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Advances in Brief

Overexpression of Histone Deacetylase 1 Confers Resistance to Sodium Butyrate–Mediated Apoptosis in Melanoma Cells through a p53-Mediated Pathway

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Abstract

Melanoma cells typically express wild-type p53, yet they are notoriously resistant to DNA-damaging agents. Here, we show that sodium butyrate (NaB), a histone deacetylase (HDAC) inhibitor, induced apoptosis in human melanoma cells in a dose- and time-dependent manner. Apoptosis was associated with HDAC1-dependent induction of Bax and acetylation of p53. Down-regulation of HDAC1 by an antisense vector sensitized the cells to NaB-induced apoptosis, whereas its overexpression conferred resistance to this agent. Increased HDAC1 levels and activity impaired NaB-mediated activation of Bax promoter and Bax protein levels. Finally, using p53-null melanoma cell line and RNA interference in cells expressing wild-type p53 protein, we show that Bax induction and NaB-mediated apoptosis is p53 dependent.

Introduction

Currently used therapies for melanoma are not effective (20% survival) to prevent metastatic spread and morbidity in patients with vertical growth phase lesions of >4.0-mm thickness. Lack of a suitable melanoma therapy may at least in part be attributed to the fact that melanoma cells are notably resistant to apoptosis.

The tumor suppressor p53 triggers apoptosis in response to a variety of stimuli, including DNA damage. Although the mechanism(s) of p53-dependent apoptosis still is not completely understood, it has been established that p53 activates transcription of proapoptotic proteins, including Bax, Bam, Puma, and Noxa (1). Additionally, p53 represses antiapoptotic proteins, such as Bcl2 (2).

Unlike other cancer types, mutation of p53 is rarely detectable in primary human melanomas (3). Interestingly, despite the presence of excess wild-type p53, these cells are extremely radioresistant and do not undergo apoptosis after exposure to DNA-damaging agents (4–6). Histone deacetylase (HDAC) inhibitors recently have been shown to be potent apoptosis inducers in a variety of cancer cells (7) and currently are being tested for their efficacy in a variety of clinical trials. Sodium butyrate (NaB) is the most common and physiologic member of HDAC inhibitors because it is formed in the colon by fermentation of dietary fibers. Although NaB and two additional HDAC inhibitors, trichostatin A and depsipeptide, induced apoptosis in uveal melanoma cells (8), their effect in epidermal melanoma has yet to be characterized.

Here, we show that NaB induced apoptosis in metastatic melanoma cells and that its activity is mediated by increased acetylation of histones H3/H4 and p53 and up-regulation of Bax. We also show that down-regulation of HDAC1 expression sensitized melanoma cells to NaB-induced apoptosis, whereas its overexpression conferred resistance. Finally, we show that the HDAC1-mediated resistance is exerted through a p53-dependent inhibition of Bax expression.

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Materials and Methods

Cell Lines, Plasmids, and Reagents. A375 melanoma cell line was obtained from American Type Culture Collection (Manassas, VA). IIB has been characterized previously (9). UCD-Mel-N cell line was established from a cutaneous malignant melanoma.[4] The SB2 cell line was isolated from a primary cutaneous lesion (10). Melanoma cell lines were grown in D/F12 medium [equal amounts of Dulbecco's modified Eagle's medium and F12 medium (Life Technologies, Inc., Rockville, MD) with 1% antibiotic and antimycotic, 2.5 μg/mL insulin, 25 μg/mL transferrin, and 250 ng/mL epidermal growth factor] supplemented with 8% fetal bovine serum.

