Histidine Triad Nucleotide-binding Protein 1 Up-regulates Cellular Levels of p27Kip1 by Targeting SceSKP2 Ubiquitin Ligase and Src*‡

Bo Cen†, Haiyang Li†, and I. Bernard Weinstein†‡†

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The one or more underlying mechanisms of the tumor suppressing activity of the histidine triad nucleotide-binding protein 1 (HINT1) are not well defined. In this study we found that HINT1 regulates cellular levels of the cyclin-dependent kinase inhibitor p27Kip1 through multiple mechanisms. Increased expression of HINT1 increases cellular levels of p27Kip1, and HINT1 knockdown with small hairpin RNA leads to decreased cellular levels of p27Kip1. HINT1 does not affect the transcription of p27Kip1, but it does inhibit proteasomal degradation of the p27Kip1 protein. HINT1 directly interacts with the SCFSKP2 ubiquitin ligase complex and inhibits the ubiquitylation of p27Kip1. Src has been shown to phosphorylate p27Kip1 and thus decrease its stability. We found that HINT1 is a negative regulator of Src transcription apparently by forming a complex with the transcription factor Sp1 on the promoter of Src. Taken together, our findings indicate that HINT1 up-regulates cellular levels of p27Kip1 by two mechanisms: 1) it inhibits its ubiquitylation by targeting the SCFSKP2 ubiquitin ligase complex, and 2) it inhibits the phosphorylation of p27Kip1 by Src via inhibiting Src expression.

HINT1 belongs to the Hint branch of the HIT (histidine triad) protein family, which is a superfamily of nucleotide hydrolases and transferases that contain an HXHXHXX motif in which X is a hydrophobic amino acid (1, 2). Our laboratory discovered the Hint1 gene and demonstrated that it functions as a haplo-insufficient tumor suppressor gene in mice (3–5). Briefly, we found that Hint1-deleted mice have a marked increase in susceptibility to chemical carcinogen-induced gastric tumors (5), mammary tumors (4), and ovarian tumors (4). In addition, with aging, Hint1-deleted mice displayed an increase in the occurrence of a variety of spontaneous tumors (4). We and other groups have made intensive efforts to elucidate the molecular mechanisms of this tumor suppressor activity. Deletion of the Hint1 gene enhances the growth of mouse embryonic fibroblasts (5), whereas overexpression of HINT1 inhibits the growth of SW480 colon cancer cells (6). Overexpression of HINT1 also triggers apoptosis in SW480 and MCF7 breast cancer cells by inducing expression of p53 and the pro-apoptotic factor Bax and concomitant down-regulation of the apoptosis inhibitor Bcl-2 (7). HINT1 has also emerged as an important regulator of gene transcription. Thus, HINT1 is present in a protein complex containing the basal transcription factor TFIIB (8). In mast cells, HINT1 binds to the microphthalmia transcription factor and the transcription factor USF2 and inhibits their transcriptional activities (9, 10). Weiske et al. (11) found that HINT1 interacts directly with Pontin and Repthin, which are direct binding partners of β-catenin and thus inhibits T-cell factor-β-catenin-mediated transcription such as transcription of cyclin D1. Studies from our laboratory indicate that HINT1 inhibits activator protein-1 transcriptional activity by binding to PSH (plenty of SH3) and c-Jun N-terminal kinase 2 (JNK2) (6). HINT1 may play a role in other cellular events such as cellular responses to ionizing radiation via interaction with the product of ATDC gene to repress fos transcription (12) and in modulating the μ-opioid receptor signaling pathway through interaction with the μ-opioid receptor and/or regulator of G protein signaling Z1 (13, 14).

Because of the frequent disturbances in cell cycle control in cancer we have recently explored whether HINT1 might also exert its tumor suppressor activity by affecting proteins that control the cell cycle. The cell cycle is tightly controlled by the activities of cyclin-dependent kinases (CDKs). In addition, the activity of CDKs is regulated by multiple mechanisms, including the binding of cyclins, phosphorylation/dephosphorylation, cellular localization, transcriptional regulation, protein degradation, and interactions with CKD inhibitors (reviewed in Ref. 15). CDK inhibitors consist of two subfamilies. One contains p21cip1, p27kip1 (hereafter referred to as p27), and p57kip2 (16–19); the other contains p16ink4a, p15ink4b, p18ink4c, and p19ink4d (20). The CDK inhibitors in the first subfamily share

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From the †Herbert Irving Comprehensive Cancer Center and ‡Department of Medicine, Columbia University Medical Center, New York, New York 10032

‡1 The abbreviations used are: CDK, cyclin-dependent kinase; KPC, Kip1 ubiquitylation-promoting complex; shRNA, short hairpin RNA; HA, hemagglutinin; GST, glutathione S-transferase; ChIP, chromatin immunoprecipitation; E2, ubiquitin carrier protein; ERK2, extracellular signal-regulated kinase 3.
similarities at their N terminus and have a broad specificity for CDKs (reviewed in Ref. 21). Cellular levels of p27 are increased in quiescent cells, decrease upon cell cycle entry, and are largely, but not exclusively, controlled by the rate of degradation of the p27 protein (22–24). Degradation of p27 is maximal in S phase following cell cycle entry and is controlled by both mitogenic and anti-mitogenic signals (for a review see Ref. 23). p27 is decreased in some human tumors, and low expression is an adverse prognostic factor (25–28). Therefore, the molecular mechanisms underlying p27 degradation are important in tumor biology. It is of interest that p27 is ubiquitylated and degraded in a proteasome-dependent manner. In the nucleus, CDK2 phosphorylates p27 at threonine 187. The modified protein is then recognized by the SCF<sup>Skip</sup> ubiquitin ligase and degraded by the proteasome (reviewed in Ref. 29). In the cytoplasm, another ubiquitin ligase KPC (Kip1 ubiquitylation-promoting complex) recognizes unphosphorylated p27 during <i>G</i><sub>i</sub> and this also leads to the proteasome-mediated degradation (30). Recently, several groups discovered that p27 can also be phosphorylated on tyrosine residues by the oncogenic protein Src, as well as its family member Lyn, and also by another non-receptor tyrosine kinase Abl, and that this results in decreased stability of the p27 protein (31, 32). The phosphorylation of p27 by Src impairs its CDK2 inhibitory action and reduces its steady-state binding to cyclinE-CDK2, thus facilitating CyclinE-CDK2-dependent p27 degradation (31, 32).

