The Histidine Triad Protein Hint1 Triggers Apoptosis Independent of Its Enzymatic Activity*1

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Jörg Weiske and Otmar Huber1

From the Department of Laboratory Medicine and Pathobiology, Charité Campus Benjamin Franklin, Hindenburgdamm 30, 12200 Berlin, Germany

Hint1 is a member of the evolutionarily conserved family of histidine triad proteins that acts as a haplo-insufficient tumor suppressor inducing spontaneous tumor formation in Hint+/- and Hint-/- mouse models. However, the molecular mechanisms for the tumor-suppressing activity are poorly defined. In this respect, we have recently shown that Hint1, by interaction with Pontin and Reptin, inhibits T-cell factor/β-catenin-mediated transcription of Wnt target genes. In this study, we have found that, after transient transfection with Hint1, SW480 and MCF-7 cells undergo apoptosis as analyzed by pro-caspase-3

The histidine triad superfamily represents an evolutionarily conserved family of proteins characterized by the sequence motif HXXHXX (H, histidine; X, hydrophobic amino acid), which is involved in nucleotide hydrolyse and/or transferase activities (1, 2). The superfamily is divided into three branches, including the histidine triad nucleotide-binding protein (Hint)2 branch, the fragile histidine triad (Fhit) branch and the galactose-1-phosphate uridyltransferase (GaIT) branch. The most prominent member of the family, the Fhit protein is encoded on chromosome 3p14.2, which represents one of the most common fragile sites in the human genome (3). During recent years, numerous studies have shown that reduced or lost expression of Fhit is associated with early preneoplastic and malignant disorders in multiple human organs, and in this context, Fhit has been ascribed a tumor suppressor function (4, 5).

Hist1, the founding member of the Hint branch, originally was identified as a 126-amino-acid protein that inhibits protein kinase C (6). The protein kinase C inhibitory activity, however, was not further confirmed. Because it was found to interact with protein kinase Cβ in a yeast two-hybrid screen, the protein was named PKCl-1 (protein kinase C-interacting protein-1) (7). Later on, x-ray structural analyses revealed that the protein forms dimers, and the histidine triad motif HXHHXHX, with X being the hydrophobic amino acid, is involved in nucleotide binding and hydrolysis (8–11). Thus the protein was renamed Hint1 (histidine triad nucleotide binding protein-1) (11). In vitro biochemical analyses have identified various nucleotides that bind to Hint1, including Ap3A, Ap3A, ADP, AMP, and AMP-NH2. However, in contrast to Fhit, Hint1 does not hydrolyze diadenosine-polyphosphates or ATP but does hydrolyze ADP and AMP-NH2 (8, 12). These observations suggest that binding of diadenosine-polyphosphates and/or hydrolysis of nucleotides is involved in the physiological activity of Hint1. However, for many years, the cellular function of Hint1 remained obscure.

The observation that Hint1 interacts with the basic helix-loop-helix microphthalmia-associated transcription factor (MITF) and the cyclin-dependent kinase 7 (Cdk7) supported a role of Hint1 in transcriptional regulation and growth control (13, 14). Hint1 suppresses the transcriptional activity of MITF and regulates target gene expression in mast cells, including RMCP-6, c-Kit receptor tyrosine kinase, lymphocyte serine protease granzyme B and tryptophan hydroxylase (13, 15). In this respect, it is of interest that binding of the diadenosine-

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2 To whom correspondence should be addressed: Institut für Klinische Chemie und Pathobiologie, Charité, Universitätmedizin Berlin, Campus Benjamin Franklin, Hindenburgdamm 30, 12200 Berlin, Germany. Tel.: 49-30-8445-2525; Fax: 49-30-8445-4152; E-mail: otmar.huber@charite.de.

3 The abbreviations used are: Hint, histidine triad nucleotide-binding protein; Ap3A, P, P' diadenosine-5'-triphosphate; Ap3A, P, P' diadenosine-5'-triphosphate; AMP-NH2, adenosine-5'-monophosphoramide; GalT, galactose-1-phosphate uridyltransferase; CAK, Cdk-activating kinase; Cdk7, cyclin-dependent kinase 7; Fhit, fragile histidine triad protein; shRNA, short hairpin RNA; HAT, histone acetyltransferase; MITF, microphthalmia-associated transcription factor; HEK, human embryonic kidney; EGFP, enhanced green fluorescent protein; PARP, poly(ADP-ribose) polymerase; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; RT, reverse transcription; ChIP, chromatin immunoprecipitation; Pipes, 1,4-piperazinediethanesulfonic acid; ELISA, enzyme-linked immunosorbent assay; TCF, T-cell factor.
polyphosphate Ap4A to Hint1 modulates the transcriptional activity of MITF in mast cells by disrupting the Hint1-MITF complex, thereby releasing the repressor Hint1 from MITF. In addition, Hint1 has been reported to interact with the product of the ATDC gene and to repress fos transcription (16, 17).

