Acetylation of p53 Protein at Lysine 120 Up-regulates Apaf-1 Protein and Sensitizes the Mitochondrial Apoptotic Pathway*

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The p53 tumor suppressor controls cell growth, metabolism, and death by regulating the transcription of various target genes. The target-specific transcriptional activity of p53 is highly regulated. Here we demonstrate that acetylation of p53 at Lys-120 up-regulates its transcriptional activity toward Apaf-1, a core component in the mitochondrial apoptotic pathway, and thus sensitizes caspase activation and apoptosis. We found that histone deacetylase (HDAC) inhibitors, including butyrate, augment Lys-120 acetylation of p53 and thus Apaf-1 expression by inhibiting HDAC1. In p53-null cells, transfection of wild-type but not K120R mutant p53 can restore the p53-dependent sensitivity to butyrate. Strikingly, transfection of acetylation-mimicking K120Q mutant p53 is sufficient to up-regulate Apaf-1 in a manner independent of butyrate treatment. Therefore, HDAC inhibitors can induce p53 acetylation at lysine 120, which in turn enhances mitochondrion-mediated apoptosis through transcriptional up-regulation of Apaf-1.

Apoptosis is a genetically programmed cell death process in multicellular organisms. It plays crucial roles in various physiological events (1). Aberrant apoptosis is involved in large cohorts of diseases, such as cancer and neurodegeneration (2–4).

Apoptosis can be initiated either extrinsically or intrinsically, depending on the nature of the death signal. Upon receiving intrinsic apoptotic stimuli, several proapoptotic proteins, such as cytochrome c (5), SMAC (second mitochondria-derived activator of caspase) (6, 7), AIF (apoptosis-inducing factor 1, mitochondria) (8), and Endo G (9), are released from mitochondria into the cytosol where Apaf-1 and caspase 9 reside. Cytochrome c interacts with Apaf-1, triggering its binding to ATP/dATP and subsequent oligomerization, forming the apoptosis complex (10, 11). As the platform for caspase activation, apoptosome recruits and activates caspase 9, which subsequently activates the downstream caspases such as caspase 3 and 7, leading to eventual apoptotic cell death.

Release of cytochrome c from the mitochondrial intermembrane space, the primary regulatory point for mitochondrial apoptosis, is controlled by Bcl-2 family proteins. Overexpression of antiapoptotic Bcl-2 family proteins such as Bcl-2, Bcl-XL, and Mcl-1 blocks cytochrome c release (12–15). Conversely, proapoptotic Bcl-2 family proteins such as Bax and Bak, as well as BH3-only proteins such as Bid, Puma, and Noxa, promote cytochrome c release (16–20). Therefore, the ratio of antiapoptotic and proapoptotic Bcl-2 family proteins determines cell fate in responses to intrinsic apoptotic signals (21).

It was reported previously that a low dose of butyrate, a well known histone deacetylase (HDAC) inhibitor against class I and IIa HDACs, can dramatically enhance the ATP/dATP-dependent caspase activation in the cell-free caspase activation system. This effect depends on protein synthesis, suggesting that butyrate regulates the mitochondrial apoptotic pathway through induction of an unidentified factor (5). In this study, by applying a series of biochemical analyses, we demonstrate that butyrate inhibits HDAC1 and thereby increases p53 acetylation at Lys-120. Lys-120-acetylated p53 subsequently stimulates the transcription of Apaf-1, leading to elevation of ATP/dATP-dependent caspase activation in vitro and mitochondrion-mediated apoptosis in cells.

Experimental Procedures
Overexpression and shRNAi Plasmids—The vector used for the construction of various different expression plasmids in this
paper was modified from Plvx-AcGFP-N1 (Clontech). We modified Plvx-AcGFP-N1 with EcoRI and NotI restriction enzymes (New England Biolabs) to replace the AcGFP region with the following sequence: ATGGCATCAATGCA-AAGCAGTATCTCAGAGGAGGAGCTGAGCTGACGGCCG-GCCGGATCTGATCCGGCGCCAGCCGTATTAAATG-GATCCGATACATACAGGAGGCTGATACAGGAGGATACCA-GAGTGGACGACGATAAGGATGACGACGATATAAGGTA.

The new plasmid was named Plvx-MycFLAG. The Apaf-1 coding sequence (CDS) region was inserted into SfiI/NotI sites. On the basis of Plvx-AcGFP-N1, the p53 CDS region was inserted into EcoRI/NotI sites.