The Bax promoter luciferase plasmid was purchased from Science Reagents (El Cajon, CA). Sense and antisense HDAC1 plasmid was constructed by subcloning a *Bgl*II-*Xho*I fragment from pOZ-HDAC1 (11) into *Bam*HI-*Xho*I site of pPCDNA3.1hygro (+) and pPCDNA3.1hygro (−), respectively. The pME18S-HDAC2 and pcDNA3-Flag-HDAC3 plasmids were a gift from Dr. Edward Seto (H. Lee Moffitt Cancer Center & Research Institute, Tampa, FL; ref. 12). Wild-type and dominant negative ($p300^{1572–1903}$) p300 were gifts of Drs. D. Livingston (Harvard Medical School, Boston, MA) and N. La Thangue (MRC National Institute of Medical Research, London, United Kingdom), respectively, and described elsewhere (13). Dr. Bert Vogelstein (The Johns Hopkins University Medical Institute, Baltimore, MD) provided p53 expression plasmid. The p53 small interference RNA (siRNA) SMARTpool along with the nonspecific control duplexes were purchased from Dharmacon, Inc. (Lafayette, CO). Oligofectamine was used for transfection of siRNA and was a product of Invitrogen (Carlsbad, CA). Anti-Bax and anti–Bcl-2 mouse monoclonal antibody was from Pharmingen (San Diego, CA); anti-p53 mouse monoclonal antibody was purchased from Oncogene (Carpinteria, CA). Anti-HDAC1, antiacetylated p53 (K373 and K382), antiacetylated histone H4, and antiacetylated histone H3 rabbit polyclonal antibodies were from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti–β-actin monoclonal antibody was from Sigma, Inc. (St. Louis, MO). Antibodies were used at a dilution recommended by the manufacturer.

Quantitation of Apoptosis. To measure apoptotic cell death, we used a cell death detection ELISA kit (Roche, Basel, Switzerland) that measures a cytoplasmic histone-bound DNA fragment (mononucleosomes and oligonucleosomes) generated during apoptotic DNA fragmentation and not free histones or DNA that could be released during nonapoptotic cell death. Briefly, 1×10^5 cells were plated in 12-well plates in triplicate. Cytosolic fractions from the cells, with or without NaB treatment, were incubated with antihistone antibody to allow the nucleosomes, released as a result of apoptosis, to bind. The bound nucleosomes were further quantitated using peroxidase-conjugated anti-DNA antibody and a colorimetric detection system. The absorbance at A_{450} is equivalent to the amount of apoptosis.

Annexin V Binding Assay. Annexin V binding was performed using a standard kit from BD Clontech (Palo Alto, CA). After rinsing cells twice with PBS, cells were resuspended in 100 μL of 1× binding buffer in a flow cytometry tube, to which 5 μL of annexin V-FITC and 5 μL of propidium iodide were added and mixed well. After a 15-minute incubation at room temperature in the dark, 400 μL of 1× binding buffer were added, and flow cytometry was performed within 15 minutes.

Histone Acetylation. Histones were isolated from melanoma cells by HCl extraction and acetone precipitation as described previously (14). Isolated histones were separated in 15% polyacrylamide gel containing 8 mol/L urea, 5% acetic acid, and 0.375% Triton X-100, or in regular SDS-PAGE.

[4] Unpublished data.

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Luciferase Assay. Cells were transfected using FuGENE 6 reagent, as specified by the manufacturer (Roche). Transfections used 0.5 μg of pGL-3 or Bax promoter-luciferase plasmids and combinations of 1.0 μg of HDAC1, p300, or p53 expression plasmids, together with a β-galactosidase expression plasmid for normalization of transfection efficiencies. Luciferase and β-galactosidase activity was measured 48 hours post-transfection (unless mentioned otherwise) using the Luciferase Assay System kit (Promega, Madison, WI). When indicated, NaB was added 5 hours after transfection and incubated for 43 hours before performing the luciferase assay.

Small Interference RNA Transfection. Cells were seeded on six-well plates and grown up to 50% confluency. On the day of transfection, the cells were washed with serum-free OPTIMEM (Invitrogen) media twice, and 800 μL OPTIMEM were added. Lyophlized siRNAs (SMARTpool and control) were dissolved in 1× siRNA buffer [20 mmol/L KCl, 6 mmol/L HEPES (pH 7.5), and 200 nmol/L $MgCl_2$] at 20 μmol/L concentration. Ten microliters of this stock were mixed gently with 175 μL OPTIMEM. Four microliters of oligofectamine were mixed separately with 11 μL OPTIMEM and kept for 10 minutes at room temperature. These two solutions then were mixed and incubated for 20 additional minutes at room temperature. The whole mixture finally was added to the cells and incubated in a CO_2 incubator for 4 hours. Five hundred microliters of regular D/F12 media supplemented with 30% fetal calf serum were added to the cells without removing the transfection reagents and incubated for 44 hours with or without NaB before harvesting.

