockdown of HDAC2 enhanced p53-DNA binding at the *c-Myc* promoter (Fig. 4D), we investigated whether HDAC2 could also affect p53-DNA binding activity at p53-induced genes using ChIP analysis. Indeed, we found that knockdown of HDAC2 together with nutlin or cisplatin treatment resulted in enhanced p53-DNA binding to the p53-responsive element within the *p21* promoter when compared with p53-DNA binding upon nutlin or cisplatin treatment alone (Fig. 6A and B; Supplementary Fig. S3A). Although the cisplatin-induced p53-p300 interaction was not greatly altered upon knockdown of HDAC2 (data not shown), the increase in p53-DNA binding was associated with a concomitant increase in binding of the coactivator p300 to the *p21* promoter (Supplementary Fig. S3B). Furthermore, knockdown of HDAC2 together with nutlin or cisplatin treatment resulted in enhanced p53-DNA binding to the p53-responsive element within the *Mdm2* promoter and to a putative p53-responsive element near the transcriptional

start site of the *DKK1* gene (Fig. 6C). Antimouse and mouse IgG_{2a} antibodies served as negative controls in the ChIP assay (Fig. 6). As an additional control, p53 was not found to bind the *GAPDH* promoter (Fig. 6C).

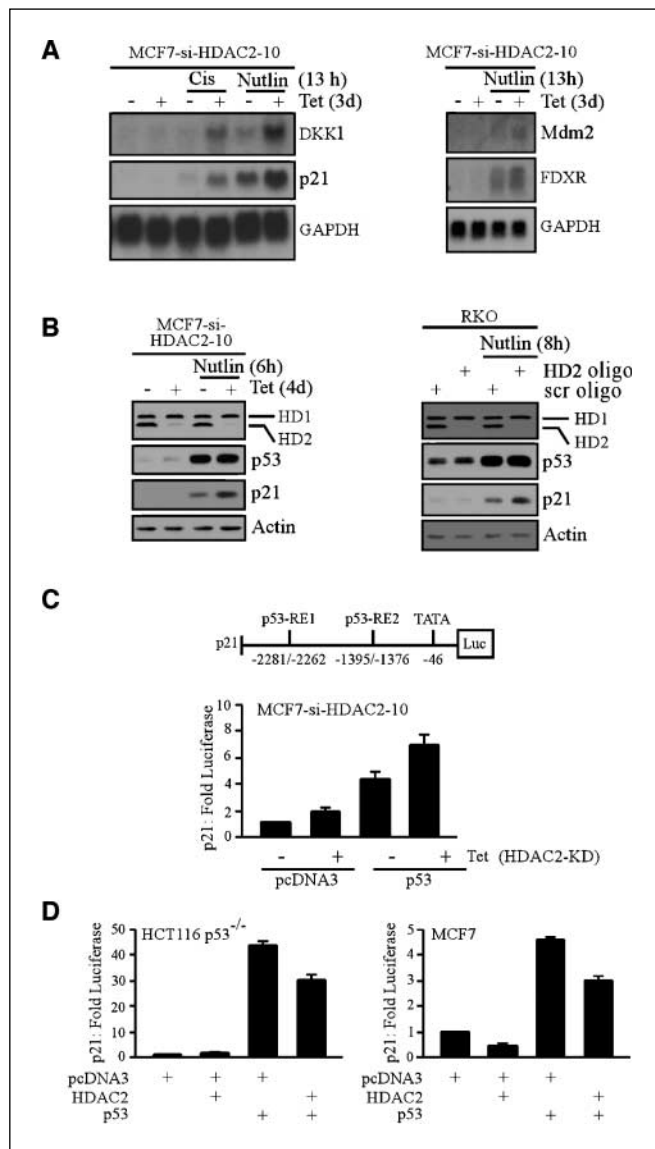


Figure 5. Knockdown of HDAC2 enhances p53 transcriptional activity for a subset of target genes. **A**, knockdown of HDAC2 enhances p53 activity to induce *DKK1*, *p21*, *Mdm2*, and *FDXR* genes. A Northern blot was prepared using total RNAs isolated from MCF7-si-HDAC2-10 cells grown with or without tetracycline for 3 d and treated with 50 $\mu\text{mol/L}$ of cisplatin or 5 $\mu\text{mol/L}$ of nutlin as indicated. The blots were probed with cDNAs derived from the *DKK1*, *p21*, *Mdm2*, *FDXR*, and *GAPDH* genes. **B**, knockdown of HDAC2 enhances the induction of *p21* by p53 in MCF7 and RKO cells. The level of HDAC1, HDAC2, p53, p21, and actin was assayed by Western blot analysis in MCF7-si-HDAC2-10 cells (*left*) treated as indicated and in RKO cells (*right*) transiently transfected with scrambled oligos or HDAC2 siRNA oligos along with treatment of nutlin as indicated. **C**, knockdown of HDAC2 activates the *p21* promoter and further enhances activation of the *p21* promoter by p53. *Top*, schematic representation of the *p21* luciferase reporter with p53-responsive elements and TATA box. *Bottom*, MCF7-si-HDAC2-10 cells were grown with or without tetracycline for 4 d and transfected with the *p21* luciferase reporter along with an empty pcDNA3 or pcDNA3 expressing p53 as indicated for 24 h. Fold increase in luciferase activity. **D**, HDAC2 diminishes the activation of the *p21* promoter by p53. HCT116 p53^{-/-} (*left*) or MCF7 (*right*) cells were transfected with the *p21* luciferase reporter along with pcDNA3, HDAC2, or p53 expression vector as indicated for 24 h. Fold increase in luciferase activity. Bars, SE.