The primers for Apaf-1 and p53 cloning were as follows: Plvx-Myc-Apaf-1-FLAG, GAATCTCTCGAGGATGATGGATGCAAAAGTCGAAATT (forward) and ATAGAATGCGGCTTCTTCAAATGCTGTTAAATAAT (reverse); Plvx-HA-p53, CCGGAATTCATGTAACCTCCTCAGACGT-GCCC (forward) and ATAGAATGCGGCTTCTTCAAATGCTGTTAAATAAT (reverse). The Apaf-1 CDS clone (the template for amplifying the Myc-Apaf-1-FLAG fragment for further plvx-Myc-Apaf-1-FLAG construction) was a gift from Dr. Xiaodong Wang (National Institute of Biological Sciences, Beijing, China). pCDNA3-HA-p53 and the template for construction of plvx-MycFLAG were gifts from Dr. Jiangang Yuan (Institute of Biophysics, Chinese Academy of Sciences, Beijing, China).

Single amino acid mutation expression plasmids were constructed on the basis of the expression plasmids mentioned above. The primers for single site mutation were as follows: HA-p53 K120Q, ATTCTGGGACAGCC-GAGGTCTGGAACCGT (forward) and TGCAAGTCACAGCTGCTGACCT (reverse). The shRNAi plasmid pCMV-FLAG-HDAC1 was gifts from Dr. Xiaodong Wang (National Institute of Biological Sciences, Beijing, China). pCDNA3-HA-p53 and the template for constructing plvx-HA-p53 and the HDAC1 overexpression plasmid pCMV-FLAG-HDAC1 were gifts from Dr. Jiangang Yuan (Institute of Biophysics, Chinese Academy of Sciences, Beijing, China).

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Cell Culture, Transfections, and Reagent Treatments— 293T, MEF, Apaf-1−/− MEF, A549, H1299, and MCF-7 cells were cultured in DMEM supplemented with 10% FBS at 5% CO2. Cells were transfected using Lipofectamine 2000 (Invitrogen) following the instructions of the manufacturer. dATP was from Roche (catalog no. 13334128) and dissolved in PBS (135 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, and 8 mM K2HPO4 (pH 7.2)) to make 1 mM stock solution. Butyrate sodium (catalog no. B5887, Sigma-Aldrich) was dissolved in PBS to make 1 mM stock solution, whereas suberoylanilide hydroxamic acid (SAHA) (catalog no. S1047, Selleck), Trichostatin A (TSA) (catalog no. S1045, Selleck), CI1994 (catalog no. S2818, Selleck), and RGFP966 (catalog no. S7229, Selleck) were dissolved in dimethyl sulfoxide to make 10 mM stock solution. LMK-235 (catalog no. S7569, Selleck) and TMP269 (catalog no. S7324, Selleck) were dissolved in dimethyl sulfoxide to make 20 mM stock solution.

Antibodies—Primary antibodies used in Western blotting analysis and immunostaining were as follows. Apaf-1 antibody (catalog no. 611364) was bought from BD Biosciences. p53 (catalog no. 2524), HDAC1 (catalog no. 5356P), caspase 9 (catalog no. 9508), caspase 3 (catalog no. 9662), GAPDH (catalog no. 5174), β-actin (catalog no. 4967), P21 (catalog no. 2946), Mcl-1 (catalog no. 5453), Bcl-XL (catalog no. 2762), caspase 7 (catalog no. 9492), XIAP (X-linked inhibitor of apoptosis protein) (catalog no. 2042), Puma (catalog no. 12450), and Noxa (catalog no. 14766) were from Cell Signaling Technology, Inc. FLAG (catalog no. A8592) was from Sigma-Aldrich. HA (catalog no. sc-7392) and Myc (catalog no. sc-40) were from Santa Cruz Biotechnology, Inc. Lys-120 p53 Ac (catalog no. ab183542) was from Abcam.

Preparation of S-100 from HeLa and 293T Cells— Intracellular soluble protein (S-100) was prepared as described previously (23).