Results

Sodium Butyrate Can Induce Apoptosis in Human Melanoma Cells. Treatment with NaB resulted in dose-dependent induction of apoptosis in UCD-Mel-N, A375, and SB2 melanoma cells (Fig. 1*A*). The lowest concentration of NaB able to induce apoptosis was found to be 1 mmol/L. Using annexin V binding assay, we confirmed these results and showed that apoptosis induced by 2 to 8 mmol/L NaB was detectable 24 hours post-treatment (Fig. 1*B*). 5 mmol/L NaB dramatically increased histone H3 and H4 acetylation within 24 hours (Fig. 1*C*). To determine whether apoptosis is mediated by HDAC inhibition, we tested the ability of Trichostatin A (TSA), a different and potent HDAC inhibitor, to induce apoptosis. TSA was able to induce apoptosis in the three melanoma cells mentioned previously within 48 hours (Fig. 1*D*). NaB-induced apoptosis coincided with induction of the proapoptotic protein Bax (Fig. 1*E* and *F, top*) in all of these cell lines. Because activation of the tumor suppressor p53 is

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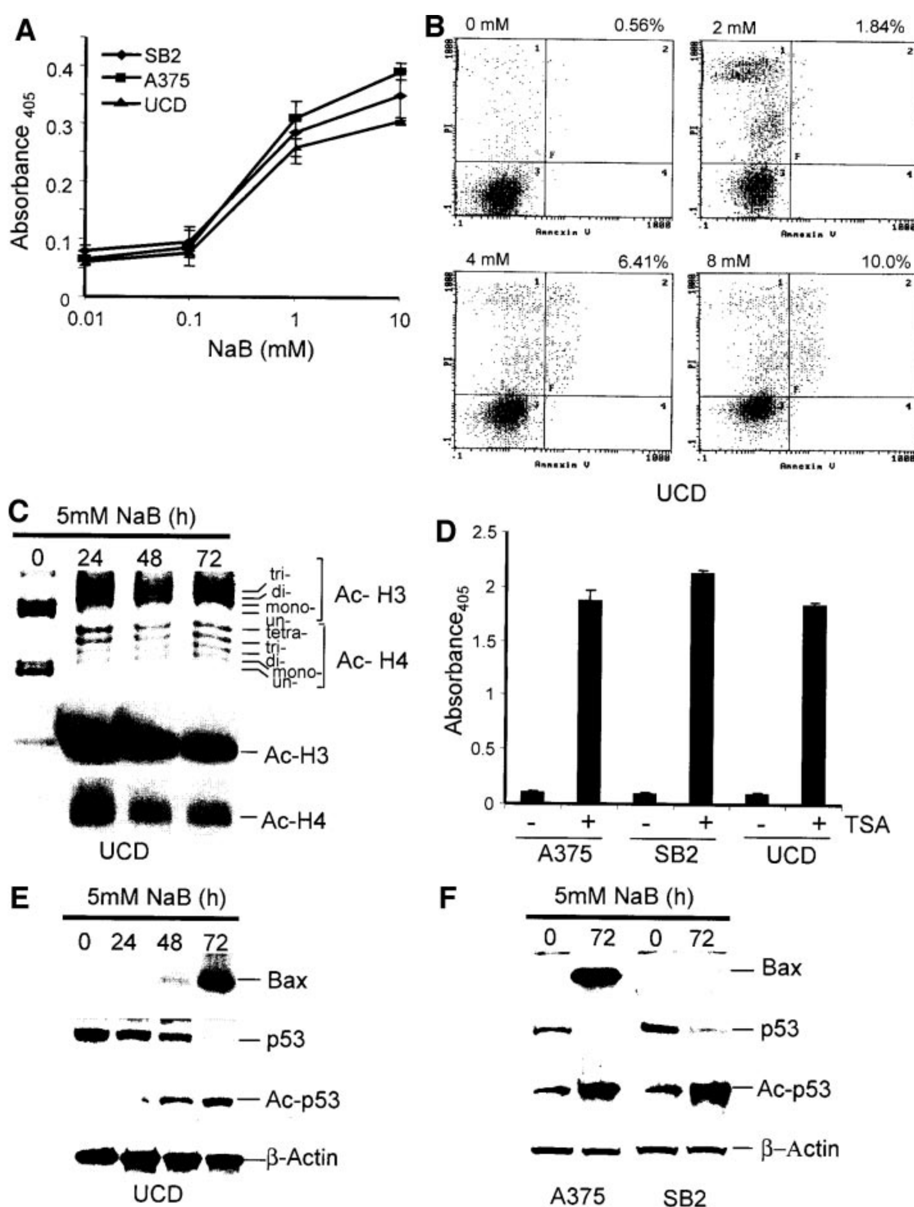