These observations and the structural homology with Fhit suggest that Hint1 might similarly act as a tumor suppressor protein. The first evidence for this function came from studies of mice with the deleted Hint1 gene (18, 19). Heterozygous and homozygous mice were not impaired in embryonic development and appeared to have normal life spans. However, N-nitrosomethylbenzylamine-treated Hint1−/− mice showed higher frequencies of squamous tumors compared with wild-type mice (19). This study now has been extended and revealed that, at the age of 2–3 years, both Hint1+/− and Hint1−/− mice exhibit an increased rate of spontaneous tumor development with comparable tumor incidence and histology (20). Similar to the observations of the previous study, Hint1+/− and Hint1−/− mice show enhanced susceptibility to tumor induction by 7,12-dimethylbenzanthracene treatment. Interestingly, in some tissues, loss of Hint1 expression was compensated by increased expression of Fhit. From these studies, Hint1 was classified as a haplo-insufficient tumor suppressor in mice (20).

Consistent with a tumor suppressor function, in the human non-small cell lung cancer cell line NCI-H522, a methylation-dependent down-regulation of Hint1 expression was observed and re-introduction of Hint1 resulted in cell growth inhibition and reduced tumorigenicity (21).

In this context, we recently reported that Hint1 interacts with Pontin and Reptin and represses β-catenin transcriptional activity by disrupting the homo- and heteromeric protein interaction between Pontin and Reptin (22). Pontin and Reptin are evolutionary highly conserved proteins that have previously been identified as direct β-catenin and TATA box-binding protein interaction partners antagonistically modulating β-catenin transcriptional activity in the Wnt signaling pathway (23–25). Both proteins are associated with various chromatin remodeling complexes, including Ino80 complexes (26, 27), the Tip60-HAT complex (28, 29), and the BA53 complex (30).

There is clear evidence now that Pontin is involved in tumorigenesis by regulating β-catenin-mediated neoplastic transformation (31). Moreover, it was shown that Pontin is required for c-Myc oncogenic transformation (32, 33).

Reduced or lost expression of tumor suppressor proteins often is associated with impaired induction of apoptosis. Because the tumor suppressor function of Hint1 was correlated with a pro-apoptotic function (34–37), we here analyzed whether the Hint1 tumor-suppressing activity is associated with regulation of apoptosis. Expression of Hint1 in MCF-7 and SW480 cells resulted in cytochrome c release, activation of caspase-3, and up-regulation of p53 levels. Moreover, expression of the pro-apoptotic factor Bax was up-regulated, whereas the anti-apoptotic factor Bcl-2 was reduced. No changes in the expression levels of the pro-apoptotic BH3-only proteins Bad and Puma were observed. In addition, we provide evidence that Hint1 together with the Tip60-HAT complex is involved in the regulation of the Bax promoter. Taken together these data clearly provide evidence that Hint1 modulates the apoptotic signaling in cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HEK293, SW480, and MCF-7 were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 5% CO2. The cell line MCF-7 Tet-On (BD Biosciences) was grown in Dulbecco’s modified Eagle’s medium containing 10 mM Hepes, 1 mM sodium pyruvate, 0.2 units/ml bovine insulin, and 0.2 mg/ml G418 and supplemented with 10% (v/v) Tet System-approved fetal bovine serum (BD Biosciences), 100 units/ml penicillin, and 100 μg/ml streptomycin.

**Plasmids**—Plasmids pCS2+Hint1, pCS2+Hint1-mycD, and pQE40-Hint1 were described previously (22). EGFP-tagged Hint1 was generated by amplification of the Hint1 cDNA with the oligonucleotide pairs 5′-CGG GAG GGT TCA GAT GGA CAG GAT AGT GCC 3′ (forward) and 5′-CGG GAG GGA TTA ACC AGG AGG CCA ATG CAT 3′ (reverse). The PCR product was cloned into BamHI-calf intestinal phosphatase-treated pEGFP-N3 vector (BD Biosciences). The plasmid pEGFP-act was obtained from Clontech, and pFLAG-CMV10-Tip60 was kindly provided by Dr. M. G. Rosenfeld (29).

Site-directed mutagenesis to generate Hint1-G105A, -S107A, -G105A/S107A, and -H112N was performed using the QuikChange™ site-directed mutagenesis kit (Stratagene). Mutations were introduced by PCR using the expression plasmid pQE40-Hint1 as the template. The following oligonucleotides were used for site-directed mutagenesis: G105A, 5′-GTT AAT GAA GGT TCA GAT GGA CAG TCT TAT TAC GTT CAT C-3′ (forward) and 5′-GGT GAA CGT GAT AGA CAG ACT GTG CAT CTG AAC CTT CAT TCA C-3′ (reverse); S107A, 5′-GAA GGT TCA GAT GGA CAG CAC GTT CAT CAC CTG AAC ACC CTT CAT TCA C-3′ (reverse); S107A, 5′-GAA GGT TCA GAT GGA CAG CAC GTT CAT CAC CTG AAC ACC CTT CAT TCA C-3′ (reverse); G105A/S107A, 5′-GTG AAT GAA GGT TCA GAT GGA CAG TCT TAT TAC GTT CAT C-3′ (forward) and 5′-CAT GGA GAT GAA CGT GAT AGA CAG CAC GTT CAT CAC CTG AAC CTT CAT TCA C-3′ (reverse); G105A/S107A, 5′-GTG AAT GAA GGT TCA GAT GGA CAG TCT TAT TAC GTT CAT CAC CTG AAC CTT CAT TCA C-3′ (reverse); H112N, 5′-GGA CAG CCT GTG CAC CAT CTG AAC CTG TAT TAC GTT CAT CAC CTG AAC CTT CAT TCA C-3′ (reverse); H112N, 5′-GGA CAG CCT GTG CAC CAT CTG AAC CTG TAT TAC GTT CAT CAC CTG AAC CTT CAT TCA C-3′ (reverse); H112N, 5′-TGG CCG ACC TAC CCC AAG AAC ATG GAG GTT AAC CTG ATA GAC AGA CTG TTC C-3′ (reverse). Sequences of the constructs were confirmed by cycle sequencing and subsequent analysis on an ABI Prism 310 genetic analyzer.

