TIP30 Induces Apoptosis under Oxidative Stress through Stabilization of p53 Messenger RNA in Human Hepatocellular Carcinoma

Jian Zhao, 1,2 Jingjing Chen, 1 Bin Lu, 1 Li Dong, 1,3 Huajing Wang, 1 Chongshan Bi, 1 Guobin Wu, 1,2 Huaizu Guo, 1 Mengchao Wu, 1,3 and Yajun Guo 1,2

1International Joint Cancer Institute and Eastern Hospital of Hepatobiliary Surgery, The Second Military Medical University; 2E-Institute of Universities, Immunology Division, Shanghai Jiao Tong University and Shanghai Center for Cell Engineering and Antibody; 3Shanghai Institute of Applied Physics, China Academy of Sciences, Shanghai, P.R. China; and 4Guangxi Cancer Hospital, Guangxi Medical University, Guangxi, P.R. China

Abstract

Reactive oxygen species (ROS) and cellular oxidant stress have long been associated with cancer. Here, we show that TIP30, also called CC3, regulates p53 mRNA stability and induces apoptosis by sensing of intracellular oxidative stress in human hepatocellular carcinoma (HCC) cells. Introduction of TIP30 induced more cell death in HepG2 cells with a high level of intracellular ROS than that in normal liver cell line, HL7702, which had low level of intracellular ROS. Treatment with an antioxidant agent attenuated TIP30-induced cell death in HepG2 cells, whereas oxidant H2O2 augmented TIP30-induced cell death in HL7702 cells. The conformation of TIP30 was altered with the formation of an intermolecular disulfide bridge under oxidative stress. TIP30 greatly enhanced p53 expression and its transcriptional activity under oxidative stress, which was probably through stabilization of p53 mRNA. TIP30 induced apoptosis and mitochondrial dysfunction were blocked by silencing of p53 expression. The nuclear import of mRNA-binding protein HuR was blocked upon TIP30 introduction, which might be due to the interruption of the association of HuR with importin b2. The elevated cytoplasmic HuR bound to p53 mRNA 3′-untranslated region, resulting in prolonged half-life of p53 mRNA. Our results suggest that TIP30 is involved in cellular oxidative stress surveillance and induces apoptosis through stabilization of p53 mRNA in HCC cells. [Cancer Res 2008;68(11):4133–41]

Introduction

Reactive oxygen species (ROS) and cellular oxidant stress have long been associated with cancer (1). Elevated ROS induced by hepatitis B virus and hepatitis C virus infection, aflatoxin B1 exposure, growth factor stimulation, and activation of oncogenes plays an important role in hepatocarcinogenesis (2). Robust ROS can induce direct damage to many important cellular constituents, such as DNA, lipids, and proteins, which results in activation of many cellular signaling cascades that regulate cell proliferation and apoptosis (2, 3). ROS-induced protein damages include conformational changes of redox-related proteins, such as transcription factors, kinases, and phosphatases, which result in alterations of enzymatic activities or protein-protein interactions (3, 4).

TIP30, also called CC3 or HTIP2, is a putative tumor suppressor. Recently, the decreased expression of TIP30 was observed in hepatocellular carcinoma (HCC) surgical specimens and cell lines (5, 6). Consistent with these observations, genetically engineered mice deficient in Tip30 gene had a high incidence of HCC and other tumors (5). These data suggest that TIP30 plays an important role in the suppression of hepatocarcinogenesis. The suppressive effects of TIP30 on tumor development could partially be ascribed to its proapoptotic property (7–9). A recent crystal structure analysis indicated the potential of TIP30 acting as a metabolic sensor linked to its proapoptotic property (10). The protein structures most closely related to TIP30 are members of the short-chain dehydrogenase reductase family. In addition, a TIP30 homodimer was present in the crystal across a 2-fold axis, which involved a disulfide bridge via Cys175 residues and a PEG 600 molecule shared between two TIP30 monomers. Because cysteine thiol-disulfide exchanges are crucial for sensing intracellular levels of ROS and signal transduction, TIP30 therefore might be oxidized with disulfide bridge formation under oxidative stress and executes its antitumor activities.