Fig. 1. Inhibition of HDAC-induced apoptosis in melanoma cells. *A.* UCD-Mel-N (▲), A375 (■), and SB2 (◆) cells were treated with increasing concentrations of NaB for 24 hours, and the nucleosomes released by apoptosis were measured by an apoptosis detection kit. *B.* UCD-Mel-N cells, treated with NaB for 24 hours, were subjected to annexin V/FITC/propidium iodide staining. Cells positive for propidium iodide and annexin V were considered apoptotic. Concentration of NaB and percentage of apoptotic cells are shown on left-top and right-top corner of each panel, respectively. *C.* Histones were isolated from UCD cells treated with NaB, followed by fractionation in either acid urea (*top*) or SDS-PAGE (*middle* and *bottom*). The acid urea gel was visualized using Coomassie staining, whereas the SDS-PAGE–separated proteins were transferred to nitrocellulose and blotted with acetylated histone H3 (*middle*) or H4 (*bottom*) antibodies. *D.* UCD, A375, and SB2 cells were treated with 300 nmol/L TSA for 24 hours, and the nucleosomes released by apoptosis were measured as in *A*. *E* and *F*. UCD (*E*), A375, and SB2 (*F*) cells were treated as indicated, and extracts were analyzed by immunoblot analysis with the indicated antibodies.

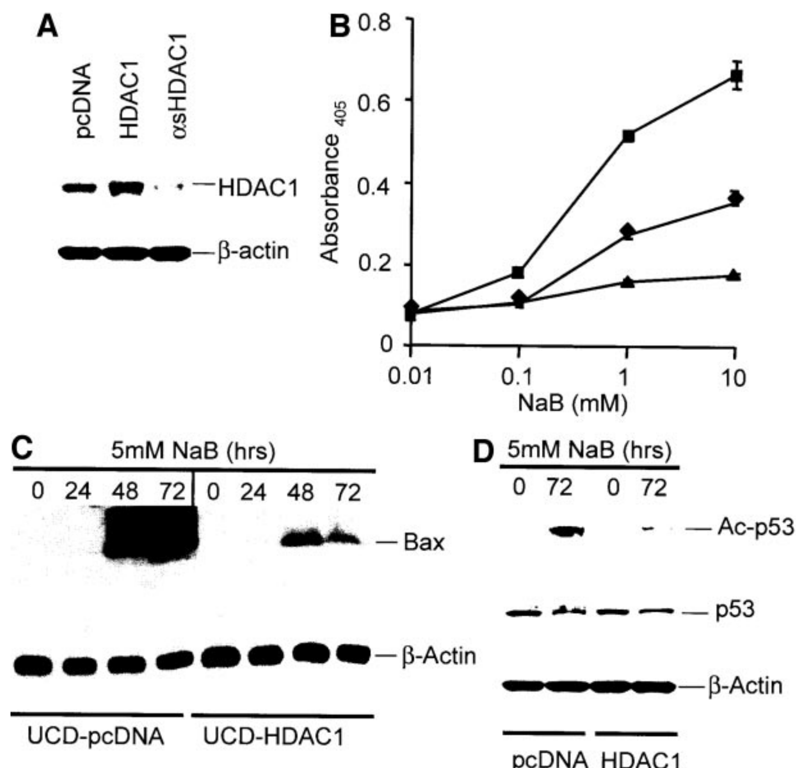

Fig. 2. Overexpression of HDAC1 conferred resistance to NaB-induced apoptosis. *A,* expression levels of HDAC1 in HDAC1- and antisense HDAC1- (asHDAC1) expressing cells. Fifty micrograms of total protein from the isolated clones were analyzed in SDS-PAGE by immunoblot analysis with anti-HDAC1 antibody. *B.* HDAC1- (▲) or asHDAC1- (■) expressing clones along with empty vector (♦) were treated with increasing concentrations of NaB for 48 hours. The extracts were assayed for apoptosis by cell death ELISA detection kit (see Materials and Methods). *C* and *D.* UCD cells expressing empty vector (pcDNA) and HDAC1 cDNA (HDAC1) were treated with 5 mmol/L NaB for the indicated time. The extracts were analyzed for Bax, p53 expression, and acetylation of p53 by immunoblot analysis. β-Actin is used as loading control.