Dual-Luciferase Reporter Assay— The plasmid encoding the Apaf-1 luciferase reporter gene was constructed by inserting the Apaf-1 promoter region into KpnI/HindIII sites of the pGL3 plasmid. The Apaf-1 promoter region was amplified from human genomic DNA using the following primers: forward, CCGGATCCACTACTGGTGTGATTTAAATG-GCTGATCTCAGAGGAGGACCTGACCTGCAGGCCG; reverse, CCCAAGCTTTACTGGACACAAAGGGAGGA. Luciferase assays were carried out using the Dual-Luciferase assay kit (Promega, Madison, WI). The quantitative PCR primers for Apaf-1 RNA level detection were as follows (from Primer Bank): forward, GTCAACGATCATGAATGCA; reverse, CGTACGCAACCCCGTGAACCA.
Mass Spectrometry Analysis In-gel Protein Digestion—In-gel digestion was performed as reported previously (24). Briefly, gel slices were cut into 1-mm³ cubes and destained with 50% acetonitrile (ACN) in 50 mM NH₄HCO₃ and dehydrated with neat ACN. Protein disulfide bonds were reduced with 10 mM DTT in 100 mM NH₄HCO₃ at 56 °C for 30 min and subsequently alkylated with 55 mM iodoacetamide in 100 mM NH₄HCO₃ at room temperature for 20 min in darkness. After dehydration by ACN, digestion was performed in a buffer containing 1.2 ng/µl trypsin at 50 mM NH₄HCO₃ (10% ACN). The enzymatic reaction was allowed to proceed overnight at 37 °C. The resulting tryptic peptides were extracted from gel cubes with 50% ACN and 5% formic acid for 20 min twice at 37 °C with constant shaking. Finally, extracted peptides were pooled and vacuum-dried for further LC-MS/MS analysis.

LC-MS/MS Analysis—Nanoflow reverse-phase LC separation was carried out on an EASY-nLC 1000 system (Thermo Scientific). The capillary column (75 µm × 150 mm) with a laser-pulled electrospray tip (model P-2000, Sutter Instruments) was home-packed with 5 µm, 100-Å Magic C18AQ silica-based particles (Michrom BioResources Inc., Auburn, CA). Tryptic peptides were dissolved in HPLC-grade water, and 200 ng of samples was loaded onto the analytical column in a single LC-MS/MS experiment. The mobile phase was comprised of solvent A (97% H₂O, 3% ACN, and 0.1% formic acid) and solvent B (100% ACN and 0.1% formic acid). The LC separation was carried out with the following gradient. Solvent B was started at 7% for 3 min and then raised to 35% over 40 min. Subsequently, solvent B was rapidly increased to 90% in 2 min and maintained for 10 min before 100% solvent A was used for column equilibration. Peptides eluted from the capillary column were electrosprayed directly onto a linear ion trap mass spectrometer (LTQ Velos Pro, Thermo Scientific) for MS/MS analysis. A data-dependent mode was enabled for peptide fragmentation. One full MS scan (m/z range, 350.00–1500.00) was followed by fragmentation of the top 10 most intense ions by collision-induced dissociation. Dynamic exclusion (with a duration of 6 s) was enabled to preclude repeated analyses of the
same precursor ions. Both the precolumn and analytical column were washed intensively between different samples to remove any carryover from previous runs.

Raw MS files from the LTQ Velos Pro were analyzed by Proteome Discoverer (version 1.4.0.288, Thermo Scientific) and searched against the *Homo sapiens* (human) protein database (Fasta file downloaded from Uniprot) by using Mascot (version 2.3.02, Matrix Science Inc., Boston, MA). Acetylation on Lys and phosphorylation on Ser, Thr, and Tyr were set as variable modifications. The resulting peptide and protein assignments were filtered to achieve a false discovery rate of 1% at both peptide and protein levels using the target decoy method.