The present study describes the discovery that HINT1 can increase cellular levels of p27 at the post-transcriptional level by inhibiting its proteasomal degradation. This appears to occur via two mechanisms: 1) inhibition of ubiquitylation of p27 by the SCF<sup>Skip</sup> ubiquitin ligase, and 2) inhibition of tyrosine phosphorylation of p27 by Src through inhibiting Src expression. These effects on p27 could further contribute to the tumor suppressor activity of HINT1.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The FLAG-tagged HINT1 expression construct p3×FLAG-Hint1 was constructed by PCR-based subcloning from pcDNA3-Hint1 (3). A 3×FLAG-Hint1 construct with altered nucleotide sequence but without alterations in amino acid coding sequence was made using PCR technology to avoid an RNA interference effect when re-introducing HINT1 expression to HINT1 shRNA SW480 cells and named 3×FLAG-Hint1-X. HA-tagged HINT1 expression construct pHANE-HA-Hint1 was previously described (6). HA-tagged p27 expression constructs were subcloned from pcDNA3-p27 (33) into the pHACE vector. The FLAG-tagged KPC1 and KPC2 were constructed by subcloning KPC1 and KPC2 cDNA from the pMX-puro-His<sub>6</sub>-FLAG-hKPC1 and the pMX-puro-hKPC2-HA (kindly provided by Dr. Keiichi Nakayama), respectively, into the p3×FLAG-7.1 vector. Several expression constructs were kind gifts from other investigators, as follows: The Sp1 expression construct pMpcSp1 from Dr. Robert Tjian, the Sp3 expression construct pCMV-Sp3/flu from Dr. Jonathan Horowitz, the His<sub>6</sub>-tagged ubiquitin expression constructs from Dr. Dirk Bohmann, GST fusion protein expression constructs and HINT1 RNA interference (shRNA) constructs from Dr. Otmar Huber, luciferase reporter pGL3-Src from Dr. Carlo Catapano, HA-Ubc3 construct from Dr. P. Renee Yew, and the Myc-Roc1 construct from Dr. Yue Xiong. His<sub>6</sub>-Ubc3 was constructed by subcloning Ubc3 cDNA from HA-Ubc3 into empty vector pET-15b (Novagen). Cycloheximide and MG132 were purchased from Sigma. PP1 was from Biomol.

**Immunoblot and Antibodies**—Lysates of total cellular proteins or immunoprecipitates were analyzed by protein immunoblotting after SDS-PAGE using the specified antibodies. Immunocomplexes were detected by enhanced chemiluminescence (Amersham Biosciences). As primary antibodies, we used p27<sup>Skip</sup> (BD Transduction Laboratories), β-actin (Sigma), HINT1 (3), HINT1 (ProteinTech Group, for chromatin immunoprecipitation assay), HA (Sigma), FLAG (Sigma), Rbx1/Roc1 (Santa Cruz Biotechnology), FAK (Cell Signaling), Cbl (Cell Signaling), Csk (Cell Signaling), Src (Cell Signaling), Src(pY418) (BIOSOURCE), ERK2 (Upstate), phospho-ERK2 (Upstate), Sp1 (Santa Cruz Biotechnology), CDK2 (Upstate), and Ubc3/CDC34 (Invitrogen).

**Co-immunoprecipitation Assay and GST Pulldown Assay**—For immunoprecipitation, cells from 6-cm plates were rinsed once in ice-cold phosphate-buffered saline and then lysed in 400 μl of immunoprecipitation buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 10% glycerol, 1 mM EDTA). The lysates were rotated for 30 min at 4 °C followed by a 10-s sonication and cleared by centrifugation at 13,000 × g for 10 min at 4 °C. Immunoprecipitation was done using EZview Red Anti-FLAG M2 Affinity gel (Sigma) or EZview Red Anti-HA Affinity gel (Sigma) or EZview Red Protein G Affinity gel (Sigma). The beads were washed three times with immunoprecipitation buffer, and the proteins were resolved on SDS-PAGE. The association of HINT1 with Roc1 or Ubc3 or Sp1 was also analyzed using agarose-immobilized GST fusion proteins. *Escherichia coli* BL21(DE3) cells harboring the plasmid encoding GST-HINT1, GST-HINT1ΔN, GST-HINT1ΔC, or GST were cultured at 37 °C with shaking. GST fusion proteins were induced by incubating the cells with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 2 h. Bacteria expressing the GST fusions were precipitated and resuspended in phosphate-buffered saline containing 0.5% Triton X-100, 2 mM EDTA (buffer A). Bacteria cell lysates were mixed with GSH-agarose beads. GST fusion proteins were immobilized on GSH-agarose by incubation at 4 °C for 30 min and washed three times with buffer A. His<sub>6</sub>-Ubc3 was expressed in *E. coli* BL21(DE3) cells and purified using an His-Bind purification kit (Calbiochem). For the binding reaction, GSH-agarose carrying the immobilized GST fusion proteins (or GST as control) was mixed with cell lysates from control 293T cells or 293T cells containing overexpressed HA-Ubc3 or Myc-Roc1 or 3×FLAG-KPC1 or 3×FLAG-KPC2, or purified His<sub>6</sub>-Ubc3 in buffer B (100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA) overnight at 4 °C. The protein-bound agarose was washed three times with buffer B. Bound proteins were eluted with 2× SDS-PAGE loading buffer.