**Antibodies**—Monoclonal anti-poly(ADP-ribose) polymerase (anti-PARP) (clone C2–10), anti-Bax (clone 6A7), anti-Bcl-2 (clone 7), and anti-p53 (clone Pab2122) antibodies were obtained from BD Biosciences. Anti-α-actinin (clone BM-752), anti-β-actin (clone AC15), and anti-FLAG-M2 antibodies were purchased from Sigma, the anti-Caspase-3 (catalog number 9662) antibody was from Cell Signaling Technology, and the anti-M30 CytoDEATH antibody was obtained from Roche Applied Science. The polyclonal anti-Hint1 antibody was described previously (22). The anti-Tip60 antibody was
purchased from Upstate Biotechnology. Horseradish peroxidase–labeled anti-mouse and anti-rabbit antibodies were purchased from Dianova. Alexa Fluor™594 goat anti-mouse IgG and Alexa Fluor™594 goat anti-rabbit IgG antibodies were obtained from Molecular Probes.

Establishment of an MCF-7 Cell Line Inducibly Expressing Hint1 Short Hairpin RNA (shRNA)—MCF-7 Tet-On cells were transfected with pRNA-Tin-H1.2/Hygro shRNA vector, shRNA-Hint1_430/431, or shRNA-Hint1_432/433. Generation of these shRNA vectors was described previously (22). Briefly, stable, transfected cells were selected in the presence of 0.5 mg/ml hygromycin B in the culture medium (see “Cell Culture”), and clones were isolated. Knockdown of Hint1 in cells was controlled by Western blotting. After selection, transfected cells were cultured in the presence of 0.2 mg/ml hygromycin B and 0.2 mg/ml G418. For induction of shRNA expression mock-, MCF-7-shRNA-Hint1430/431-, and MCF-7-shRNA-Hint1_432/433-transfected cells were cultured with 1 μg/ml doxycycline (BD Biosciences) and lysed after 48 h as described above.

Transient Transfections and Reporter Gene Assays—Transfection of MCF-7 Tet-On cells was performed with Lipofectamine™plus (Invitrogen) according to the manufacturer’s recommendations. SW480 and MCF-7 cells were Nucleofected® using the Cell Line Nucleofector® Kit V (Amazka) following the manufacturer’s instructions. Hint1 protein levels were analyzed in cell lysates by Western blotting and quantified on a FujiFilm LAS-1000 imager resulting in a 13.24 ± 1.60-fold (n = 4) and 15.38 ± 3.04-fold (n = 4) excess of Hint1 compared with control-transfected cells in SW480 and MCF-7 cells, respectively. Reporter gene assays were performed with luciferase (constant light signal) reporter gene assay and the chemoluminescent light signal reporter gene assay and the chemoluminescent reporter gene assays using a p53 luciferase construct kindly provided by Dr. S. Sukomar (The Johns Hopkins University). transcriptional regulation of p53 was analyzed in transfection experiments are presented for all reporter gene standards.

To verify the dimeric structure of the recombinant Hint1-His6 proteins, gel filtration chromatography was performed on a Δkta purifier system using a Superdex™ 75HR 10/30 column (Amersham Biosciences). The proteins were eluted with gel filtration buffer (300 mM NaCl, 5 mM MgCl2, 20 mM imidazole, pH 8.0) and monitored at 280 nm. Lysozyme (14 kDa) and carbonic anhydrase (29 kDa) were used as molecular mass standards.