**ChIP—ChIP was performed as described on the Abcam web site.**

**FIGURE 2. Butyrate induces p53-dependent transactivation of Apaf-1.** A, 293T cells were seeded at a density of $1 \times 10^6$ cells/10-cm dish on day 0 and treated with 20 mM butyrate sodium solution on day 1 for 12, 24, and 48 h. Whole cell lysates were prepared at each time point and normalized. Aliquots of 10 μg of intracellular total proteins were subjected to Western blotting analysis against Apaf-1, p53, p21, Mcl-1, Bcl-xl, caspase 9 (Casp-9), caspase 3, caspase 7, XIAP, and β-actin. B, the mRNA level of Apaf-1 was increased by butyrate (20 mM, 12 h). C, on day 0, 293T cells were seeded at a density of $1 \times 10^6$ cells/10-cm dish. On day 1, each dish of cells was transfected with 2 μg of plasmids encoding the Apaf-1-luciferase reporter gene and tk-rellina reporter. On day 2, the cells were treated with butyrate for 12 h, and luciferase activity was measured subsequently as described under "Experimental Procedures." Shown are means ± S.E. of normalized firefly/Renilla luciferase activity. D, on day 0, 293T cells were plated at a density of $1 \times 10^6$ cells/10-cm dish. On day 1, cells were transfected with 2 μg of plasmids encoding HA-tagged p53 or vector (Vec) control as labeled. On day 2, transfected cells were treated with either butyrate sodium solution or PBS for 12 h, immediately followed by preparation of intracellular total protein. Intracellular levels of Apaf-1, HA-tagged p53, and endogenous p53 as well as GAPDH were visualized by Western blotting analysis as indicated. E, human non-small-cell lung carcinoma H1299 cells were plated at a density of $5 \times 10^6$ cells/10-cm dish on day 0 and treated with butyrate sodium or PBS on day 1. After 12 h, intracellular soluble protein S-100 was collected and normalized to a concentration of 1 μg/μl. Aliquots of 27 μg of proteins were incubated with a final concentration of 1 mM dATP for 3 h at 30 °C in a total volume of 30 μl. The reaction was terminated by adding 6 μl of 6× SDS loading buffer, followed by boiling at 95 °C for 10 min. Aliquots of 10 μg were subjected to Western blotting analysis of caspase 9 and β-actin. F, on day 0, H1299 cells were plated at a density of $5 \times 10^6$ cells/10-cm dish and treated with or without different concentrations of butyrate sodium solution as indicated for 12 h on day 1. Total cell lysates were collected, and aliquots of 10 μg of protein were subjected to Western blotting analysis of Apaf-1 and GAPDH. G, H1299 cells were seeded at a density of $5 \times 10^6$ cells/10-cm dish on day 0, transfected with 2 μg of vector or plasmids encoding HA-p53 as described in F and then treated with butyrate sodium solution for 12 h, followed by UV irradiation ($2 \times 10^5$ μJ/cm²). 8 h after UV irradiation, the cells were harvested and co-stained with Annexin V/PI. Apoptotic cells were determined by flow cytometry.
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ward) and GGAAAATGCATGGTTAAAAT (reverse); AIP1, TAATCCCCAGGCTTTGGGAAG (forward) and TGCGAGA-ACCTGGATCTAGCAA (reverse); and GAPDH, GTATTCC-CCCCAGGTTTACAT (forward) and GGAGTGAGTGGAAG-ACAGAA (reverse).

**Immunofluorescence**—Cells grown on coverslips were fixed in 4% paraformaldehyde with 0.1% Triton X-100 at room temperature for 15 min. Alexa 488- and Alexa 568-conjugated highly cross-absorbed goat anti-mouse and goat anti-rabbit IgG were used as secondary antibodies. The resulting samples were observed at room temperature under a TCS SP2 laser-scanning confocal microscope with a ×100, 1.4 numerical aperture Apo oil immersion lens on an upright microscope (DM IRBE, Leica) or under an IX 71 inverted fluorescent microscope (Olympus) equipped with a ×60, 1.4 numerical aperture Apo oil immersion lens (Leica). Images were acquired by Leica confocal software and DP controller software (Olympus). Image processing was performed in DP Manager (Olympus) and Photoshop (CS2, Adobe).

**Western Blotting**—Samples were prepared by adding SDS-PAGE loading buffer to the cell lysate and boiling for 5 min at 95 °C. After gel electrophoresis, the proteins were transferred onto a Magna nitrocellulose supported transfer membrane (Merck Millipore) and probed with primary antibodies at 4 °C for 8 h. HRP-conjugated donkey anti-mouse (Jackson ImmunoResearch Laboratories) and goat anti-rabbit IgG antibodies (Sigma-Aldrich) were used as secondary antibodies. The final results were visualized with a SuperSignal West Pico kit (Thermo Scientific).

**Production of Recombinant Apaf-1 Protein in a Baculovirus Expression System**—The Apaf-1 expression plasmids were transformed into DH10Bac *Escherichia coli* cells (Life Technologies). The recombinant viral DNA and bacmids were purified according to the Bac-To-Bac baculovirus expression procedure (Life Technologies) and confirmed by PCR analysis. The DNA was then subjected to transfect the Sf21 cell by using the Cell FECTIN reagent (Life Technologies). The cells were grown in IPL41 medium supplemented with 10% fetal calf serum, 2.6 g/liter tryptophane phosphate, 4 g/liter yeastolate, 0.1% Pluronic F-68 plus penicillin (100 units/ml), streptomycin (100 mg/ml), and fungizone (0.25 g/ml). The expression of recombinant protein was analyzed by Western blotting. The virus stocks were amplified to 100 ml and used to infect 1 liter of Sf21 cells at a density of 2 × 10⁶ cells/ml. The infected cells were harvested after 40 h for Apaf-1 by centrifugation and resuspended in 5 volumes of buffer A (20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 0.1 mM PMFS). The resuspended cells were lysed in buffer A by homogenization. After centrifugation at 10,000 × g for 1 h, the supernatant was loaded onto a 3-ml nickel affinity column. After equilibrating the column with 20 ml of buffer A, the column was eluted with buffer A containing 250 mM imidazole. The eluted Apaf-1 protein was stored in multiple aliquots at −80 °C.