known to play a direct role in the apoptotic pathway (15) and acetylation of p53 increases its transcriptional activity (12), we tested whether the levels of p53 protein and/or its acetylation are altered by NaB. We observed that this agent caused a gradual decrease of p53 protein level in UCD cells (Fig. 1*E, second panel from top*). However, acetylation of Lys-373 and/or Lys-382 of p53 steadily increased throughout the duration of the experiment (Fig. 1*E, third panel from top*). Interestingly, p53 acetylation preceded the induction of Bax, suggesting that p53 mediates NaB-induced apoptosis. Similar down-regulation of total p53 and elevation of Lys-373/382 acetylation of p53 were observed in A375 and SB2 cells at the time tested (72 hours; Fig. 1*F, second and third panels*).

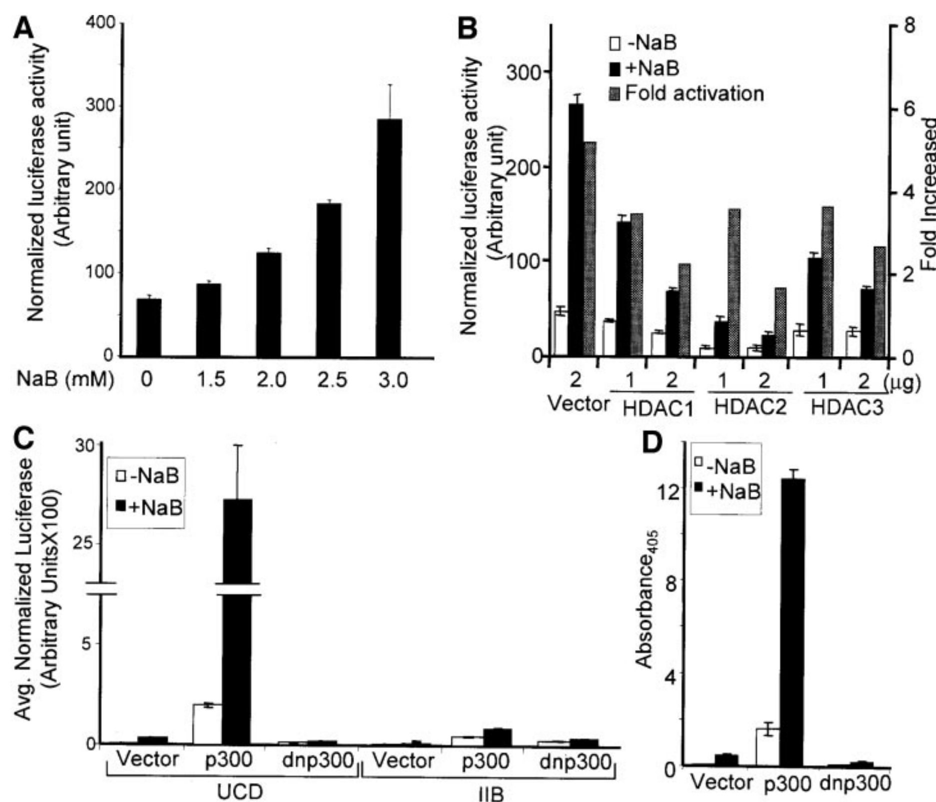

Fig. 3. HDAC overexpression counteracts NaB-mediated activation of Bax promoter. *A.* UCD-Mel-J cells were transfected with Bax promoter-luciferase plasmid and treated with increasing concentrations of NaB for 43 hours. The luciferase activities were normalized against an internal β-galactosidase. *B.* UCD cells were transfected with the indicated plasmids and treated with 3 mmol/L NaB for 32 hours. The normalized luciferase activity is plotted. *C.* UCD and IIB cells were treated as in *B*, and normalized luciferase values were plotted. The results shown are representative of three independent experiments. *D.* UCD cells were transfected with the indicated plasmids and treated with 3 mmol/L NaB for 32 hours, and apoptosis was measured as described previously.