Immunofluorescence Microscopy—Cells were grown for 24 h on glass coverslides and subsequently transfected with 2.0 μg of pEGFP-Hint1 with Lipofectamine™plus (Invitrogen) according to the manufacturer’s recommendations. After 72 h, the cells were washed with PBS and fixed in ice-cold methanol for 10 min at −20 °C. Subsequently, the cells were gently washed in PBS again, blocked with 0.1% (v/v) goat serum in PBS for 30 min at room temperature, and incubated with mouse anti-M30 CytoDEATH (1:100) for a further 30 min at room temperature. After three washes with PBS, the cells were incubated with Alexa Fluor™594 goat anti-mouse IgG (1:1000) for 30 min. To stain the nuclei, the cells were treated with 4′,6-diamidino-2-
phenylindole (0.1 μg/ml) for 5 min at room temperature. Coverslides were mounted with ProTaqs Mount Fluor (Biocyt GmbH and Co. KG). Analysis and photography were performed on a confocal laser-scanning microscope (LSM 510 META, Zeiss, Jena, Germany) with a Plan-Neofluar objective (40×/1.3 oil) at excitation wavelengths of 543 nm (anti-M30 CytoDEATH), 488 nm (EGFP), and 405 nm (4,6-diamidino-2-phenylindole), respectively. Details on the microscope setup can be obtained upon request. The figures were prepared with Adobe Photoshop CS2 software without any adjustments.

**Bax, Bcl-2, Bad, Puma, and p53 Expression**—Total RNA and protein were isolated from Hint1- and mock-transfected SW480, MCF-7, and MCF-7 Tet-On cells with the NucleoSpin®RNA II kit or NucleoSpin®RNA/protein (Macherey-Nagel). Semiquantitative PCR was performed with the OneStep reverse transcription (RT)-PCR kit (Qiagen) according to the manufacturer’s instructions (95 °C, 20 s; 60 °C, 20 s; 72 °C, 60 s). For human Bax, Bcl-2, p53, and β-actin, the following oligonucleotide pairs were used: Bax (forward, 5′-GGC CCA CCA GCT CTG AGC AGA-3′ and reverse, 5′-GCC ACG TGG GGC TGG CCA CAA ATG AAG-3′); Bcl-2 (forward, 5′-GTT GAG GAG CTC TTC AGG GAC-3′ and reverse, 5′-AGG CAC CCA GGG TGA TGG AAG-3′); Bad (forward, 5′-CAG TGA CCT TCG CTC CAC AAT-3′ and reverse, 5′-ATG GTG-3′ and reverse, 5′-ATG CTG ATG CTT GCA TCA GCC GTC-3′); p53 (forward, 5′-AAG GAT GCC CAG GCT GGG AAG-3′ and reverse, 5′-CCG GGA TCC TCA GTC GTC AGG CCC TTC TTC-3′); and β-actin (forward, 5′-GCC TG AAT TAG GTG-3′ and reverse, 5′-ATG GTG-3′). The PCR products were analyzed on a 8% polyacrylamide gel. Quantification and data analysis were performed on a FujiFilm LAS-1000 imager with the Image Gauge, version 3.2, software. Bax, Bcl-2, and p53 mRNA levels were always normalized using β-actin or α-actinin as the reference. Mean values obtained from RT-PCRs performed with RNAs from three independent preparations are presented.

**Cytochrome c Release**—Cytochrome c release was assayed with the Cytochrome c Release Apoptosis assay kit (Calbiochem) according to the manufacturer’s recommendations.

**Chromatin Immunoprecipitation (ChIP)**—ChIP was performed according to the procedure described by Nowak et al. (39) and Weinmann and Farnham (40) with minor modifications. HEK293 cells were cultured as described above and fixed with 2 mM disuccinimidyl-glutarate for 45 min at room temperature. Subsequently, the cells were washed twice with PBS. Chromatin was cross-linked for 10 min at room temperature using 1% (v/v) formaldehyde and washed twice with ice-cold PBS. The cross-linking reaction was stopped by incubation with glycine at a final concentration of 0.125 M for 5 min at room temperature. After incubation with trypsin, the cells were scraped from the cell culture dish and washed twice with PBS. The cell pellet was resuspended in lysis buffer (5 mM Pipes, pH 8.0, 85 mM KCl, and 0.5% (v/v) Nonidet P-40), incubated on ice for 10 min, and homogenized with a Dounce homogenizer. Subsequently, nuclei were pelleted by centrifugation (3500 × g, 5 min, 4 °C) and resuspended in nuclei buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, and 1% (w/v) SDS). Nuclei were disrupted by sonication with three 20-s pulses in a UP 50H sonicator (Hiielscher Ultraschall Technologie) at a setting of cycle 0.5 and amplitude 30%, yielding genomic DNA fragments with a bulk size of 200–1000 bp. For ChIP, 50 μg of DNA was diluted 4-fold in immunoprecipitation buffer (16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl, 1.2 mM EDTA, 1.1% (v/v) Triton X-100, and 0.01% (w/v) SDS). For each immunoprecipitation, the diluted fraction was precleared by the addition of 20 μl of protein A beads (50% slurry of protein A-Sepharose CL4B in nuclei buffer containing 0.1% (w/v) bovine serum albumin and 0.4 mg/ml salmon sperm DNA) for 15 min at 4 °C. After centrifugation (20,800 × g, 5 min, 4 °C), the supernatant was incubated overnight at 4 °C with anti-Hint1 (5 μg, affinity-purified), anti-Tip60 (5 μg), or anti-GFP (5 μg) antibodies. Immune complexes were precipitated by adding 30 μl of blocked protein A-Sepharose CL4B beads for 1 h at 4 °C. Precipitates were serially washed twice (2700 × g, 2 min, 4 °C) with 300 μl of low salt buffer (20 mM Tris-HCl, pH 8.1, 150 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) Triton X-100, and 2 mM EDTA), high salt buffer (500 mM NaCl, 20 mM Tris-HCl, pH 8.1, 0.1% (w/v) SDS, 1% (v/v) Triton X-100, and 2 mM EDTA), LiCl buffer (20 mM Tris-HCl, pH 8.1, 250 mM LiCl, 1% (v/v) Nonidet P-40, 1% (w/v) deoxycholate, and 1 mM EDTA), and twice with 300 μl of TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). Chromatin complexes were eluted by incubation for 15 min with 200 μl of elution buffer (1% (v/v) SDS and 0.1 M NaHCO3) under constant agitation. After centrifugation (20,800 × g, 3 min, 4 °C), the supernatant was transferred to a new tube. This step was repeated and both supernatants were combined. All buffers were supplemented with Complete™ EDTA-free protease inhibitor mixture (Roche Diagnostics). Cross-linking was reversed by an overnight incubation at 65 °C, and DNA was purified as described previously (40). For subsequent PCR analysis, 2 μl of the extracted DNA (50 μl) were used as a template for 30 cycles of amplification. The following primers were used to detect the Bam promoter: forward, 5′-CAT GCC TGT AAT CCC AGC-3′; reverse, 5′-ATG CTT CCA GGC AGG ACG-3′. PCR products were analyzed on a 8% polyacrylamide gel.