**Reporter Gene Assays**—SW480 cells (3 × 10<sup>5</sup> cells per well) were seeded into 12-well plates and transfected the second day using the Lipofectin reagent (Invitrogen), with different expression plasmids (1 μg each) together with 10 ng of pRL-Luc
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(renilla luciferase) and 0.1 μg of the reporter plasmid pGL3-Src. After incubation for 40 h, the cells were lysed with passive lysis buffer (Promega, Madison, WI). Luciferase activities present in cellular lysates were assayed with the Lumat LB 9507 luminometer as specified by the manufacturer (EG&G Berthold) and normalized to the Renilla luciferase activity to compensate for variability in transfection efficiencies.

Ubiquitylation Assays—The in vivo ubiquitylation assay was conducted as described previously (34). The lysis buffer contains 10 mM N-methylmaleimide, 1 mM dithiothreitol, 0.1% SDS. HEK293T cells in 60 mm plates were transfected with His6-ubiquitin, HA-p27 expression plasmids, 3×FLAG-Hint1 using the calcium phosphate reagents. Thirty hours after transfection, the cells were then treated with 10 μM MG132 for an additional 6 h. The cells from each plate were harvested and split into two aliquots, one for use in Western blot and the other for detection of ubiquitylated proteins using nickel-nitrilotriacetic acid beads (Qiagen) followed by Western blot with anti-p27 antibody. The in vitro ubiquitylation reaction mixture contained, in a volume of 15 μl, 50 mM Tris-HCl (pH 7.6), 5 mM MgCl2, 10% (v/v) glycerol, 1 mM dithiothreitol, 10 mM phospho-creatine, 100 μg/ml bovine serum albumin, 1 mg/ml methylated ubiquitin, 1 μM ubiquitin aldehyde, 1 pmol of E1, 50 pmol of Ubc3/Cd3c34, 1 μl of Skp2 lysate (containing Skp2, Skp1, Rbx1, and Cul1), 2 μM Cdk2-cyclin E, 50 nm Cks1, and 1 μl of [35S]p27. Following incubation at 30 °C for 60 min, samples were subjected to SDSA PAGE followed by PhosphorImager scanning.

Real-time PCR and Reverse-transcription PCR—Total RNA was extracted with an Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare). cDNA was synthesized from 3 μg of total RNA for each sample using the iScript Select cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. cDNA was diluted from 20 μl to 60 μl with ddH2O. Real-time PCR was performed with primers chosen to extend products at least three independent experiments. Control experiments run in the absence of reverse transcriptase and/or RNA demonstrated no product amplification.

For reverse-transcription PCR, total RNA was isolated from SW480 cells using TRIzol reagent as recommended by the manufacturer (Invitrogen). cDNA was amplified from 1 μg (10 ng for β-actin) of total RNA using a Superscript One-Step reverse transcription-PCR with the platinum TaqDNA polymerase system (Invitrogen)). Primer sets used were: p27-forward, 5′-TGCAACCAGCGATTCTCTACTCTCAAA-3′; p27-reverse, 5′-CAAGCAGTGTATGTCTGATAAC-3′; Hint1-forward, 5′-CGGATGGCGATGAGATGG-3′; and Hint1-reverse, 5′-CTTTATTGCAGCCAGATCA-3′. As an internal control, we used β-actin, forward, 5′-GACCTGACTGACTACCTC-3′; reverse, 5′-GACGCCAGGTAGC-3′. The annealing temperature was 72 °C. After 30 cycles, PCR products were analyzed by agarose gel electrophoresis.

ChIP Assays—ChIP was performed using the ChIP-IT™ Express Kit (Active Motif) according to the manufacturer's instructions with minor modifications. In brief, HEK293T cells were fixed with 2 μM disuccinimidylglutarate for 45 min at room temperature. Subsequently, the cells were washed twice with phosphate-buffered saline before fixation with 1% (v/v) formaldehyde. Nuclei were disrupted by sonication with eight 20-s pulses at 30% output in a Sonifier Cell Disruptor (Heat Systems-Ultrasonics, Inc., Plainview, NY), yielding genomic DNA fragments with a bulk size of 200–1000 bp. For each ChIP 15 μg of DNA was incubated overnight at 4 °C with 5 μg of anti-HINT1 antibody, anti-Sp1 antibody, or rabbit normal IgG. For subsequent PCR analysis, 5 μl of the extracted DNA (100 μl) was used as a template for 40 cycles of amplification. The following primers were used to detect the Src promoter: GC1-forward, 5′-CCTGAGCAGCTTAGAGT-3′; GC1-reverse, 5′-GAGGGAGAAGGAGAAGGC-3′; GA2-forward, 5′-GGC TTCCTTCTCCGCTCTC-3′; GA2-reverse, 5′-GACCTTAG AGGATCCCCG-3′. PCR products were analyzed on a 2% agarose gel.

Statistical Analysis—All assays were repeated at least three times. The results of quantitative studies are reported as mean ± S.D. Differences were analyzed by Student's t test. p < 0.05 was regarded as significant, and such differences are indicated in the figures by an asterisk.