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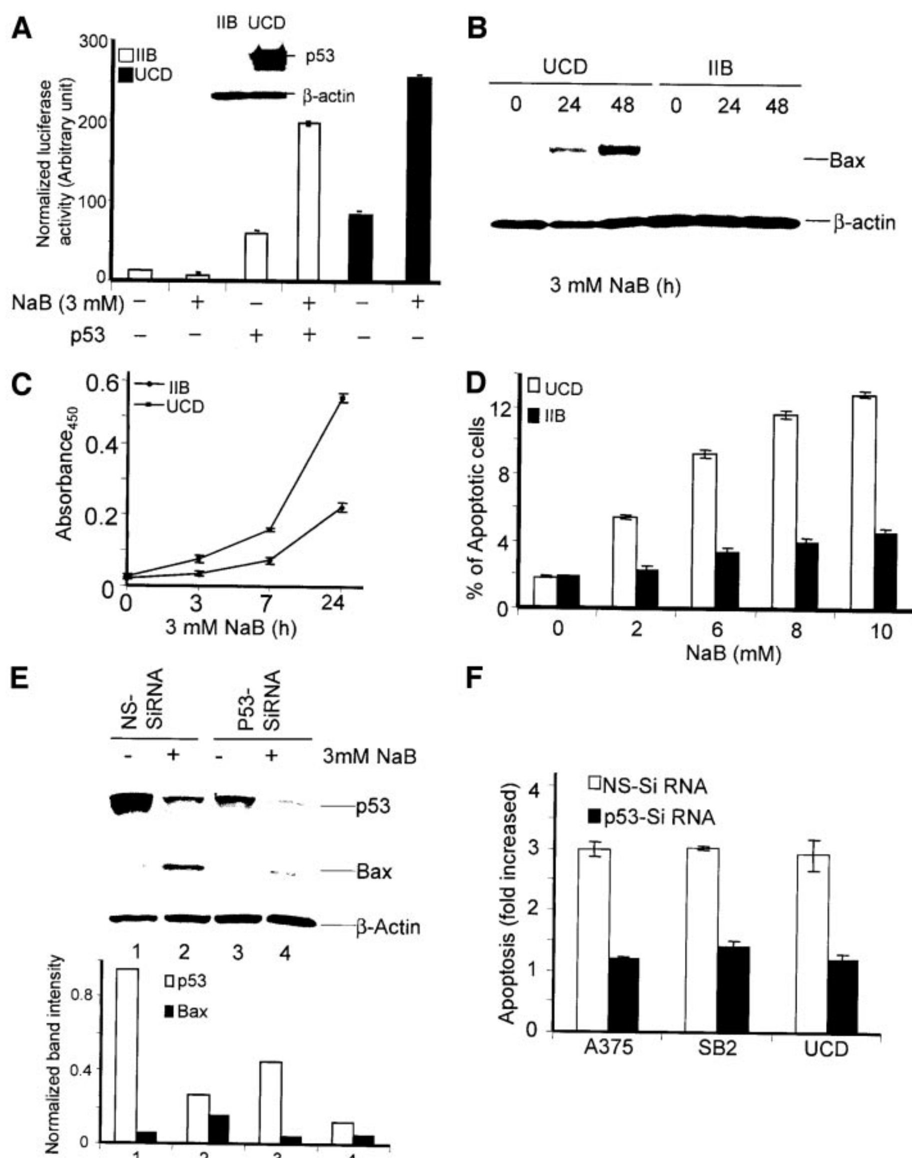