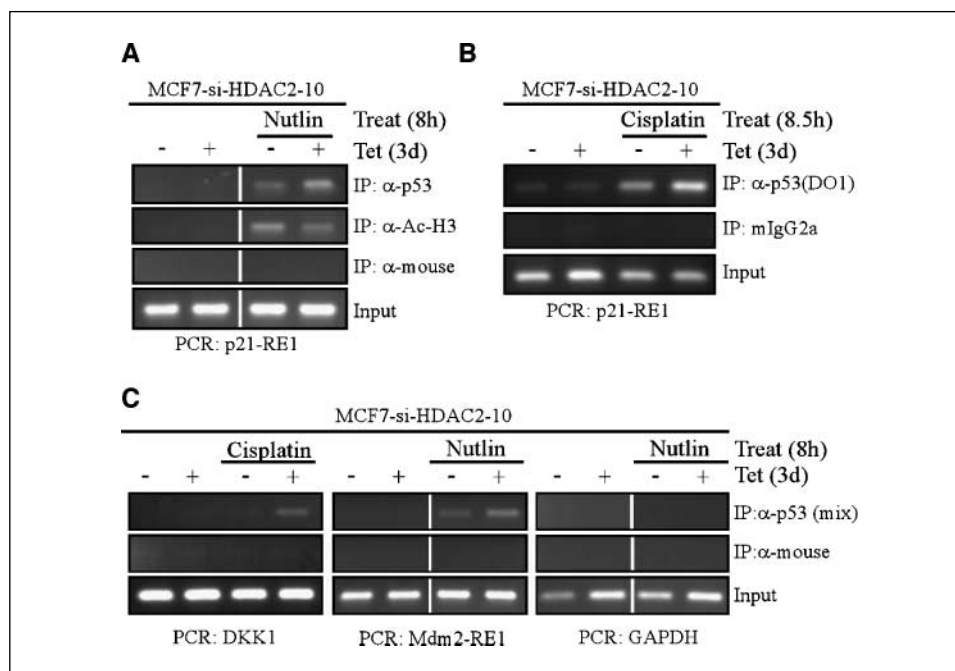


Figure 6. HDAC2 inhibits p53-DNA binding. *A* and *B*, knockdown of HDAC2 enhances p53-DNA binding at the endogenous p21 promoter upon treatment with nutlin (*A*) and cisplatin (*B*). ChIP assay was done using MCF7-si-HDAC2-10 cells grown with or without tetracycline for 3 d and treated with 5 $\mu\text{mol/L}$ of nutlin (*A*) or 50 $\mu\text{mol/L}$ of cisplatin (*B*) as indicated. Chromatin was immunoprecipitated with anti-p53 (either a mixture of DO1, PAb-1801, PAb-240, and PAb-421 or DO1 alone), anti-Ac-histone H3, control anti-mouse, or control mouse IgG_{2a} antibodies as indicated. p53-RE1 within the *p21* gene was amplified by PCR. *C*, knockdown of HDAC2 enhances p53-DNA binding at *DKK1* and *Mdm2* genes. ChIP assay was done as described above. A potential p53-responsive element near the transcriptional start site of the *DKK1* gene and p53-RE1 within the *Mdm2* gene were amplified by PCR. The GAPDH promoter was amplified and served as an additional negative control for p53-DNA binding.

Although it is well accepted that the binding of an activator facilitates the hyperacetylation of core histones at some promoters (34), surprisingly, we found that the level of histone H3 acetylation did not parallel the increase in binding of p53 and p300 to the *p21* promoter. When compared with the unstressed condition, we found that the level of histone H3 acetylation increased upon treatment with nutlin or cisplatin regardless of HDAC2 status (Fig. 6A; Supplementary Fig. S3A). However, the level of histone H3 acetylation was diminished upon knockdown of HDAC2 together with nutlin or cisplatin treatment compared with nutlin or cisplatin treatment alone (Fig. 6A; Supplementary Fig. S3A). Thus, knockdown of HDAC2 together with cisplatin or nutlin treatment correlated with increased p53- and p300-binding and decreased histone H3 acetylation at the p21 promoter. This suggests that a corepressor, such as HDAC2, exerts a unique effect on the promoter for gene regulation.