RESULTS

High Cellular Levels of HINT1 Are Associated with High Cellular Levels of p27—Several tumor suppressor proteins, including Rb, p27, and p53, play important roles in regulating the cell cycle. Therefore, we examined if HINT1, as a tumor suppressor, also plays a role in cell cycle regulation. Indeed, we found that expression of FLAG-tagged HINT1 in SW480 cells markedly increased cellular levels of the p27 protein but not that of CDK2, a binding partner of p27, when compared with SW480 cells transfected with only the vector (Fig. 1A, lanes 1 and 2). Similar results were obtained when HA-tagged HINT1 was expressed in MCF7 cells (Fig. 1A, lanes 3 and 4). Expression of HINT1 shRNA in SW480 cells led to decreased expression of p27 but again no change was observed in CDK2 expression.
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FIGURE 1. The cellular level of HINT1 correlates with the cellular level of p27. A, HINT1 up-regulates p27 expression. Lane 1, Western blot analysis of endogenous p27, CDK2, HINT1, and β-actin expression in SW480 cells transiently transfected with an empty vector. Lane 2, analysis of the same cells after transient transfection with a 3×FLAG-HINT1 plasmid. Lane 3 and 4, similar analysis of MCF7 cells stably transfected with an empty vector or an HA-HINT1 plasmid. Lane 5 and 6, similar analysis of SW480 cells stably transfected with a control shRNA or a HINT1 shRNA construct. The expression of transfected HINT1 was detected using anti-FLAG and anti-HA antibodies. The results of p27 expression from three independent experiments were plotted after quantitation using NIH ImageJ v1.41 software with normalization to the corresponding β-actin control. *, significant differences with respect to control (p < 0.05). A representative experiment is shown. B, comparison of HINT1 and p27 or Src levels in various colon cell lines. Cell lysates from the normal colon cell lines CCD-112CoN, CCD-841CoN; the colon cancer cell lines HT29 and SW480, as well as SW480 cells with reduced endogenous HINT1 expression, were subject to Western blot analysis for p27, HINT1, and Src. β-Actin was used as a loading control.

levels (Fig. 1A, lanes 5 and 6). Image quantitation confirmed that these changes are statistically significant (Fig. 1A). We also found that two normal colon cell lines, CCD-112CoN and CCD-841CoN, which expressed high levels of HINT1, also expressed high levels of p27, whereas HT29 and SW480 colon cancer cells, which expressed low levels of HINT1, expressed relatively low levels of p27 (Fig. 1B). Furthermore, further decreasing the level of HINT1 in SW480 cells with HINT1 shRNA caused a further decrease in p27 (Fig. 1B, last lane). Similar results were obtained on repeated Western blots. A representative experiment is shown.

HINT1 Does Not Alter the Transcription of p27—To determine the level at which HINT1 regulates p27 expression, we performed reverse transcription-PCR assays. SW480 cells were transiently transfected with an empty vector or a FLAG-HINT1 construct (Fig. 2A, left panel) or stably transfected with a control shRNA construct or a HINT1 shRNA construct (Fig. 2A, right panel). Neither overexpression nor knockdown of HINT1 resulted in significant changes in the levels of p27 mRNA (Fig. 2A). Data from real-time PCR, which is a more quantitative method to detect mRNA abundance, also showed no significant difference in the abundance of p27 mRNA before and after HINT1 overexpression or knockdown (Fig. 2B). To assess possible effects of HINT1 on the transcriptional activity of the p27 promoter, we performed luciferase reporter assays. The full-length p27 promoter was linked to a luciferase reporter. This reporter (p27PF) was co-transfected into SW480 cells with increasing amounts of HINT1 or Sp1 expression plasmids. We did not observe any effect of HINT1 on the activity of the p27 promoter (Fig. 2C). Sp1, which is known to act as a cis-transcription factor on the p27 promoter (35), served as a positive control in these studies. Taken together, these findings indicate that the up-regulation of p27 by HINT1 occurs at a post-transcriptional level.

HINT1 Inhibits Proteasome-mediated p27 Degradation and Reduces the Ubiquitylation Level of p27—p27 is a short-lived protein and its cellular level is regulated by the proteasome-dependent system (24, 36). Thus, we found that treatment of SW480 cells with the proteasome inhibitor MG132 increased cellular levels of p27 (Fig. 3A). HINT1 overexpression increased the basal level of p27, and this was augmented when the cells were also treated with MG132 (Fig. 3A, left panel). When HINT1 expression was inhibited with a shRNA this partially impaired the ability of MG132 to increase the levels of p27 compared with the control shRNA (Fig. 3A, right panel). Similar results were obtained in three independent studies. Image quantitation confirmed that these changes of p27 levels are statistically significant (Fig. 3A).

We then investigated the effect of transfection of a FLAG-tagged HINT1 on the half-life of the p27 protein in SW480 cells by treating the transfected cells with cycloheximide to block de novo protein synthesis and following the levels of the remaining endogenous p27 protein at various time intervals. As a control, the cells were transfected with an empty vector (Fig. 3B). In the control cells the half-life of the p27 protein was ~2 h, but when the cells were transfected with a HINT1 construct the half-life was ~4 h. Therefore, the half-life of the p27 protein was clearly increased by HINT1. Furthermore, in SW480 cells stably transfected with a control shRNA construct, the half-life of the p27 protein was again ~2 h, but when the cells were transfected with a HINT1 shRNA construct the half-life dropped to ~1 h (Fig. 3B). Taken together, the results in Fig. 3 (A and B) suggested that HINT1 inhibits proteasome-mediated degradation of p27, either by inhibiting the ubiquitylation of p27 or the function of the proteasome. Unlike p27, HINT1 has a relatively long half-life (~6 h, Fig. 3B). Neither treatment with MG132 for 2 h nor treatment with cycloheximide for 4 h had significant effects on cellular levels of HINT1 in SW480 cells or 293T cells (data not shown).