Although gene expression is crucially modulated by transcription, the essential contributions of posttranscriptional events are becoming increasingly recognized. Unlike transcription modulation that relied upon new molecular synthesis, mRNA turnover is primarily controlled as an early immediate response to various cellular stimuli through the association of RNA-binding proteins with specific RNA sequences (11, 12). The RNA-binding protein HuR prominently regulates gene expression through binding to mRNAs that contain AU-rich elements (ARE) located in the 3′-untranslated regions (3′-UTR: refs. 13, 14). HuR has emerged as a key regulator of genes that are central to the stress response, cell growth, and proliferation, such as p21, c-fox, c-myc, p53, cyclin A, and cyclin B1 (13, 14). HuR is shown to promote the stability of target transcripts and to enhance target mRNA translation in response to agents causing DNA damage (15, 16). HuR is predominantly nuclear in unstimulated cells and translocates to the cytoplasm in response to various stimuli, which are intimately linked to its effects on target mRNAs (14, 17). Importin β, also known as transportin, was found to participate in the nuclear import of HuR (18, 19). TIP30 was recently found to form complex with multiple karyopherins of the importin β family in a RanGTP-insensitive manner, by which it inhibited nuclear import and induced apoptosis (9). Thus, TIP30 might regulate HuR distribution through interaction with importin β.

Note: Jian Zhao and Jingjing Chen contributed equally to this work.

Requests for reprints: Yajun Guo, International Joint Cancer Institute, Second Military Medical University, 800 Xiang Yin Road, New Building 10th-11th Floor, Shanghai 200433, P.R. China. Phone: 86-21-25070241; Fax: 86-21-25074349; E-mail: yjguo@smmu.edu.cn.

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We have previously found that Tip30 might induce apoptosis through elevation of p53 protein expression, which was not due to stabilization of p53 protein (6). In this study, we show that Tip30 is oxidized with formation of intermolecular disulfide bridge under oxidative stress, resulting in cytoplasmic accumulation of HuR, stabilization of p53 mRNA, and induction of mitochondria-mediated apoptosis.

Materials and Methods

Cell culture and chemicals. Human HCC cell lines HepG2 and Hep3B; three established HCC cell lines CH-Hep-1, CH-Hep-3, CH-Hep-4 (20); and a normal liver cell HL7702, were maintained in DMEM containing 10% fetal bovine serum, under an atmosphere of 5% CO₂ at 37°C. N-αcetyl-l-cysteine (NAC) and actinomycin D were purchased from Sigma-Aldrich.

Transfection of small interfering RNA. p53 short hairpin (shRNA; V2HS_93615) inserted in pSM2 retrovector virus was purchased from Open Biosystems. p53 shRNA and control shRNA (not matching any known human gene) were transfected into HepG2 cells and screened under 3 μg/mL puromycin (Merck). The stably transfected cell clones were obtained by limiting dilution and confirmed by Western blot analysis using anti-p53 antibody (DO-1, Santa Cruz Biotechnology). HuR short interfering RNA (siRNA) AACAGCGUAGAGCGCUUGAG (16), Tip30 siRNA CCGGCGA-GAG/GGCUCCUAA (21), and control siRNA AAGUAGCGUCCUUGA were transfected with Lipofectamine 2000 (Invitrogen).

Plasmid construction. The NH₂-terminal His-tagged Tip30 cDNA was obtained by PCR amplification from pcDNAs-Tip30 construct and subcloned into pcDNA3 (Invitrogen) in BamHI-XhoI sites. The NH₂-terminal His-tagged importin β2 cDNA was obtained by PCR amplification from the cDNA pool of normal liver cell line HL7702 and subcloned into pcDNA3 in KpnI-XhoI sites. An overlap extension PCR method was used to introduce site-specific substitutions within the Tip30 open reading frame. The Tip30ΔI78A, F188A, G190A (Tip30M6) cDNA was subcloned into pcDNA3 in BamHI sites. The NH₂-terminal His-tagged Tip30ΔI78A, ΔS114, Tip30ΔC123A, and Tip30ΔT172A cDNA was subcloned into pcDNA3 in BamHI-XhoI sites. Construction of Ad-GFP and Ad-TIP30 adenovirus was carried out as described previously (6).