Fig. 4. NaB-mediated apoptosis in melanoma cells depends on p53. *A.* IIB or UCD-Mel-J cells were transiently transfected with the indicated plasmid(s), followed by a 3-hour treatment with 3 mmol/L NaB. The luciferase activity was normalized against an internal β-galactosidase. The result shown is representative of three independent experiments; *A, inset*. Fifty micrograms of extracts from UCD or IIB cells were analyzed in SDS-PAGE for p53 expression by immunoblot analysis with a p53 antibody. *B.* UCD or IIB cells were treated with 3 mmol/L NaB for the indicated time, and total extracts were analyzed with immunoblot analysis with an anti-Bax antibody. *C.* UCD (■) or IIB cells (10^6 cells; ◆) were treated with 3 mmol/L NaB for the indicated time points. The extracts were assayed for apoptosis using the cell death ELISA detection kit. *D.* UCD or IIB cells were treated with the indicated concentrations of NaB for 24 hours and subjected to annexin V/FITC binding assay. The percentages of apoptotic cells are plotted. *E.* UCD cells were transfected with control nonspecific siRNA or p53 siRNA and treated with 3 mmol/L NaB for 43 hours. The p53 and Bax expression was estimated by immunoblot analysis. The Bax and p53 levels were estimated by densitometric scanning and normalized for β-actin levels (bar chart). *F.* UCD, A375, and SB2 cells were transfected with control (NS-siRNA) or p53-specific siRNA (p53-siRNA) and treated with NaB as in *E.* Apoptosis was quantified by nucleosome estimation as before. The experiment is representative of three independent sets.

Overexpression of HDAC1 Confers Resistance for NaB-Induced Apoptosis. To test whether induction of apoptosis specifically results from HDAC inhibition, we used two strategies: overexpression of HDAC1 and down-regulation of HDAC1 levels by antisense mRNA (Fig. 2*A*). Treatment of these cell lines and a control cell line (harboring the empty vector pcDNA) with increasing concentration of NaB for 48 hours showed that overexpression of HDAC1 (Fig. 2*B, triangles*) increased resistance to apoptosis, whereas antisense mRNA sensitized the cells to NaB-induced apoptosis (Fig. 2*B, squares*). Importantly, induction of the proapoptotic protein Bax also was impaired in the HDAC1-overexpressing cells (Fig. 2*C*). This raised the possibility that Bax levels are directly responsible for NaB-mediated apoptosis. To correlate acetylation of p53 and Bax induction with HDAC1 levels, we asked whether HDAC1 overexpression could impair acetylation of p53. Empty vector and HDAC1-overexpressing UCD cells were treated with 5 mmol/L NaB for 72 hours. As expected, overexpression of HDAC1 impaired NaB-induced acetylation of p53 at 72 hours (Fig. 2*D, top*). Interestingly, HDAC1 overexpression did not cause appreciable changes in NaB-mediated down-regulation of total p53 (Fig. 2*D, middle*).

NaB-Mediated Activation of Bax Promoter Is Repressed by HDAC1. To determine whether NaB regulates Bax expression, we transiently transfected UCD cells with a Bax-promoter reporter construct (16). Five hours post-transfection, the cells were treated with increasing concentrations of NaB and incubated for an additional 43 hours. A luciferase assay showed that the Bax promoter activity was increased twofold to fourfold by such treatment (Fig. 3*A*). To determine whether increased levels of HDAC1 could counteract this effect, we cotransfected UCD cells with the Bax-promoter luciferase plasmid together with increasing amounts HDAC1 expression plasmid or with an empty vector, followed by NaB treatment as described previously. Overexpression of HDAC1 decreased the basal and NaB-induced Bax promoter activity in a dose-dependent manner (Fig. 3*B*). We also tested whether HDAC2 and HDAC3, two additional members of the class I family of HDACs, could have similar effects. Fig. 3*B* showed that HDAC2 and HDAC3 also were effective in counteracting the NaB-induced Bax promoter activation in a dose-dependent manner. HDAC2 was found to be most effective among the HDACs tested. Conversely, overexpression of the histone acetyltransferase p300, superinduced basal and NaB-mediated Bax promoter activation within 32 hours. Interestingly, this superinduction was not seen in

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p53-null IIB cells, indicating p53 dependence. Supporting this hypothesis, overexpression of a dominant negative p300, which lacks histone acetyltransferase activity but maintains an intact p53-binding domain (13), reduced Bax promoter activity and completely suppressed NaB-mediated induction (Fig. 3*C*). Collectively, these results suggest that histone acetyltransferases and deacetylases are mediators of Bax promoter activity in melanoma cells. The counteracting effects of p300 and dominant negative p300 on Bax promoter activity can further be extended to NaB-induced apoptosis (Fig. 3*D*).