To examine whether HINT1 inhibits the ubiquitylation of p27, HA-p27 was co-transfected with His-ubiquitin into 293T cells due to the reason that we were not able to produce high quality ubiquitylated p27 in SW480 cells, and the cells were
then treated with 10 μM MG132 for 6 h, and extracts were prepared and analyzed for ubiquitylation of p27 (Fig. 3C), as described under “Experimental Procedures.” The middle lane displays a series of bands of ubiquitylated p27. The right lane indicates that the amount of ubiquitylated p27 was significantly reduced by HINT1 as confirmed by image quantitation from three independent experiments (Fig. 3C). HINT1 itself is not ubiquitylated under the same experimental conditions (data not shown), which rules out the possibility of competition for ubiquitin between p27 and HINT1.

HINT1 Is a Component of the SCF<sup>SKP2</sup> Ubiquitin Ligase Complex but Not the KPC Ubiquitin Ligase Complex—To investigate how HINT1 reduces the ubiquitylation level of p27, we examined if HINT1 forms a complex with the SCF<sup>SKP2</sup> ubiquitin ligase protein complex that ubiquitylates p27. We first performed GST pulldown assays. GST, a GST-full-length HINT1 fusion protein (GST-HINT1), a GST-HINT1 N-terminal half fusion protein (GST-HINT1<sub>N</sub>C), and a GST-HINT1 C-terminal half fusion protein (GST-HINT1<sub>C</sub>N) were expressed in E. coli BL21 strain and purified to 95% purity (Fig. 4A). 293T cells were transiently transfected with HA-tagged CDC34/Ubc3 (hereafter referred to as Ubc3), a component of the SCF<sup>SKP2</sup> complex that serves as an E2-conjugating enzyme (24), or Myc-tagged Roc1/Rbx1 (hereafter referred to as Roc1), another component of the SCF<sup>SKP2</sup> complex that recruits Ubc3 (37). Cell lysates were then incubated with the purified GST fusion proteins. We found that HA-Ubc3 bound to GST-HINT1 and GST-HINT1<sub>N</sub>C but not to GST or GST-HINT1<sub>C</sub>N. Myc-Roc1 bound to GST-HINT1, GST-HINT1<sub>N</sub>, and GST-HINT1<sub>C</sub> (Fig. 4A). Therefore, the N-terminal half of HINT1 mediates binding of HINT1 to the Ubc3. GST-HINT1<sub>N</sub> and GST-HINT1<sub>C</sub> share an acidic motif (EDDDE) localized in the middle of HINT1 that may mediate the binding of HINT1 to Roc1. By using purified Ubc3 protein from bacteria we demonstrated that GST-HINT1 but not GST directly binds to Ubc3 (Fig. 4A).

We also co-transfected FLAG-tagged HINT1 with HA-Ubc3 into 293T cells and found that HINT1 co-immunoprecipitated with Ubc3 (Fig. 4B). HINT1 also pulled down Myc-tagged Roc1 (Fig. 4B). Co-immunoprecipitations were done in reverse to confirm these findings (data not shown). The interaction between HINT1 and Roc1 survived exposure to a high stringency wash buffer (with detergent), whereas the interaction between HINT1 and Ubc3 could only be detected with a low stringency wash buffer (without detergent, data not shown). Two truncation mutants of HINT1, N<sub>20</sub> and C<sub>17</sub>, which lack the N-terminal 20 residues and C-terminal 17 residues of HINT1, respectively, were also able to co-immunoprecipitate with Roc1 (supplemental Fig. S1A), suggesting that the central region of HINT1 is responsible for binding to the SCF<sup>SKP2</sup> complex. Endogenous HINT1 was also found to co-immunoprecipitate with respect to HINT1 (p < 0.05). C, HINT1 does not activate the p27 promoter. Transient transfection assays were performed in SW480 cells using a full-length p27 promoter linked to luciferase (p27PF). Increasing amounts of HINT1 and Sp1 expression constructs were co-transfected with p27PF, as indicated. Duplicates of each sample were included in each experiment. Three independent experiments gave similar results. *, significant stimulation by Sp1 (p < 0.05).
**FIGURE 3. HINT1 inhibits proteasome-mediated p27 degradation.** A, SW480 cells were transiently transfected with an empty vector or the 3×FLAG-HINT1 construct (left panel), or SW480 cells were stably transfected with control shRNA and Hint1 shRNA constructs (right panel). The cells were then treated with 4 μM MG132 for an additional 6 h. Extracts were then studied by Western blot analysis using anti-p27, anti-β-actin, anti-FLAG, and anti-HINT1 antibodies. The results of p27 expression from three independent experiments were plotted after quantitation using NIH ImageJ v1.41 software with normalization to the corresponding β-actin control. *, significant differences with respect to control (p < 0.05). A representative experiment is shown.

B, HINT1 extends the half-life of p27. SW480 cells were transiently transfected with empty vector or 3×FLAG-Hint1 construct (upper panel), or SW480 cells were stably transfected with control shRNA and HINT1 shRNA constructs (lower panel). After 40 h, cells were incubated with fresh medium containing 40 μM cycloheximide for the indicated times. Cell lysates were subject to Western blot analysis with antibodies for p27, CDK2, HINT1, β-actin, and FLAG. The results of p27 were plotted after quantitation using NIH ImageJ v1.41 software with normalization to the corresponding β-actin control. A representative experiment is shown.