Intracellular ROS assay. ROS level was analyzed by flow cytometry using an H₂O₂-sensitive fluorescent dye, dichlorodihydrofluorescein diacetate (DCF-DA, Sigma-Aldrich), as described previously (22).

RNA extraction and real-time PCR. Total RNA was isolated from cells using an RNeasy Mini Kit (Qiagen). Genomic DNA was removed from total RNA before cDNA synthesis using the RNase-Free DNase Set for DNase cocktail (Sigma-Aldrich; ref. 26). After centrifugation at 14,000 rpm for 20 min, either rabbit polyclonal anti-His antibody or IgG (1.5 mg antibody/μg extract) and protein A-agarose (Invitrogen) were added to the supernatant cytoplasmic lysates and incubated at 4°C overnight. The beads were washed thrice in the same lysis buffer and the proteins remaining on beads were analyzed by immunoblotting with relevant antibodies.

Microscopy and fluorescence resonance energy transfer analysis. The Tip30 cDNA was subcloned into pECFP-N1 and pEYFP-C1 (Clontech Laboratories) in EcoRI-BamHI sites, making the Tip30-CFP and YFP-TIP30 fusion construct, respectively. HepG2 cells were cotransfected with pECFP-N1-Tip30 and pEYFP-C1-Tip30 and imaged on a Zeiss LSM 510 confocal laser-scanning microscope (Carl Zeiss) as described previously (27, 28).

In vitro transcription. Total RNA from normal liver cell HL7702 was reverse transcribed, and the cDNAs generated were used as templates for PCR amplification of the coding region and 3′-UTR of p53 mRNA as described previously (16). The 5′ primers contained the T7 RNA polymerase promoter sequence (TTT): CCAAGCTTCTAATACGACTCACTATAGGGAGA. To prepare the coding region (encompassing positions 252 to 1439), oligonucleotides (TTT) ATGGAGAGGCCGCTAGCTCATTAGC were used. To prepare the 3′-UTR template (encompassing positions 1421 to 2629), oligonucleotides (TTT) TGACTCAGACTGACATTCTCC and TGGCAGCAAAATTTTATGTTAATAAGAGATC were used. PCR-amplified products were resolved on agarose gels, purified, and used as templates to transcribe biotinylated RNA in vitro by using T7 RNA polymerase (Invitrogen) in the presence of biotin-cytidine 5′-triphosphate, and purified.

RNA-protein binding assays. Biotin pull-down assays were performed as described previously (16). Six micrograms of biotinylated transcripts were incubated with 120 μg of cytoplasmic lysate for 30 min at room temperature. Complexes were isolated with streptavidin agarose (Invitrogen), and pull-down material was analyzed by Western blotting with anti-HuR and anti-actin antibodies.

Immunoprecipitation of endogenous HuR-mRNA complexes, used to assess the association of endogenous HuR with endogenous p53 and p21 mRNA, was performed as described previously (16, 25). About 120 μg of mRNA-protein complex lysate were used for immunoprecipitation in the presence of excess (30 μg) immunoprecipitated antibody [either mouse monoclonal anti-HuR antibody 3A2 or IgG (1) for 4 h at room temperature. RNA in immunoprecipitated material was used in RT-PCR reactions to detect the presence of p53 and p21 mRNA.

Coimmunoprecipitation assay. Cell extracts were prepared by sonicating in 25 mmol/L Tris C1 (pH 7.4), 100 mmol/L NaCl, 0.15% NP40, 0.25 mmol/L EDTA, and 10% glycerol, supplemented with protease inhibitor cocktail (Sigma-Aldrich; ref. 26). After centrifugation at 14,000 rpm for 20 min, either rabbit polyclonal anti-His antibody or IgG (1.5 mg antibody/μg extract) and protein A-agarose (Invitrogen) were added to the supernatant cytoplasmic lysates and incubated at 4°C overnight. The beads were washed thrice in the same lysis buffer and the proteins remaining on beads were analyzed by immunoblotting with relevant antibodies.
CFP channel, acceptor YFP channel, and FRET channel under identical conditions. The image obtained with the FRET channel was evaluated using Carl Zeiss AxioVision FRET software (Carl Zeiss) and FRET was calculated using the acceptor ratio correction method (27).