**Cell Apoptosis Assay**—Cells after treatment were stained with PI/Annexin V (cell apoptosis detection kit, TransGen Bio-tech Co., Ltd.) followed by flow cytometry analysis. The results were analyzed by FlowJo software (TreeStar Inc.).

**Statistical Analyses**—Statistical analyses were performed using SPSS software. Statistical significance was calculated by Student’s *t* test.

**Results and Discussion**

The HDAC Inhibitor Butyrate Enhances dATP-dependent Caspase Activation in Vitro and Sensitizes to UV Radiation-induced Apoptosis in Vivo—Previous studies indicated that cytosolic fraction from cells treated with the HDAC inhibitor butyrate manifests much stronger caspase activation in response to dATP in *vitro* (5). To determine the underlying mechanisms, HeLa and MEF cells were treated with 20 mM butyrate for 12 h, and then cell lysates were prepared and incubated with 0.3 or 1 mM dATP at 30 °C for 1 h. Subsequently, Western blotting analysis against caspase 9 was performed to visualize caspase activation. We found that, in both HeLa and MEF cells, butyrate treatment dramatically increased caspase 9 activation when the cell lysates were incubated with dATP (Fig. 1A, compare lanes 2–4 with lanes 6–8 and lanes 10–12 with lanes 13–15). Consistent with this *in vitro* observation, butyrate treatment of HeLa, MEF, and 293T cells sensitized UV-induced apoptosis, as monitored by Annexin-V and PI co-staining coupled with flow cytometry analysis (Fig. 1, B–D).

On the basis of the fact that caspase activation triggered by dATP in *vitro* is executed through dATP/ATP-dependent interaction of three core factors, Apaf-1, caspase 9, and cytochrome *c* (27), we examined whether butyrate-enhanced
caspase activation is dependent on Apaf-1. As shown in Fig. 1E, Apaf-1−/− MEFs were treated with butyrate, the cell lysates were prepared, and different concentrations of recombinant Apaf-1 were incubated with cell lysates in the presence of 1 mM dATP. To our surprise, in the presence of recombinant Apaf-1, butyrate-treated cell lysates did not show any enhancement of dATP-dependent caspase activation (Fig. 1E, compare lanes 1–5 with lanes 6–10), suggesting that the endogenous Apaf-1 gene is required for the effect of butyrate to enhance caspase activation.

**Butyrate Induces p53-dependent Transactivation of Apaf-1**—Apaf-1, caspase 9, and cytochrome c are required for dATP-dependent caspase activation in the cell-free system (27). To unravel the biochemical mechanism of how butyrate enhances dATP-dependent caspase activation, the expression levels of Apaf-1 and caspase 9 after butyrate treatment in 293T cells were examined by Western blotting analysis. As shown in Fig. 2A, butyrate increased Apaf-1 expression substantially, started at the 12-h time point, and peaked at the 24-h time point, whereas caspase 9 remained unchanged (Fig. 2A). In addition, the levels of other apoptosis-related proteins such as downstream effector caspases and their inhibitor XIAP and certain anti-apoptotic members of the Bcl-2 family of proteins all stayed consistent upon butyrate treatment (Fig. 2A). Although the p53 level was unchanged during butyrate treatment, the transcriptional target of p53, p21, was up-regulated at the 24-h time point after butyrate treatment (Fig. 2A). These results suggest that p53, one of the well defined transcription factors for Apaf-1 (28–30), may be involved in this process.

We performed reverse transcription quantitative PCR to detect the Apaf-1 mRNA level after butyrate treatment and found it increased by 2.5-fold compared with the control (Fig. 2B). To further ascertain that butyrate enhances Apaf-1 expression through transcriptional regulation, a luciferase reporter assay was performed. As shown in Fig. 2C, only when luciferase cDNA was fused with the Apaf-1 promoter sequence could butyrate stimulate luciferase expression (by ~2-fold).