C, HINT1 reduces the ubiquitylation level of p27. 293T cells were transfected with or without plasmids encoding HA-p27, His6-ubiquitin, and 3×FLAG-HINT1, as indicated. 30 h after transfection, the cells were treated with 10 μM MG132 for an additional 6 h prior to being harvested. Cell lysates were incubated with nickel-nitrilotriacetic acid beads. The beads were then washed intensively before Western blot analysis using anti-p27 antibody. The results of p27 ubiquitylation from three independent experiments were plotted after quantitation using NIH ImageJ v1.41 software with normalization to the corresponding p27 expression. *, significant differences with respect to control (p < 0.05). A representative experiment is shown.
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Because HINT1 co-immunoprecipitated with both Ubc3 and Roc1, we further explored the binding relationship between HINT1, Ubc3, and Roc1. We co-transfected HA-Ubc3 and Myc-Roc1 into control shRNA SW480 cells and into HINT1 shRNA SW480 cells. Immunoprecipitated Ubc3 specifically co-precipitated Roc1 in control shRNA SW480 cells. Interestingly, in HINT1 shRNA SW480 cells, a comparable amount of Ubc3 co-precipitated much more Roc1 than in control shRNA SW480 cells. Re-introduction of HINT1 by overexpression in HINT1 shRNA SW480 cells dramatically reduced the amount of co-precipitated Roc1 (Fig. 4D). These findings suggest that in vivo HINT1 can interfere with the interaction between Roc1 and Ubc3, thus impairing the recruitment of Ubc3 by Roc1.

To further determine whether the observed binding to the Skp2 complex is indeed functionally relevant to the inhibition of p27 ubiquitylation we performed an in vitro ubiquitylation assay. Full-length HINT1 inhibited the ubiquitylation of p27 in vitro (Fig. 4E). Whereas the N-terminal half of HINT1 (GST-HINT1A) retained this activity, the C-terminal half of HINT1 (GST-HINT1N) lacked this activity (Fig. 4E). The inhibition by either full-length HINT1 or the N-terminal half of HINT1 was very reproducible, although it was only ~30% revealed by quantitation (data not shown). It is less than the inhibition (~50%) we observed in an in vivo ubiquitylation assay (Fig. 3C) indicating there might be additional factors assisting the function of HINT1 in vivo. This result is consistent with our previous finding that the N-terminal half of HINT1 mediates binding of HINT1 to Ubc3 (Fig. 4A).

Another ubiquitin ligase complex KPC formed by KPC1 and KPC2 also can degrade p27 at the G1 phase. In contrast to the SCFSkp2 complex, which is a nuclear ubiquitin ligase, KPC1 and KPC2 are exclusively cytoplasmic proteins (30). We found that the HINT1 protein resides both in the nucleus and cytoplasm of all the cell lines used in this study (data not shown). Because we did not detect any interactions between HINT1 and KPC1 or...
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Because an elevated level of Src expression and activity in KM12C colon cancer cells caused similar changes in morphology and vimentin expression (38), we examined Src expression in the HINT1 knockdown SW480 cells. Indeed, HINT1 shRNA SW480 cells expressed a higher level of the Src protein than the control shRNA cells. The level of phosphorylated Src (Y418), i.e. activated Src, was also increased, and the level of Src kinase (Csk), a negative regulator of Src kinase activity, was decreased in the HINT1 knockdown cells. The level of phosphorylated ERK2 (p42) was also increased, whereas the total level of ERK2 was not altered. ERK2 is known to be constitutively activated in Src-transformed cells (39). However, there was no change in the expression of focal adhesion kinase or Cbl (Fig. 5B). A duplicate study produced similar results (data not shown). These findings suggested that Src signaling can be inhibited by HINT1 in vivo.

HINT1 Inhibits Src Expression—Our finding that knockdown of HINT1 led to an increased cellular level of the Src protein (Fig. 5B) suggested that HINT1 may be a negative regulator of Src expression. To further explore this, SW480 cells were transiently transfected with a vector control or HINT1 expression construct. Indeed, Src protein expression was inhibited by HINT1. Furthermore, when HINT1 was reintroduced into HINT1 shRNA cells, the elevated level of Src was reduced (Fig. 6A). Similar results were obtained in a duplicate study (data not shown). The inverse association between cellular levels of HINT1 and Src is not confined to transfected cells, because two normal colon epithelial cell lines, CCD-112CoN and CCD-841CoN, that express relatively high levels of HINT1 have very low levels of Src; whereas two colon cancer cell lines, HT29 and SW480, that have relatively low levels of HINT1 have relatively high levels of Src (Fig. 1B). Furthermore, further decreasing the level of HINT1 in SW480 cells with HINT1 shRNA caused a further increase in Src (Fig. 1B, last lane). However, other differences between the normal and colon cancer cell lines may explain the differences in level of Src expression.

Because HINT1 did not affect the transcription of p27 (Fig. 2), we investigated the effect of HINT1 on cellular levels of Src mRNA using real-time PCR assays. Overexpression of HINT1 in SW480 cells led to a ~40% decrease in the level of Src mRNA (Fig. 6B, left panel). HINT1 knockdown SW480 cells displayed a ~3-fold increase in Src mRNA when compared with the control SW480 cells (Fig. 6B, right panel). We also used an Src promoter luciferase reporter pGL3-Src (40) in transient transfection reporter assays to determine the effects of HINT1 on Src transcription. pGL3-Src was co-transfected with vector control, HINT1, Sp1, or Sp3 into the control shRNA SW480 cell line and into the HINT1 shRNA SW480 cell line (Fig. 6C). Sp1 was used because it is known to play an important role in activating the Src promoter (40, 41). This was apparent in the present study, because Sp1 but not Sp3 clearly activated the pGL3-Src reporter in both cell lines (Fig. 6C). These effects were especially striking in the HINT1 shRNA cells and co-transfection with HINT1 markedly inhibited the stimulation of promoter activity by Sp1. The basal reporter activity obtained in the absence of Sp1 and exogenous HINT1 (p < 0.05) was also greater in the HINT1 shRNA cells (Fig. 6C).