**Detection and quantitation of apoptosis.** Apoptosis was examined by assessing nuclear changes indicative of apoptosis using the DNA-binding dye Hoechst 33342 (Sigma-Aldrich) under an OLYMPUS IX71 fluorescence microscope (OLYMPUS). Apoptotic nuclei were identified by condensed chromatin, contiguous to the nuclear membrane, and nuclear fragmentation of condensed chromatin.

**Measurement of mitochondrial membrane potential.** Cells were incubated with tetramethylrhodamine ethylester (TMRE) by MitoShift Kit (Trevigen) and observed under fluorescence microscope.

**Statistical analysis.** Statistical analysis was performed using the Analysis ToolPack provided by Microsoft Excel. A Student’s two-sample t test, assuming unequal variances, was used to determine the equality of the means of two samples. Results were considered statistically significant at $P < 0.05$.

**Results**

**TIP30 induces cell death under oxidative stress.** The intracellular ROS level was much higher in five HCC cells tested than that in normal liver cell HL7702 (Fig. 1A). Infection of Ad-TIP30 caused significant increase of cell death in HepG2 cells, which had high intracellular ROS level compared with Ad-GFP infection (Fig. 1B). On the other hand, no obvious difference was found in HL7702 cells that had low intracellular ROS level (Fig. 1C).

Treatment with the antioxidant NAC greatly suppressed TIP30-induced cell death in HepG2 cells (Fig. 1B). In contrast, TIP30-induced cell death significantly increased in HL7702 cells with the addition of $\text{H}_2\text{O}_2$ (Fig. 1C). These data suggest that TIP30-induced cell death might be triggered by oxidative stress.

**TIP30 is oxidized under oxidative stress.** A recent crystal structure analysis indicated the potential of TIP30 acting as a metabolic sensor linked to its proapoptotic property (10). We then investigated the redox forms of the TIP30 in normal liver cell HL7702 using the cysteine-trapping method (29, 30). Inspection of the Western blotting under nonreducing conditions revealed a faint anti-TIP30 stained higher molecular weight band at various low doses of $\text{H}_2\text{O}_2$, whose presence correlated with TIP30 oxidation (Fig. 2A). The absence of this band under reducing conditions indicated a probable intermolecular disulfide linkage of TIP30 and high molecular weight proteins (Fig. 2A). We further analyzed the redox forms of His-tagged TIP30 in HepG2 cells, in which TIP30 was almost undetectable (6), by immunoblotting with an anti-His antibody. Again, a distinct slower mobility band was found after exposure to various concentrations of $\text{H}_2\text{O}_2$ (Fig. 2B).

To further investigate which cysteine(s) take part in the formation of intermolecular disulfide linkage(s) in response to ROS, three TIP30 mutants were constructed as shown in Fig. 2C. The mutant containing Cys$^{172}$ substituted into alanine lost its ability to form oxidized TIP30 band. The data indicates the formation of a TIP30 mixed-disulfide, involving Cys$^{172}$, under oxidative stress.

Figure 1. TIP30 induces cell death under oxidative stress. A, HCC cells and normal liver cell HL7702 were incubated with 10 $\mu$mol/L DCF-DA for 20 min at 37°C. Fluorescence was measured using flow cytometry (excitation at 488 nm, emission at 515–545 nm) and the mean fluorescence intensity was used to quantify the response. Columns, mean from three independent experiments; bars, SD. *, $P < 0.05$. HepG2 cells (B) or HL7702 cells (C; $2 \times 10^5$ per well in 24-well plates) were infected with Ad-TIP30 and Ad-GFP as the control at a multiplicity of infection (MOI) of 10 in the presence or absence of 75 $\mu$mol/L NAC (B) or 100 $\mu$mol/L $\text{H}_2\text{O}_2$ (C), in triplicate. The cell death was evaluated by trypan blue staining at the indicated time postinfection. Points, mean from three independent experiments; bars, SD. Expression of TIP30 was confirmed by Western blotting with anti-TIP30 antibody.
To investigate whether TIP30 forms homodimer under oxidative stress in living cells, we conducted FRET assay (27, 28). The cells coexpressing TIP30-CFP and YFP-TIP30 were selected by double-colored fluorescence (Fig. 2D). Upon H\textsubscript{2}O\textsubscript{2} exposure, the enhanced fluorescence from cytoplasm was clearly detected in the FRET channel after correction for bleed-through of CFP and YFP. Evaluation of FRET using acceptor ratio analysis depicted increased FRET efficiency (62.9%), indicating a direct interaction between two TIP30 monomers under oxidative stress. The interaction between TIP30-CFP and YFP-TIP30 was disrupted within 30 minutes upon antioxidant NAC treatment. Together, these data show the formation of intermolecular disulfide linkage and molecular conformation alteration of TIP30 under oxidative stress.