**Analysis of Apoptosis by Cell Death Detection ELISA**—To determine and quantify apoptosis induced by overexpression of Hint1 in SW480 and MCF-7 cells, DNA fragmentation was measured with Cell Death Detection ELISA™ Plus (Roche Diagnostics). Cell lysates were obtained 48 h after transfection with wild-type or mutant Hint1 constructs, and the ELISA was performed according to the manufacturer’s recommendations. The absorbance at 405 nm was measured using a 96-well plate reader (Spectra Max 340PC).

**RESULTS**

**Expression of Hint1 Induces Apoptosis**—To investigate a possible involvement of Hint1 in apoptosis, human SW480 colon cancer cells and human MCF-7 breast carcinoma cells were transiently Nucleofected® with a Hint1 expression vector, and after 72 h, the cell lysates were examined by Western blot analysis with a monoclonal anti-poly(ADP-ribose) polymerase (PARP) antibody. Cleavage of PARP, a 116-kDa protein involved in DNA repair, is a characteristic marker in the detec-
tion of apoptotic cells (41, 42). As shown in Fig. 1A, in Hint1-transfected cells, full-length PARP was efficiently cleaved, and the characteristic apoptotic 89-kDa cleavage product was detectable. In addition, proteolytic activation of pro-caspase-3 was detectable by Western blotting with an anti-caspase-3 antibody (Fig. 1B). To more directly show that those cells that were transfected and overexpressed Hint1 undergo apoptosis, staining with the anti-M30 CytoDEATH antibody was performed. This antibody specifically detects cytokeratin-18 fragments generated by caspases after induction of apoptosis. Fragmentation of cytokeratin-18 was detectable in MCF-7 cells transiently transfected with Hint1-EGFP (Fig. 1C, e–h) but not in cells transfected with EGFP-actin (enhanced green fluorescent protein) alone (Fig. 1C, a–d). Cleavage of cytokeratin-18 was also observed in HEK293 and SW480 cells transfected with Hint1 (not shown). These data suggest that overexpression of Hint1 triggers apoptosis.

**Hint1 Induces Changes in the Expression of p53 and Bcl-2 Family Proteins**—p53 is a key regulator of apoptosis, and mutations or defective upstream regulation of p53 contributes to tumorigenesis (43). With respect to the tumor suppressor function of Hint1, we investigated whether p53 is involved in Hint1-triggered apoptosis. Expression of p53 was analyzed at the mRNA and protein level in MCF-7 and SW480 cells transiently transfected with Hint1 and compared with mock-transfected cells. In RT-PCR experiments, p53 mRNA expression was increased by a factor of 1.53 ± 0.04 and 1.9 ± 0.03 in MCF-7 and SW480 cells, respectively (Fig. 2, A and B). At the protein level, a 2.49 ± 0.40-fold (MCF-7) and 3.71 ± 0.33-fold (SW480) up-regulation of p53 was detected by Western blot analyses with a monoclonal anti-p53 antibody (Fig. 2, C and D).

Bax and Bcl-2 have been implicated as major players in the control of apoptotic pathways in a wide range of different cell types, and p53 was demonstrated to be an upstream inducer of Bax expression (44). To determine whether Hint1 overexpression and the consequential enhanced expression of p53 is associated with a modulation of the apoptosis-related proteins Bax and Bcl-2, Western blot analyses with monoclonal anti-Bax and anti-Bcl-2 antibodies were performed with cell
Hint1 Triggers Apoptosis

A

MCF-7

cycles 20 25 30 35

β-actin

p53

Hint1 - + - +

SW480

cycles 20 25 30 35

β-actin

p53

Hint1 - + - +

B

rel. exp.

p53

Hint1 - + - +

MCF-7

SW480

C

MCF-7

α-actinin

p53

Hint1 - +

SW480

α-actinin

p53

Hint1 - +

D

rel. exp.