In view of the above results we then examined if HINT1 interacts with Sp1 using pulldown assays with GST fusion proteins. Lysates of 293T cells were incubated with GST, GST-
FIGURE 6. **HINT1 is a negative regulator of Src expression.** A, HINT1 inhibits Src protein expression. Extracts from SW480 cells transiently transfected with the empty vector (lane 1) or transiently transfected with the 3×FLAG-HINT1 plasmid (lane 2) or stably transfected with a control shRNA construct (lane 3) or stably transfected with a HINT1 shRNA construct (lane 4) or the stable cell line from lane 4 transiently transfected with the 3×FLAG-HINT1-X plasmid as described under "Experimental Procedures" (lane 5) were examined by Western blot analysis with an anti-Src antibody, an anti-β-actin antibody, and an anti-HINT1 antibody. B, HINT1 inhibits expression of Src mRNA. The same experiment was conducted as described in Fig. 2 except that Src mRNA expression was determined by real-time PCR as described under "Experimental Procedures." *, significant differences with respect to HINT1 and Src comparing the control and HINT1 overexpression cells, and the control shRNA versus the HINT1 shRNA cells. C, HINT1 inhibits Sp1-dependent Src promoter activation. An Src promoter luciferase reporter (pGL3-Src) was co-transfected into SW480 cells with HINT1-, Sp1-, and Sp3-expressing plasmids, as indicated. *, significant differences between the control shRNA cells and the HINT1 shRNA cells under basal conditions and after stimulation with Sp1. It also designates that HINT1 caused significant inhibition of the activity stimulated by Sp1 in both cell types. D, HINT1 forms a complex with Sp1 on the Src promoter. GST pulldown assays were conducted as described in Fig. 4A, except that cell lysates from non-transfected 293T cells were incubated with the GST fusion proteins. An anti-Sp1 antibody was used to detect the interaction between HINT1 and endogenous Sp1. ChIP was done with anti-HINT1 and anti-Sp1 antibodies. GC1 and GA2 are reported Sp1 binding sites on Src promoter. The amplicons from GC1 primers and GA2 primers contain GC1 and GA2, respectively.
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**FIGURE 7.** HINT1 up-regulates cellular levels of p27<sup>kip1</sup> by targeting SCFSKP2 ubiquitin ligase and Src. A, SW480 cells were transfected as indicated. 40 h later cells were treated with 10 μM PP1 for 6 h prior to Western blot analysis with indicated antibodies. B, a hypothetical model based on the results obtained in this study in which HINT1 inhibits p27 degradation by targeting the SCFSKP2 complex and also Src. According to this model HINT1 binds to Rbx/Roc1 and thus interferes with its recruitment of CDC34/Ubc3 to the SCFSKP2 complex, thus inhibiting the ubiquitylation of p27. In addition, HINT1 acts at Sp1 sites on the Src promoter to inhibit the transcription of Src, thus decreasing cellular levels of the Src protein.

HINT1, GST-HINT1ΔN, or GST-HINT1ΔC. We found that the endogenous Sp1 protein in these lysates bound to GST-HINT1 and GST-HINT1ΔC but not GST alone or GST-HINT1ΔN (Fig. 6D, left panel). The latter result indicates that the N terminus (residues 1–72), but not the C terminus (residues 66–126), plays a critical role in the binding of HINT1 to Sp1. Further study is required to map the exact Sp1 binding site on HINT1.

The involvement of HINT1 in transcriptional regulation of Src was further investigated in chromatin immunoprecipitation assays. Two Src promoter fragments, both of which contain a Sp1 binding site, were precipitated with the anti-HINT1 and anti-Sp1 antibodies from lysates of 293T cells. No binding was found in control experiments employing no DNA, no antibody, or normal rabbit IgG (Fig. 6D, right panel). An Src promoter fragment that does not contain an Sp1 site was not pulled down by either anti-Sp1 or anti-HINT1 antibody (data not shown). Therefore, it appears that HINT1 plays a direct role in inhibiting Sp1 stimulation of Src transcription by forming a complex with Sp1 on the Src promoter.

A previous study indicated that inhibition of Src can cause increased cellular levels of p27 (31). Therefore, we examined the effects of PP1, which inhibits the kinase activity of Src, on cellular levels of the p27 protein in SW480 cells when used alone or in cells transfected with HINT1 (Fig. 7A). Both PP1 treatment and increased expression of HINT1 increased the level of p27, and the combination had an even greater effect. As previously shown (Fig. 3B), treatment with HINT1 shRNA caused a decrease in the level of p27. It is of interest that combined treatment with this shRNA plus PP1 partially reversed this effect (Fig. 7A).