**TIP30 enhances p53 expression and its transactivation activity under oxidative stress.** Introduction of TIP30 resulted in 23.9% increases of p53 mRNA compared with mock treatment in HepG2 cells harboring wild-type p53 gene, which was augmented by the addition of H\textsubscript{2}O\textsubscript{2} and attenuated by the addition of NAC (Fig. 3A). Western blotting confirmed the regulatory effects of TIP30 on p53 (Fig. 3A). Moreover, treatment with H\textsubscript{2}O\textsubscript{2} alone caused only slight increase of p53 mRNA in TIP30-deficient HepG2 cells, but a significant increase was found in TIP30 wild-type HL7702 cells (Fig. 3A and B). Depletion of TIP30 greatly attenuated H\textsubscript{2}O\textsubscript{2}-induced p53 up-regulation in HL7702 cells (Fig. 3B). Consistent with the expression of p53, the transactivation activity of p53, as detected by p21, puma, mdm2, and bax mRNA expression, was enhanced upon ectopic expression of TIP30 in HepG2 cells, which was significantly augmented by oxidant H\textsubscript{2}O\textsubscript{2} and blocked by antioxidant NAC (Fig. 3C). The data suggest that oxidative stress promotes TIP30-induced p53 up-regulation, and TIP30 is required for p53 elevation under oxidative stress.

**TIP30 induces a p53-dependent mitochondria-mediated apoptosis in HepG2 cells.** To investigate whether TIP30-induced cell death was p53 dependent in HepG2 cells, we generated a HepG2 cell line stably expressing p53 shRNA. Depletion of p53 greatly attenuated TIP30-induced apoptosis (Fig. 4A). Moreover, introduction of TIP30 resulted in a significant loss of mitochondrial membrane potential in HepG2 cells, but not in Hep3B cells harboring p53 gene null (ref. 6; Fig. 4B). Depletion of p53 blocked TIP30-induced mitochondrial membrane potential decrease in HepG2 cells (Fig. 4B).

Bax is a direct transcription target of p53 and translocates to mitochondria when receiving death signals (31, 32). The mitochondrial fraction of Bax was significantly enhanced in HepG2 cells but not in Hep3B cells by TIP30 transduction (Fig. 4C). Consistent with this, release of cytochrome c and Smac from the mitochondria to the cytosol and the activation of procaspase-9 were detected in HepG2 cells but not in Hep3B cells (Fig. 4C). Moreover, TIP30-induced mitochondria accumulation of Bax and redistribution of...
cytochrome c and Smac were abolished by p53 depletion (Fig. 4D). These data show that TIP30 induces a p53-dependent mitochondria-mediated apoptosis.

**TIP30 up-regulates p53 expression through stabilization of p53 mRNA.** We have previously shown that elevation of p53 by TIP30 was not due to stabilization of the p53 protein (6). The transcriptional activities of p53 promoter did not change following TIP30 transduction in HepG2 and Chang liver cells (data not shown). Instead, assays were carried out to measure the stability of p53 mRNA by monitoring the rate of p53 mRNA clearance in HepG2 cells where transcription was halted by actinomycin D. Introduction of TIP30 significantly enhanced the stability of p53 mRNA from half-life of 6 to 7 hours in untreated or Ad-GFP–treated populations to over 10 hours in Ad-TIP30–treated cells (Fig. 5A). Addition of H2O2 significantly enhanced TIP30-induced stabilization of p53 mRNA, whereas NAC treatment reduced the stabilization (Fig. 5A). Thus, TIP30 might enhance p53 expression through stabilization of p53 mRNA under oxidative stress.