Hint1 - + - +

MCF-7

SW480

FIGURE 2. Hint1 up-regulates expression of the tumor suppressor p53. MCF-7 and SW480 cells were transiently nucleofected with pCS2+ Hint1 or an empty vector. A, semiquantitative RT-PCR was performed with total RNA using p53- and β-actin-specific primers. After the indicated number of cycles, the PCR products were analyzed by agarose gel electrophoresis. B, the amount of PCR products was quantified on a FujiFilm LAS-1000 system. The signal intensity for p53 and β-actin at cycle 30 was set to 1. The transcript levels of p53 were normalized using β-actin transcript levels. The average of three independent RT-PCR experiments with total RNA isolated from independent transfections is presented. C, cell lysates obtained from the same transfected cells as described in panel A were analyzed with a monoclonal anti-p53 antibody by Western blot analysis. Signals were quantified by chemoluminescence imaging on a FujiFilm LAS-1000 system. The signal intensity for p53 and β-actin was used as the loading control. D, the average of three Western blot analyses of MCF-7 and SW480 cell lysates obtained from independent transfections is presented. rel. exp. relative expression.

lysates 72 h after transfection. In Hint1-transfected MCF-7 and SW480 cells, an increase in the pro-apoptotic protein Bax (3.46 ± 0.19-fold in MCF-7 cells; 1.96 ± 0.16-fold in SW480 cells) and a decrease in the anti-apoptotic/pro-survival protein Bcl-2 (32.6 ± 2.5% in MCF-7 cells; 45.4 ± 2.3% in SW480 cells) was detectable (Fig. 3, A and B). Consistent with these observations, RT-PCRs performed with RNAs obtained from the same transfected cells that were used for protein isolation revealed an increase in Bax (1.9 ± 0.1-fold and 2.0 ± 0.1-fold in MCF-7 and SW480 cells, respectively) and a decrease of Bcl-2 mRNA levels (0.72 ± 0.08-fold and 0.45 ± 0.1-fold in MCF-7 and SW480 cells, respectively) (Fig. 3, C and D). In contrast, no changes in the RNA levels of the pro-apoptotic BH3-only proteins Bad and Puma were detectable (supplemental Fig. 1).

Activation of the pro-apoptotic protein Bax has previously been shown to result in an increase of mitochondrial outer membrane permeability and release of apoptogenic factors such as cytochrome c (45). Accumulation of cytochrome c in the cytosolic fraction and a decrease in the mitochondrial fraction was observed in Hint1-transfected MCF-7 and SW480 cells. In control-transfected cells, cytochrome c was only detectable in the mitochondrial fraction (Fig. 4).

If Hint1 is indeed involved in modulation of p53 and expression, silencing of Hint1 by shRNAs should affect p53 RNA and protein levels. To knock down Hint1, shRNAs previously designed and analyzed for their interfering activity were used (22). Vectors expressing shRNA-Hint1 (shRNA-Hint1430/431) or an inactive shRNA (shRNA-Hint1432/433) were stably expressed in MCF-7 Tet-On cells, and the inducible knockdown of Hint1 was verified by immunoblotting. Doxycycline-induced cells transfected with vector or inactive shRNA-Hint1432/433 did not show changes in Hint1 protein expression (Fig. 5A). To investigate the effect of Hint1 knockdown on p53 expression, Western blot and semiquantitative RT-PCR analyses were performed with lysates and RNAs obtained from cells cultured in the presence or absence of doxycycline. Induction of shRNA-Hint1430/431 results in reduced p53 protein levels. In cells transfected with empty vector or non-functional shRNA-Hint1432/433, p53 levels were unchanged (Fig. 5B). Furthermore, p53 mRNA was down-regulated in doxycycline-treated cells expressing shRNA-Hint1430/431, whereas no changes were detectable in cells transfected with empty vector or control shRNA-Hint1432/433 (Fig. 5C). Expression of Bcl-2 was not altered after the addition of doxycycline either at the protein (Fig. 5D) or mRNA (not shown) level. In contrast, knockdown of Hint1 resulted in a strong reduction of Bax protein and mRNA (Fig. 5, D and E).

These observations suggested that Hint1 is directly or indirectly involved in transcriptional regulation of p53 and Bax expression. In this respect, a dose-dependent increase of the luciferase activity was detectable in reporter gene assays with a p53-luciferase construct in Hint1-transfected HEK293 cells. Moreover, co-transfection of shRNA-Hint1430/431 reduced the reporter gene activity (Fig. 6).