Subsequently, we assessed whether butyrate-induced transcriptional activation of Apaf-1 was dependent on p53. Butyrate treatment increased the Apaf-1 level in 293T cells, although the protein level of p53 was not altered (Fig. 2A). Notably, transfection of HA-tagged p53 increased the basal expression level of Apaf-1. As shown in Fig. 1E, Apaf-1−/− MEFs were treated with or without 20 mM butyrate sodium solution, and then S-100 was prepared and normalized to 1 μg/μl to perform the HA immunoprecipitation as described under “Experimental Procedures.” The immunoprecipitates were subjected to SDS-PAGE. The gel was stained with Coomassie Brilliant Blue, and the bands indicated were sliced out for MS analysis. β, MS posttranslational modification analysis of acetylation of the Lys-120 site of the HA immunoprecipitates after 4 and 12 h of butyrate treatment. αa, amino acids. C, 293T cells were treated with butyrate sodium solution for different time courses as indicated and total cell lysates were prepared and subjected to Western blotting analysis of Apaf-1, ac-Lys-120 of p53 and β-actin, respectively. D and E, MCF-7 cells were treated with or without butyrate sodium solution for 12 h, fixed, and immunostained with antibodies against total p53 (red), Lys-120-acetylated p53 (green), and DAPI (blue) as described under “Experimental Procedures.” F, 293T cells were treated with or without butyrate as indicated for 12 h. ChIP was performed using an antibody against Lys-120-acetylated p53 (p53 K120 Ac) to analyze binding of Lys-120-acetylated p53 with the promoter regions of the indicated genes. IgG was used as the negative control antibody. **, p < 0.01; ***, p < 0.001.
Apaf-1, and butyrate treatment further elevated Apaf-1 expression (Fig. 2D). Importantly, in the p53-null cell line H1299, butyrate did not affect dATP-dependent caspase activation (Fig. 2E) or the expression level of Apaf-1 (Fig. 2F), whereas transfection of exogenous HA-tagged p53 in H1299 cells restored the response of Apaf-1 expression to butyrate (Fig. 2G). In addition, butyrate sensitized UV-induced apoptosis in the H1299 cell line stably transfected with HA-p53 but remained completely ineffective in the H1299 parental cell line (Fig. 2H). On the basis of these results, we conclude that butyrate enhances dATP-dependent caspase activation in vitro and sensitizes UV-induced apoptosis in vivo through p53-driven transactivation of Apaf-1.

**Structurally Unrelated HDAC Inhibitors Exert a Similar Effect as Butyrate**—Butyrate belongs to the short aliphatic acid group of HDAC inhibitors and preferentially inhibits class I HDACs (31). To address whether other classes of HDAC inhibitors could also increase expression of Apaf-1, A549, 293T, H1299 as well as H1299 cells transfected with exogenous p53 were treated with SAHA and TSA, two other pan-inhibitors of HDACs (both hydroxamate derivatives). As shown in Fig. 3A, SAHA and TSA induced up-regulation of Apaf-1 in A549 and 293T cells harboring p53 expression but were completely ineffective in the p53-null H1299 cell line (Fig. 3A). When exogenous p53 was expressed in H1299 cells, the response of Apaf-1 expression to SAHA and TSA was restored (Fig. 3A). In addition to Apaf-1, all three tested HADC inhibitors also increased the expression level of the BH3-only proteins Puma and Noxa (Fig. 3B), both defined p53 transcriptional targets (19, 20).

**Butyrate Induces Acetylation of p53 at Lysine 120**—Because the expression level of p53 was constant up to 48 h after butyrate treatment, we reasoned that modification of p53 might be responsible for the transcriptional activation of Apaf-1. The functional significance of p53 acetylation has been firmly established (32, 33). To identify butyrate-dependent modification on p53, we pulled down HA-tagged p53 from control or butyrate-treated 293T cells stably expressing HA-p53 (Fig. 4A). The HA-p53 protein band was subjected to mass spectrometry analysis. As shown in Fig. 4B, acetylation of Lys-120 was greatly increased by butyrate treatment (1.8-fold at the 4-h time point and 7-fold at the 12-h time point; Fig. 4B), a modification that occurs rapidly after DNA damage (34, 35). Levels of other acetylation sites of p53 did not show any obvious change upon butyrate treatment (data not shown).

The enhancement of acetylation of p53 at Lys-120 was further confirmed by Western blotting (Fig. 4C) and immunoflu-
orescence (Fig. 4E) using an antibody specifically against Lys-120-acetylated p53. Interestingly, in control cells, p53 was evenly distributed in the cytosol and nucleus, whereas, after butyrate treatment, significant nuclear accumulation of p53 was observed (Fig. 4D). In contrast to total the p53 pool, Lys-120-acetylated p53 was exclusively localized in the nucleus (Fig. 4E), indicating that Lys-120 acetylation trapped p53 in the nucleus.