Use of biotinylated p53 transcripts in RNA pull-down assays revealed an association of the p53 mRNA 3’¶-UTR with HuR in lysates prepared from Ad-GFP–infected cells (Fig. 5B). The association significantly increased when using lysates prepared from Ad-TIP30–infected cells (Fig. 5B). In contrast, no such complexes were found when using biotinylated p53 coding region transcript (devoid of AU-rich regions). The *in vivo* association of endogenous p53 mRNA and HuR in HepG2 cells was analyzed through immunoprecipitation of HuR under the conditions that preserved its association with target mRNAs (25). RT-PCR analysis revealed the presence of endogenous p53 mRNA in the material immunoprecipitated with anti-HuR antibodies, but not with nonspecific antibodies (IgG1; Fig. 5C). The p53 mRNA was more abundant in Ad-TIP30–infected cell lysates (Fig. 5C). The association of HuR with p21 mRNA, which also contains ARE in its 3’¶-UTR, was also enhanced upon introduction of TIP30 (Fig. 5C). These findings show that TIP30 promotes the interaction of HuR with p53 and p21 mRNA 3’¶-UTR.

We then used a siRNA targeting the *HuR* mRNA, which caused >50% decreased expression of *HuR* mRNA (Fig. 5D), to examine the role of HuR in regulation of p53 mRNA expression following TIP30 transduction. TIP30-induced p53 up-regulation and cell death was greatly attenuated by HuR depletion in HepG2 cells (Fig. 5D). These findings reveal that HuR contributes to the increased stability of p53 mRNA and cell death upon TIP30 introduction.

Figure 3. TIP30 elevates p53 expression under oxidative stress. A, HepG2 cells with p53 wild-type were infected with Ad-TIP30 or Ad-GFP at MOI 10, or untreated as control, in the presence or absence of oxidant H2O2 (100 µmol/L) or antioxidant NAC (75 µmol/L). The cells were collected 12 h after treatments and the relative p53 mRNA expression was analyzed with quantitative RT-PCR. p53 protein expression was analyzed by Western blotting with anti-p53 monoclonal antibody (DO-1). B, normal liver cell HL7702 was transfected with siTip30 or siNon at 200 nmol/L, or untreated (UT) as control; 24 h later, the cells were treated with 100 µmol/L H2O2. The cells were collected 24 h after oxidant supplement and the relative p53 mRNA expression was analyzed with quantitative RT-PCR. p53 protein expression was analyzed by Western blotting with anti-p53 antibody. C, the relative p21, bax, puma, and mdm2 mRNA expression was analyzed with quantitative RT-PCR in HepG2 cells treated as in A. Columns, mean from three independent experiments; bars, SD. *, P < 0.05.
Figure 4. TIP30 induces a p53-dependent mitochondria-mediated apoptosis. A, HepG2 cells stably transfected with shp53 or shNon were infected with Ad-TIP30 or Ad-GFP at MOI 10. Apoptosis was determined with Hoechst staining. Photographs are representative images from each group as indicated under a fluorescence microscope. Columns, average number of Hoechst staining cells from five microscopic views. The expression of p53 was analyzed by Western blotting with anti-p53 antibody. B, HepG2 and Hep3B cells, as well as shRNA stably transfected HepG2 cells were infected with Ad-TIP30 or Ad-GFP at MOI 10. Mitochondrial potential was detected by a MitoShift kit 12 h after infection. In healthy cells, the TMRE dye accumulates in the mitochondria and appears bright red by fluorescence microscopy; however, in apoptotic cells, the mitochondrial membrane potential collapses and the TMRE dye cannot accumulate within the mitochondria. Adenovirus-infected cells (green, left) with the corresponding TMRE staining profile (red, middle) and phase-contrast images (right) were observed under a fluorescence microscope. Arrows, the corresponding TMRE staining of adenovirus-infected cells. C, cytosolic and mitochondrial subcellular fractions were isolated from HepG2 and Hep3B cells infected with Ad-TIP30 at various times after infection, and then subjected to SDS-PAGE and immunoblotted with the indicated antibodies. D, shRNA stably transfected HepG2 cells were infected with Ad-TIP30 or Ad-GFP, or untreated. The mitochondrial fractions were extracted 24 h postinfection and subjected to Western blotting with the indicated antibodies.
TIP30 induces cytoplasmic accumulation of HuR through interaction with importin β2 under oxidative stress. To further investigate the mechanism by which TIP30 promotes the association of HuR with p53 mRNA 3’-UTR, the expression of HuR was examined. Whole-cell HuR levels did not change with ectopic TIP30 expression in HepG2 cells. In contrast, its cytoplasmic presence significantly increased upon TIP30 introduction (Fig. 6A). This cytoplasmic accumulation of HuR was enhanced by addition of H2O2 and blocked by NAC treatment (Fig. 6B).