Based on the observations that (i) Hint1 binds to Pontin and Reptin (22) and (ii) Pontin and Reptin are components of the
Tip60/HAT complex (28), we wanted to know whether Hint1 is associated with the Tip60/HAT complex. HEK293 cells were transiently transfected with FLAG-Tip60 and Hint1-myc6, and immunoprecipitation experiments were performed with the anti-myc (9E10) monoclonal antibody. Indeed FLAG-Tip60 readily co-precipitated, as detected by Western blotting with anti-FLAG-M2 monoclonal antibody. Moreover, in immunoprecipitations with an anti-Tip60 antibody, endogenous Tip60 was found associated with Hint1-myc6 (Fig. 7, A and B).

In this context, the recently reported observation that Tip60 favors the expression of some pro-apoptotic p53 target genes (46) suggests that Hint1 may be involved in the p53-mediated transcriptional regulation of Bax (47). To investigate this, ChIP experiments were performed. Indeed, a Bax promoter fragment containing the p53-binding site was precipitated with the anti-Hint1 antibody in mock-transfected and Hint1-transfected cells. In control experiments without antibody or using IgG or anti-GFP, only background binding was detectable (Fig. 7C). However, we could not find Tip60 associated with the Bax promoter in a ChIP using an anti-Tip60 antibody (not shown), similar to observations reported previously (46). Taken together, these results suggest that Hint1, together with the Tip60/HAT complex, at least in part is involved in the modulation of p53-dependent regulation of Bax transcription. The detailed molecular mechanisms have to be elucidated.

**Hint1 Triggers Apoptosis Independent of Its Enzymatic Activity** — Next, we examined whether the Hint1 enzymatic activity is necessary for the induction of apoptosis. Specific amino acids, suggested to be involved in the enzymatic activity by x-ray structural analysis (48–50), were exchanged by site-directed mutagenesis. To analyze these mutant Hint1 proteins (Hint1-H112N, -G105A, -S107A, and -G105A/S107A) for their enzymatic activity, they were expressed with an N-terminal His6 tag and purified by nickel-nitrilotriacetic acid chromatography. Enzymatic activity was quantified by HPLC using AMP-NH2 as a substrate. In these assays, only Hint1-H112N (mutated in the central histidine residue within the histidine triad) was enzymatically "dead" (supplemental Fig. 2), whereas the other mutated proteins were only partially impaired in their activity (not shown). From x-ray structural analysis, it is known that Hint1 forms dimers (10, 11, 48). To prove that the introduced mutations do not impair dimerization, gel filtration chromatography was performed. Both wild-type and all mutant Hint1 proteins formed dimers in these assays (supplemental Fig. 3).

When transfected into SW480 or MCF-7 cells, both wild-type and mutant Hint1 proteins did not differ in their ability to induce apoptosis in a dose-dependent way as measured by a cell death assay. The expression of Bax and Bcl-2 in Hint1-transfected cells was monitored by Western blotting. A and B, after 72 h, cell lysates were analyzed by Western blotting with anti-Bax and anti-Bcl-2 antibodies. The presented data are representative of three independent experiments. C and D, Bax mRNA levels are up-regulated, whereas Bcl-2 is down-regulated as shown by RT-PCR performed on total RNA using Bax-, Bcl-2-, and β-actin-specific primer pairs. Data present a representative of three independent experiments.
death detection ELISAPlus quantifying DNA fragmentation (Fig. 8A). Consistent with these observations, the enzymatically dead Hint1-H112N mutant was able to induce p53 and Bax expression and to down-regulate Bcl-2 expression (Fig. 8, B and C). Interestingly, all mutant Hint1 proteins were also able to suppress TCF/β-catenin-mediated transcription in reporter gene assays (supplemental Fig. 4).

DISCUSSION

The structural similarity between Hint1 and Fhit suggests that Hint1 might also act as a tumor suppressor. However, until recently, functional studies to prove this hypothesis had been missing. Analysis of Hint1<sup>−/−</sup> and Hint1<sup>−/−/−</sup> mice provided the first evidence that Hint1 is a novel tumor suppressor affecting tumor susceptibility in multiple tissues (19, 20). Interestingly, the loss of one Hint1 allele appears sufficient to sensitize cells to carcinogen-induced tumorigenesis (20), defining it as a haplo-insufficient tumor suppressor. Moreover, expression of Hint1 in a non-small cell lung cancer cell line was shown to inhibit cell growth and to slow down tumor growth in nude mice (21). In line with a tumor suppressor role, we recently reported that Hint1 represses TCF/β-catenin-mediated transcription by binding to Pontin and Reptin, two β-catenin-associated AAA+ superfamily proteins found in multiple chromatin remodeling complexes (22).