HDAC1 Represses Bax Promoter Activity in a p53-Dependent Manner. The p53-mediated induction of the Bax promoter activity is reduced by overexpression of HDACs (17). We reasoned that the antagonistic effect of HDAC1 on NaB-induced apoptosis might be mediated through down-regulation of p53 activity and consequent repression of the Bax promoter. To test this hypothesis, we used a p53-null melanoma cell line IIB-Mel-J and a melanoma cell line expressing wild-type p53 (UCD; Fig. 4*A, inset*). Whereas 3 mmol/L NaB induced Bax promoter activity by 3.3-fold in UCD cells, the same treatment in IIB-Mel-J cells resulted in virtually no induction by NaB treatment (Fig. 4*A*). Importantly, overexpression of p53 was sufficient to increase basal and NaB-induced Bax promoter activity.

To determine whether NaB-mediated induction of Bax protein level is p53 dependent, UCD and IIB cells were treated with 3 mmol/L NaB for the indicated periods (Fig. 4*B*), and protein extracts were analyzed by immunoblot analysis using an anti-Bax antibody. As expected, induction of Bax by NaB was severely impaired in IIB (Fig. 4*B*) and correlated with decreased apoptosis as measured by ELISA (Fig. 4*C*) and annexin V binding assay (Fig. 4*D*). To confirm impaired apoptosis results directly from p53 deficiency, we down-regulated p53 in UCD cells using siRNA. A pool of four different p53 siRNAs reduced basal p53 levels by 47% (compare *Lanes 1* and *3*; Fig. 4*E, top*), which were further decreased by NaB treatment to 13% (*Lanes 2* and *4*; Fig. 4*E*). In agreement with the data discussed previously, induction of Bax by NaB was impaired by 50% in presence of p53 siRNA (Fig. 4*E, middle*). Similar effects of p53-siRNA on apoptosis were observed in A375, SB2, and UCD cells (Fig. 4*F*).

Discussion

The presence of excess wild-type p53 in the majority of human melanoma cells and their notorious resistance to apoptosis have puzzled researchers for many years. Here, we show that NaB at >1 mmol/L concentration induced apoptosis in at least three human melanoma cell lines. This concentration is comparable with that reported for other nonmelanoma cell lines, including colorectal carcinoma cells (18) and leukemic lymphoblasts (19). This effect is not NaB specific because another HDAC inhibitor, TSA, also could induce apoptosis at nanomolar concentration. Notably, NaB increased p53 acetylation within 24 hours, whereas the total level of p53 began to decrease at 48 hours. Decrease of p53 levels by HDAC inhibitors has been observed by others (20) but is not yet understood.

Importantly, induction of Bax by HDAC inhibitors appears to be cell type specific. Trichostatin A or NaB induced Bax and down-regulated Bcl2 level in hepatoma (21), oral, and gastric carcinoma cell lines (20) and metastatic melanoma (this study). However, HDAC inhibitors did not induce Bax in uveal melanoma (8) or in human glioma cell lines (6). Interestingly, the maximum levels of stable overexpression of HDAC1 protein attained in isolated melanoma clones were only twofold above controls, indicating that higher levels of HDAC1 could be deleterious for cell survival. However, a twofold increase of HDAC1 was sufficient to confer resistance to NaB-induced apoptosis. Conversely, an antisense HDAC1 vector reduced HDAC1 levels by twofold to threefold. Collectively, these results suggest that a critical level of HDAC1 is important to maintain cellular homeostasis.

Increased resistance to apoptosis in HDAC1-overexpressing clones was associated with impaired Bax expression. Our experiments using Bax-promoter luciferase assays also showed that overexpression of all three members of the class I HDAC family (HDAC1, HDAC2, and HDAC3) repressed Bax promoter activity, whereas inhibition of HDAC activated it. This is in agreement with a previous report showing that coexpression of HDAC1 reduced the activation potential of p53 on Bax promoter (17). Down-regulation of p53 function relies largely on the COOH-terminal region of p53 containing the basic lysine residues Lys-373 and Lys-382. NaB increased p53 acetylation at Lys-373 and/or Lys-382 (Fig. 1*E*). These results suggest that p300 and HDAC levels play a key role in regulation of apoptosis in melanoma cells and suggest that HDAC could be a potential target for novel therapies in the management of melanoma.

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