Discussion

HDAC inhibitors, which have been shown to inhibit proliferation and to induce apoptosis and cellular senescence (13–15, 35, 36), are emerging as promising cancer therapies (9). Here, we have identified that HDAC2 is a critical target for HDAC inhibition. We showed that inducible knockdown of HDAC2 induced G₁ arrest, inhibited cellular proliferation, and induced cellular senescence. Our data is consistent with recent studies which have shown that transient knockdown of HDAC2 inhibits proliferation and induces apoptosis (37, 38). Importantly, we found that the proliferative defect induced upon knockdown of HDAC2 was partly p53-dependent. In addition, we showed that knockdown of HDAC2 lead

to the repression of *c-Myc* that was partly dependent on p53. Because *c-Myc* is required for cellular proliferation in some cell types (39–41), it is likely that the repression of *c-Myc* plays an important role in the proliferation defect upon knockdown of HDAC2. Furthermore, through our microarray study, we identified and confirmed that claudin-1 (*CLDN1*), a component of the tight junction, was induced upon knockdown of HDAC2 (Supplementary Fig. S4A and B). Because *CLDN1* expression has been found to be increased in senescent compared with proliferating mammary epithelial cells (42), perhaps *CLDN1* plays a role in the senescence induced upon knockdown of HDAC2. Taken together, these data suggest that HDAC2 promotes cellular proliferation and prevents cellular senescence and is thus a critical target of HDAC inhibitors.

The function of HDAC2 as a corepressor is well established. HDAC2 plays an important role in transcriptional repression by the mSin3a and NURD complexes as well as by transcription factors such as Mad, YY1, p53, and others (27, 43–46). In this study, we have found that HDAC2 negatively regulates p53 transcriptional activities such that HDAC2 not only inhibits the ability of p53 to trans-activate but also to trans-repress a subset of genes. We showed that knockdown of HDAC2 enhanced p53 induction of *p21*, *DKK1*, *Mdm2*, and *FDXR*, but not *IGFBP3* or *AQP3*. Furthermore, knockdown of HDAC2 repressed *c-Myc* and *cyclin B1*. Investigation into the mechanism revealed that enhanced trans-activation and trans-repression by p53 was due to augmented p53-DNA binding activity but not alterations in p53 stability or acetylation.

The precise mechanism by which HDAC2 inhibits p53-DNA binding remains unclear. Several hypotheses exist (*a*) HDAC2 directly inhibits p53-DNA binding activity, (*b*) HDAC2 deacetylates p53 at unknown lysine residue(s) to inhibit p53-DNA binding

activity, or (c) HDAC2 modulates the chromatin structure which affects p53-DNA binding activity. Because HDAC2 has been found to directly interact with p53 (27), the first hypothesis is formally possible. Evidence suggests that acetylation of COOH-terminal lysine residues in p53 enhances p53-DNA binding activity *in vitro* and *in vivo* (47–49). Although cisplatin-induced acetylation of lysines 373 and 382 was not altered upon knockdown of HDAC2, HDAC2 may act on the 18 other lysine residues within p53. Thus, the second hypothesis is formally possible. However, several lines of evidence suggest that p53 acetylation and stabilization are linked (6, 28). Because p53 stability was not affected upon knockdown of HDAC2, p53 acetylation may not be responsible for the augmented p53-DNA binding activity. Unexpectedly, we found that the level of histone H3 acetylation was diminished upon knockdown of HDAC2. The decrease in histone H3 acetylation correlated with the increase in p53-DNA binding activity. Thus, our data may support the third hypothesis in which HDAC2, although a deacetylase, affects p53-DNA binding through the modulation of histone acetylation and chromatin composition. In line with this, HDAC2 has been shown to interact with topoisomerase II, which plays an important role in the chromatin structure (45). Furthermore, *Myc* has recently been identified to influence global chromatin structure whereby lack of *Myc* led to a striking decrease in overall histone acetylation (50). Perhaps knockdown of HDAC2 induces an initial p53-independent repression of *c-Myc*, which then triggers a feed-forward loop of decreased histone H3 acetylation

and increased p53-DNA binding activity. Regardless, the reduced acetylation of histone H3 may enhance the electrostatic interactions between p53 and chromatin, thereby stabilizing the p53-DNA interaction.

In summary, we showed that knockdown of HDAC2 inhibited cellular proliferation in a manner that partly depended on p53, induced cellular senescence, and augmented p53-dependent transactivation and trans-repression of a subset of target genes. This is the first report, to our knowledge, to show that HDAC2 negatively regulates p53-DNA binding activity. Future studies need to address the precise mechanism by which HDAC2 inhibits p53-DNA binding as well as whether HDAC2 affects the DNA binding activity of mutant p53 or other p53 family proteins. Because the expression of HDAC2 has been found to be increased in cancers (37, 38), our study suggests that HDAC2 is a critical target of HDAC inhibitors in cancer therapies.

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