**DISCUSSION**

This report provides the first evidence that HINT1 is a novel regulator of cellular levels of p27. Our studies suggest that HINT1 achieves this function at the post-transcriptional level through two mechanisms. First, HINT1 inhibits the ubiquitylation of p27 by binding to the SCFSKP2 ubiquitin ligase complex and interfering with recruitment of the E2 enzyme CDC34/Ubc3 by Rbx1/Roc1. Second, Src-induced down-regulation of the stability of p27 is inhibited, because HINT1 inhibits the expression of Src. These effects, shown schematically in Fig. 7, may contribute to the known tumor suppressor activity of HINT1 (4). We should emphasize that the results obtained in our studies are not simply artifacts of overexpression of HINT1 and other proteins, because they were complemented by shRNA knockdown studies, by comparisons of cell lines, and evidence for co-immunoprecipitation of the endogenous proteins HINT1 and Roc1.

p27 is a short-lived protein, and its degradation is controlled by the ubiquitin-proteasome system (24). In the nucleus, CDK2 phosphorylates p27 at threonine 187. The p27 protein is then recognized by the SCFSKP2 ubiquitin ligase and degraded by the proteasome (29). The proto-typical SCF complex contains an F-box protein (SKP2 in the case of p27), Skp1, Cul-1, Rbx1/Roc1, and Cks1 (42–44). HINT1 appears to be a new member of this protein complex. However, unlike other members of this complex, HINT1 inhibits the ubiquitylation of p27, thus increasing its stability (Fig. 3). We obtained evidence that, as a component of the SCFSKP2 complex, HINT1 interferes with the interaction between the ubiquitin E2 enzyme Ubc3 and its recruiter Roc1 (Fig. 4E). This would result in fewer molecules of Ubc3 recruited by Roc1 so that the ubiquitylation level of p27 would drop. Indeed, we found that HINT1 does inhibit the ubiquitylation of p27 (Fig. 3C). Ultimately, HINT1 increases the cellular level of the p27 protein (Fig. 1), which is also reflected in the prolonged half-life of p27 (Fig. 3B). Because HINT1 impairs the interaction of Ubc3 with Roc1 (Fig. 4D), and both the interaction between Ubc3 and Roc1 and the interaction between HINT1 and Roc1 are very strong (both interactions survived a high-stringency wash buffer, data not shown), while the interaction between HINT1 and Ubc3 is relatively weak (this interaction could only survive a low stringency wash buffer, data not shown), it appears that the HINT1–Roc1 interaction and the Ubc3–Roc1 interaction are mutually exclusive. We should, however, emphasize that further studies are required to more precisely determine how HINT1 inhibits the function of the SCFSKP2 complex. Although HINT1 directly binds to Ubc3 (Fig. 4A) we also must point out that the data presented in Fig. 4 (A and B) do not exclude the possibility that HINT1 binds Roc1 via adaptor molecules, for example, Ubc3. Nevertheless our results provide strong evidence that HINT1 is part of the SCF protein complex, which is a novel finding.
studies using purified proteins are required to explore the binding relationships between these proteins. KPC is another ubiquitin ligase that degrades p27 in the cytoplasm at the G1 phase (30). We did not detect any interaction between HINT1 and KPC1 or KPC2 (supplemental Fig. S1B) suggesting that HINT1 specifically acts on the nuclear SCFSKP2 complex but not the KPC complex. An additional potential mechanism of HINT1 regulation of p27 might be its ability to associate with p27 itself and thus prevent proteasomal degradation. This, however, does not appear to be the case, because we could not detect any direct interactions between p27 and HINT1 (data not shown).

HINT1 itself does not demonstrate positive transcriptional activity. However, there is increasing evidence that HINT1 is an important player in the transcriptional regulation of several genes by forming protein complexes with other transcription factors (6, 7, 9, 11, 45). In the current studies, we demonstrated that HINT1 acts as a negative cofactor for the transcription factor Sp1 on the Src promoter, thus inhibiting Src transcription (Fig. 6). Thus we discovered a new binding partner of HINT1. However, we are not certain that HINT1 directly interacts with Sp1, which requires further study. Whether HINT1 plays a role in other Sp1-dependent transcriptional events remains to be discovered. Interestingly, HINT1 does not alter the transcriptional activity of the p27 promoter even though it contains two Sp1 binding sites (Fig. 2). The basis for this selectivity for Sp1 sites is currently unclear. It is of interest to determine how HINT1, a 13-kDa and relatively small protein, binds to several different proteins (see the introduction). Current findings indicate that the C terminus of HINT1, which mediates homodimer formation and nucleotide binding and hydrolysis activity, is not responsible for its interaction with Roc1 (Fig. 4A and supplemental Fig. S1A), Sp1 (Fig. 6D), or Pontin (11). The one or more precise motifs in the N-terminal half of HINT1 that make contacts with these binding partners remain to be identified. Nor have we been able to identify a HINT1 binding consensus sequence among its interaction partners (some of which may be indirect binding partners). More advanced three-dimensional structures may provide answers to these questions.

Src was the first proto-oncogene described and was among the first molecules in which tyrosine kinase activity was documented. Src and Src family kinases act at points of integration, relaying signals from cell surface receptors to the nucleus. As such, Src mediates several different cell fate decisions. Src activation has been associated with proliferation, survival, differentiation, and motility (46–48). Increased Src levels and/or activity have been observed in many human cancers when compared with adjacent normal tissues, including colorectal, breast, prostate, lung, and ovarian carcinomas (49–51), and are often associated with increased disease stage (52). Thus a better understanding of the regulation of Src expression and kinase activity will aid in the design and future evaluation of therapeutic strategies that target Src function. We found that HINT1 is a novel endogenous negative regulator of the expression of Src (Fig. 6). Because Src can decrease the stability of p27 by directly phosphorylating it (31, 32), the ability of HINT1 to inhibit Src expression (Fig. 6) would counteract this effect, thus further contributing to the up-regulation of cellular levels of p27 by HINT1 (Fig. 7). The remarkable ability of HINT1 to up-regulate cellular levels of the tumor suppressor p27 and to also down-regulate Src expression may contribute, at least in part, to its tumor suppressor activity.

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