It has been reported that Lys-120 acetylation of p53 could increase its binding to the promoter regions of Bax and Puma (35). To determine whether Lys-120-acetylated p53 also enriches in the Apaf-1 promoter region after butyrate treatment, we performed a ChIP assay using an antibody specific for Lys-120-acetylated p53 and subsequently analyzed the enrichment of p53 on several known p53-binding promoters as well as on negative controls, including the GAPDH promoter (Fig. 4F). In response to butyrate treatment, Lys-120-acetylated p53 was enriched about 5-fold in the Apaf-1 promoter, similar to the Noxa and Puma promoters. This result suggests that the Lys-120 acetylation of the p53 site plays an important role in butyrate-induced transactivation of apoptotic genes, including Bax and Puma, as reported previously (34, 35), and Noxa and Apaf-1, as we demonstrate here. Consistent with previous reports (34, 35), the promoter regions of the p21, Mdm2, and AIP1 genes, which are non-apoptotic targets of p53, did not enrich Lys-120-acetylated p53 upon butyrate treatment.

Lys-120 Acetylation of p53 Accounts for the Up-regulation of Apaf-1—To further evaluate the role of Lys-120 acetylation of p53 in butyrate-induced Apaf-1 up-regulation, we transfected wild-type p53, the K120R mutant (incapable of Lys-120 acetylation), or the K120Q mutant (mimicking Lys-120 acetylation) into p53-null H1299 cells. The cells were subsequently treated with butyrate. As shown in Fig. 5A, butyrate treatment failed to up-regulate Apaf-1 and Noxa in parental

FIGURE 6. HDAC1 is the functional target for butyrate to induce Lys-120 acetylation of p53 and elevate the expression level of Apaf-1. A, 5 × 10⁶ HeLa, A549, 293T, and H1299 cells were seeded in 10-cm cell culture dishes on day 0 and treated with dimethyl sulfoxide, 10 μM C994, or 1 μM RGFP966 for 12 h. Cell lysates were collected for Western blotting analysis of Apaf-1 and β-actin. B, 5 × 10⁶ 293T, HeLa, A549, and H1299 cells were seeded in 10-cm dishes on day 0 and treated with dimethyl sulfoxide, 10 μM TMP269, 10 μM LMK-235, or butyrate as indicated for 12 h. Cell lysates were collected for Western blotting analysis of Apaf-1 and β-actin. C, 293T cells were treated with HDAC1 knockdown (KD) or scramble knockdown as indicated. mRNA levels of Apaf-1 were subsequently quantitated by quantitative RT-PCR. Vec, vector. D, 1 × 10⁶ 293T cells were plated in 10-cm dishes on day 0 and transfected with 2 μg of vector plasmids or plasmids encoding FLAG-HDAC1 as indicated on day 1. On day 3, the cells were treated with butyrate sodium solution for 12 h. Cell lysates were collected and subjected to Western blotting analysis of Apaf-1, HDAC1, FLAG, and GAPDH. E, 293T cells were plated at a density of 1 × 10⁶ in 10-cm dishes on day 0 and transfected with 4 μg of PLKO-Scramble plasmids or PLKO-HDAC1-shRNA plasmids on day 1. On day 4, the transfected cells were split into new dishes cultured in the presence of 2 μg/ml puromycin. Stable cell lines were established after the fifth passage. Cell lines with stable knockdown of HDAC1 and its control cell line (Scramble) were treated with butyrate sodium solution for 12 h, immediately followed by protein preparation. Aliquots of 10 μg of total cell lysates were subjected to Western blotting analysis of ac-Lys-120 p53, Apaf-1, HDAC1, and β-actin. (F) HDAC1 KD stable line established as described in panel C was rescued by transfecting plasmids encoding shRNA-resistant HDAC1 (R-HDAC1). Total cell lysate from 293T, HDAC1 KD and HDAC1 KD rescued with R-HDAC1 cell lines were subjected to Western blot analysis against Apaf-1, HDAC1 and β-actin. G, scramble and HDAC1-shRNA cell lines were treated with butyrate sodium solution for 12 h and then subjected to UV irradiation (2 × 10⁵ μJ/cm²). 8 h after irradiation, cells were harvested and co-stained with Annexin V/PI. Apoptotic cells were quantified by flow cytometry. *** p < 0.001.
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H1299 cells but was able to do so when cells were transfected with wild-type p53. Remarkably, in cells expressing K120R mutant p53, butyrate was incapable of increasing Apaf-1 expression (Fig. 5A). On the contrary, transfection of the acetylation-mimicking mutant K120Q increased expression of Apaf-1 even in the absence of butyrate treatment, and butyrate treatment could not further increase the Apaf-1 level under this specific genetic background (Fig. 5A). These results suggest that, among all of the reported acetylated sites on p53, only acetylation of Lys-120 is responsible for butyrate-stimulated Apaf-1 up-regulation.