To investigate whether TIP30 competes with HuR for forming complex with importin β2, we constructed a TIP30 mutant (TIP30F187A, F188A, G189A), named TIP30M6, in which the amino acids (residues 95-98, 141-142, and 187-189 of TIP30) predicted to interact with importin β2 were substituted into alanine (10). As shown in Fig. 6C, TIP30M6 lost the capability to induce cytoplasmic HuR accumulation. Coimmunoprecipitation analysis showed that HuR interacted with His-importin β2 by immunoprecipitation with anti-His antibody in the cytoplasmic extracts of HepG2 cells. When TIP30 was introduced, accompanying with the interaction of TIP30 with importin β2, the interaction of importin β2 with HuR was decreased (Fig. 6D). On the other hand, TIP30M6, which was unable to form complex with importin β2, did not block the interaction of importin β2 with HuR (Fig. 6D). Moreover, treatment with NAC blocked the interaction of TIP30 with importin β2 (Fig. 6D). Thus, TIP30 competes with HuR for the formation of complex with importin β2 and causes the cytoplasmic accumulation of HuR under oxidative stress.

**Discussion**

We have previously reported that introduction of TIP30 into TIP30-decreased HCC cells inhibited cell growth and sensitized HCC cells to chemotherapy drugs (6). In this study, we show that TIP30-induced cell death is triggered by oxidative stress. TIP30-induced cell death was more effective in ROS-increased HCC cells than that in ROS-low normal liver cells. Treatment with antioxidant NAC attenuated TIP30-induced cell death in HCC cells. Thus, TIP30 may act as a ROS sensor and plays an important role for elimination of ROS-increased cells. Given that most cancer cells produce more ROS than do normal cells (33, 34), the selective

**Figure 5.** TIP30 enhances binding of HuR with p53 transcripts and p53 mRNA stability. A, HepG2 cells were either untreated or treated with Ad-TIP30 or Ad-GFP at MOI 10. Alternatively, HepG2 cells were infected with Ad-TIP30 in the presence or absence of H2O2 (100 μmol/L) or NAC (75 μmol/L). p53 mRNA stability was calculated 24 h later. RNA was prepared at the time indicated after actinomycin D (2 μg/mL) treatment and subjected to quantitative RT-PCR analysis. Points, mean of three independent experiments; bars, SD. B, cytoplasmic lysates prepared from HepG2 cells infected with either Ad-TIP30 or Ad-GFP were incubated with biotinylated p53 mRNA 3’-UTR or CR for 30 min at 25 °C. The resulting complexes were pulled down using streptavidin-coated beads and immunoblotted with anti-HuR antibody. β-Actin, a protein lacking RNA-binding activity, served to verify the specificity of this assay. C, lysates from HepG2 cells infected with either Ad-TIP30 or Ad-GFP were used for immunoprecipitation in the presence of anti-HuR antibody or nonspecific IgG1. RNA in the immunoprecipitated material was used in RT-PCR reactions to detect the presence of p53 and p21 mRNA and the resulting PCR products were visualized in agarose gels. D, HepG2 cells were transfected with siHuR or siNon; 24 h later, the cells were infected with Ad-TIP30 or Ad-GFP at MOI 10. At 16 h postinfection, the expressions of p53 and HuR mRNA were analyzed by quantitative RT-PCR and cell death was evaluated by trypan blue staining.
killing of ROS-increased HCC cells by TIP30 highlights its potential in the treatment of cancer.