Here we extended our studies on the molecular mechanism involved in the tumor suppressor function of Hint1 and provide evidence that Hint1 is involved in apoptosis. Over-expression of Hint1 in MCF-7 and SW480 cells induces apoptosis as shown by PARP cleavage, pro-caspase-3 cleavage resulting in caspase-3 activation, M30 CytoDEATH staining, cytochrome c release, and a DNA fragmentation assay. We did not observe this effect in cells that were transfected with Hint2. To further characterize Hint1 involvement in apoptosis, the expression of apoptosis-related proteins, such as p53, Bax, Bcl-2, Bad, and Puma, was analyzed. Consistent with a pro-apoptotic function of Hint1, an up-regulation of p53 and Bax and a down-regulation of Bcl-2 were observed on the protein and RNA level. Bad and Puma mRNA were not changed. In contrast, Fhit was reported not to modulate p53, Bax, and Bcl-2 protein expression levels (35). This either may be explained by the use of different cell lines in our study or suggests that, although highly similar in structure, Hint1

3 C. Wirths, J. Weiske, and O. Huber, unpublished observations.
and Fhit differ in the mechanism triggering apoptosis (51). The observed down-regulation of p53 and Bax expression at the RNA and protein level in response to the knockdown of Hint1 by shRNA suggests that normal levels of Hint1 are required for base-line expression of p53 and Bax, and affected Hint1 function may impair apoptosis.

Previous studies provided evidence that Hint1 is involved in transcriptional regulation (13–15, 22). Our p53-luciferase assays suggest that Hint1 is also involved in the regulation of p53 transcription. However, it remains to be investigated whether Hint1 directly regulates p53 transcription or whether the change in expression is an effect mediated by other factors. In this respect, the recent findings that p53 and Tip60 interact and together with Reptin/TIP49b are required for DNA damage-induced apoptosis and the observation that Tip60 together with p53 is involved in the regulation of Bax expression (46) suggest thatHint1 may be involved in the regulation of Bax expression. Evidence for this hypothesis is provided by the observation that Hint1 is associated with Tip60 in co-immunoprecipitation experiments and the finding that Hint1 is associated with the Bax promoter in ChIP experiments. Otherwise, Hint1 may regulate p53 activity by binding to Cdk7 (14) within the trimeric Cdk-activating kinase (CAK) complex, which phosphorylates p53 (52). In addition, it was reported that p53 inhibits CAK-mediated phosphorylation of Cdk2 (53).

Hint1 enzymatic activity as an adenosine-5′-monophosphoramide was shown to support the activity of the yeast Cdk7 homolog Kin28 (12). In this respect, we investigated whether the enzymatic activity of Hint1 is required to trigger apoptosis. Site-directed mutagenesis of histidine 112 to asparagine (H112N), representing the central histidine residue within the histidine triad, resulted in an AMP-NH2-hydrolase dead enzyme (10, 11, 48) that still formed dimers, as shown by gel filtration chromatography. Overexpression of this mutated Hint1-H112N protein induced apoptosis at rates comparable with wild-type protein. Similar observations were reported for a mutant Fhit gene with a central histidine 96 to asparagine (Fhit-H96N) mutation. In in vitro and in vivo experiments, Fhit-H96N was not impaired in its tumor-suppressing activity (54–56). It was suggested that binding of ApmA (but not hydrolysis) is important in this respect; however, this remains to be experimentally proven (2). Interestingly, the repressive effect of Hint1 on TCF/β-catenin transcriptional activity also does not depend on the Hint1 enzymatic activity.

FIGURE 6. Hint1 is involved in the regulation of p53 transcription. A, HEK293 cells were transiently transfected with a p53-luciferase construct and increasing amounts of Hint1. Luciferase activity was measured 42 h after transfection. pCH110 (β-galactosidase) was used for normalization. B, transcription from the p53 promoter is reduced in the presence of shRNA directed against Hint1. Data represent the mean of four independent transfections, each measured in duplicate. rel. activity, relative activity.

FIGURE 7. Hint1 forms a complex with Tip60 and is associated with the Bax promoter. A, HEK293 cells were transiently transfected with FLAG-tagged Tip60 and myc-tagged Hint1. The cells were lysed 42 h after transfection, and protein complexes were immunoprecipitated (IP) with an anti-myc (9E10) monoclonal antibody and analyzed by Western blotting (IB) with the anti-FLAG-M2 antibody. B, HEK293 cells were transfected with Hint1-mycα and endogenous Tip60 was precipitated with an anti-Tip60 antibody. Associated Hint1-mycα was detected on a Western blot with the anti-myc (9E10) antibody. C, chromatin immunoprecipitation assay on the Bax promoter (p53 binding-site) using affinity-purified anti-Hint1 antibody. Ab, antibody.
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Taken together, Hint1, similar to Fhit, triggers apoptosis, suggesting that other histidine triad protein family members may also share this function. However, Hint1 and Fhit appear to differ in the molecular mechanisms involved in the induction of apoptosis. Moreover, the pro-apoptotic activity of Hint1 may contribute to its tumour suppressor function. In this context, Hint1 appears to modulate transcriptional regulation, cell cycle control, and induction of apoptosis.

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Note Added in Proof—During the reviewing process of this manuscript Martin et al. reported that Hint2 acts as an apoptotic sensitizer and is frequently down-regulated in hepatocellular carcinoma. (Martin, J., Magnino, F., Schmidt, K., Piguet, A. C., Lee, J. S., Semela, D., St-Pierre, M. V., Ziemiecki, A., Cassio, D., Brenner, C., Thorgersson, S. S., and Dufour, J. F. (2006) Gastroenterology 130, 2179–2188.)

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