We subsequently examined the effect of Lys-120 acetylation of p53 on apoptosis. In the parental H1299 cell line, butyrate had no impact on UV-induced apoptosis (Fig. 5B). When the cells were transfected with wild-type p53, butyrate could increase UV-induced apoptosis as in MEF, HeLa, and 293T cells, which express wild-type p53 (Fig. 1, B–D). In H1299 cells transfected with K120R mutant p53 (Lys-120 acetylation-defective), butyrate was not able to synergize UV-induced apoptosis. On the other hand, transfection of acetylation-mimicking mutant K120Q p53 increased UV-induced apoptosis in the absence of butyrate treatment, and butyrate treatment did not further enhance cell death.

Butyrate Targets HDAC1 to Elevate p53-mediated Apaf-1 Expression and Subsequent Apoptosis—There are about 20 HDACs, classified into five different groups: class I, IIA, IIB, III, and VI (36). Butyrate inhibits class I (HDAC1, 2, 3, and 8) and IIA HDACs (HDAC4, 5, 7, and 9). Previous reports have discrepancies regarding whether HDAC1 (37) or HDAC5 (38) is responsible for Lys-120 deacetylation of p53. To further determine the specific HDAC whose inhibition by butyrate accounts for Apaf-1 transactivation, we tested the effect of several specific HDAC inhibitors in various p53-positive cells. CI994, a specific inhibitor against HDAC1 (39), increased expression of Apaf-1 to a similar extent as butyrate, whereas RGFP966, which specifically inhibits HDAC3 (40), was almost inactive (Fig. 6A). Furthermore, TMP269, which specifically inhibits class IIA HDACs (HDAC5 included) (41), and LMK235, which specifically inhibits HDAC4 and HDAC5 (22), had little effect on Apaf-1 protein level compared with butyrate (Fig. 6B). Therefore, our results support the conclusion that Lys-120 deacetylation of p53 is specifically catalyzed by HDAC1, at least in the multiple cell types examined in our experiment. Importantly, it is also possible that HDAC1 and HDAC5 might mediate distinct biological contexts to drive Lys-120 deacetylation of p53. Indeed, in a previous study that supports a dominant role of HDAC5 (38), a rather different stimulus (DNA damage) was used to trigger p53 activation.

Because specific HDAC1 inhibitors can increase Apaf-1 expression, we reasoned that overexpression of HDAC1 should have the opposite effect on the intracellular level of Apaf-1 compared with butyrate. Indeed, overexpression of FLAG-tagged HDAC1 dramatically decreased expression of Apaf-1 (Fig. 6D). The effect of HDAC1 on butyrate-induced Apaf-1 up-regulation was further validated by a loss-of-function strategy. RNAi knockdown of endogenous HDAC1 increased the expression level of Apaf-1 as well as acetylation of p53 at Lys-120 to a similar extent as butyrate treatment (Fig. 6, C and E).

Treatment with butyrate on top of HDAC1 knockdown had little, if any, effect on Apaf-1 expression (Fig. 6E), indicating that HDAC1 is the major functional target for butyrate-induced Apaf-1 up-regulation. As expected, the effect of endogenous HDAC1 knockdown on Apaf-1 expression could be neutralized by ectopic expression of shRNA-resistant HDAC1 (Fig. 6F). Consistently, in 293T cells transfected with scrambled shRNA, butyrate treatment synergized UV-induced apoptosis (Fig. 6G). When HDAC1 was knocked down by shRNA, both the basal level and UV-induced apoptosis were increased, whereas the effect of butyrate on apoptosis was completely diminished (Fig. 6G).

Taken together, this study demonstrates that butyrate can induce p53 acetylation at Lys-120 via inhibition of a specific HDAC, HDAC1, in various cell types. The acetylation of p53 at Lys-120 subsequently gives rise to transcriptional up-regulation of Apaf-1, a crucial component in the intrinsic apoptotic pathway, and thus sensitization of mitochondrion-mediated apoptosis.

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