The mechanism for activation of TIP30 under oxidative stress is probably through conformation change of TIP30 by the formation of intermolecular disulfide linkage. A higher molecular weight band stained by anti-TIP30 antibody was found in both endogenous TIP30 protein and ectopically expressed TIP30 under oxidative stress in the nonreduced condition. A directed intermolecular interaction between two TIP30 proteins was confirmed by the FRET assay. The formation of intermolecular disulfide linkage of TIP30 under oxidative stress might be crucial for sensing the cellular redox state and contribute to its proapoptotic effects.

The p53 tumor suppressor regulates cellular homeostasis through induction of cell cycle arrest, apoptosis, and cellular senescence in response to various types of stress. A previous study showed that p53 was activated under oxidative stress, inducing either antioxidant genes or pro-oxidant gene expression depending on the severity of stresses (22). The precise mechanism by which p53 is activated by oxidative stress is unknown. Oxidative stress could cause p53 protein oxidation directly with a conformation modulation (35), or p53 protein deacetylation by interaction with the human orthologue of yeast Sir2, which is a NAD⁺-dependent histone deacetylase (36). The relatively low frequency of p53 gene mutation in HCC (~30%) indicates that defects in the p53 pathway also play important roles in hepatocarcinogenesis.

Here, we show evidences that TIP30 may be another regulator involved in p53 pathway. First, introduction of TIP30 into Tip30-deficient HepG2 cells promoted endogenous p53 expression and enhanced transactivation activities of p53 on its target genes. This effect was enhanced by oxidant and attenuated by antioxidant. Second, knockdown of Tip30 by siRNA blocked H₂O₂-induced p53 up-regulation. Finally, TIP30-induced mitochondria damage was p53 dependent.

Recently, evidence has emerged revealing that p53 mRNA stability and translation are also strong regulators of p53 expression (16, 37–39). Here, we show that TIP30 enhances p53 expression through stabilization of p53 mRNA under oxidative stress. Given the existence of AREs located in the 3' UTR of p53 mRNA and affinity of RNA binding protein HuR for the p53 mRNA (16), we found that TIP30 induced cytoplasmic accumulation of HuR and enhanced the interaction of HuR with p53 3'-UTR. TIP30 was previously found to form a complex with karyopherins of the importin β family, by which TIP30 inhibited nuclear transport and induced apoptosis (9). Interestingly, the members of importin family were identified as mediators for HuR nuclear import (18, 19). Structure docking study and coimmunoprecipitation analysis presented that TIP30 was located toward the COOH terminus of importin β2, acting as an antagonist for cargo proteins binding to importin β (9, 10). In the present study, we showed evidences that TIP30 competed with HuR to interact with importin β2 under oxidative stress and caused cytoplasmic accumulation of HuR.
Besides p53 mRNA, HuR was found to interact with another ARE-containing mRNA, p21 mRNA, and elevate its expression upon TIP30 induction. However, no obvious differences were found in the binding of HuR with cyclin A, cyclin B1, c-myc, and c-fos mRNA after TIP30 induction (data not shown). Recent studies have suggested that ARE-binding proteins may be modulated to recognize only a subset of AREs in given physiologic conditions or in a given cell type. H2O2 treatment decreased HuR binding to several target mRNAs (those encoding p53, p21, and ProTa mRNAs (15, 16, 41) but decreased binding to the cyclin D1 mRNA (42). The selectivity of HuR binding with target mRNAs after TIP30 induction still needs to be investigated.

In summary, we show that TIP30 plays an essential role in sensing increases of ROS and translating ROS signals into p53-dependent apoptosis pathway. The proapoptosis properties of TIP30 under oxidative stress might be critical for eliminating ROS-increased cells, avoiding the further DNA damage and the risk of transformation and cancer development.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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