

New Molecular Bridge between RelA/p65 and NF- κ B Target Genes via Histone Acetyltransferase TIP60 Cofactor^{*S}

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Background: TIP60 is involved in transcriptional regulation acting as a coactivator or corepressor. Also, TIP60 acetylates histones to modulate chromatin remodeling.

Results: TIP60 enhances transcriptional activity of RelA/p65, which is the active subunit of NF- κ B through their physical interaction.

Conclusion: TIP60 is critical cofactor of RelA/p65 for transcription of NF- κ B target genes.

Significance: TIP60 is involved in the NF- κ B pathway in hepatocarcinoma cells.

The nuclear factor- κ B (NF- κ B) family is involved in the expressions of numerous genes, in development, apoptosis, inflammatory responses, and oncogenesis. In this study we identified four NF- κ B target genes that are modulated by TIP60. We also found that TIP60 interacts with the NF- κ B RelA/p65 subunit and increases its transcriptional activity through protein-protein interaction. Although TIP60 binds with RelA/p65 using its histone acetyltransferase domain, TIP60 does not directly acetylate RelA/p65. However, TIP60 maintained acetylated Lys-310 RelA/p65 levels in the TNF- α -dependent NF- κ B signaling pathway. In chromatin immunoprecipitation assay, TIP60 was primarily recruited to the *IL-6*, *IL-8*, *C-IAP1*, and *XIAP* promoters in TNF- α stimulation followed by acetylation of histones H3 and H4. Chromatin remodeling by TIP60 involved the sequential recruitment of acetyl-Lys-310 RelA/p65 to its target gene promoters. Furthermore, we showed that up-regulated TIP60 expression was correlated with acetyl-Lys-310 RelA/p65 expressions in hepatocarcinoma tissues. Taken together these results suggest that TIP60 is involved in the NF- κ B pathway through protein interaction with RelA/p65 and that it modulates the transcriptional activity of RelA/p65 in NF- κ B-dependent gene expression.

The inducible transcription factor (TF)² family nuclear factor κ B (NF- κ B) consists of dimeric complex proteins involved in diverse processes such as controlling cell proliferation, apoptosis, differentiation, and inflammation (1–3). In mammals, there exist five family members, RelA/p65, c-Rel, RelB, p105/p50 (NF- κ B1), and p100/p52 (NF- κ B2), that homo- or het-

erodimerize to form transcriptionally active or repressive complexes (4, 5). NF- κ B is expressed ubiquitously, and the primary NF- κ B member responsible for transcriptional activation of target genes is the subunit RelA/p65 (6). In unstimulated cells, most NF- κ B complexes are sequestered in their inactive forms by interactions with inhibitors of κ B (I κ B) proteins in the cytoplasm (3, 7). After stimulation, I κ B is phosphorylated by the I κ B kinase (IKK) complex and rapidly undergoes ubiquitination and proteasome-dependent degradation (8–10). The NF- κ B subunits are then released and accumulate in the nucleus to regulate target gene transcription (11, 12).

After nuclear translocation, NF- κ B complexes utilize various strategies to achieve proper TF binding when recognition sites are buried in chromatin. Previous researchers proposed that TFs can bind to nucleosomal DNA in a cooperative manner (13, 14). This has been confirmed by *in vivo* studies showing that transcriptional activator Pho4 can bind to the *PHO5* promoter before nucleosome disassembly (15). In other cases, chromatin remodeling complexes can further stimulate binding of TFs to the nucleosomal sites (16). In a large scale screening of the human genome, high levels of histone H3K4/79 methylation and H3 acetylation were found to be prerequisites for binding of the transcription factor Myc, which implies that chromatin modifications can actually regulate TF binding (17).

Transcriptional activation of NF- κ B involves the association of NF- κ B with various cofactors including histone acetyltransferase (HAT) p300/CBP and the nuclear receptor coactivators SRC3/Rac3 and SRC1/N-CoA1 (18–21). These cofactors are thought to promote the rapid formation of preinitiation and reinitiation complexes by bridging sequence-specific activators to the basal transcription machinery, thereby facilitating multiple rounds of transcription (20, 21). Enhancement of NF- κ B transcriptional activity requires the factor acetyltransferase activity of p300/CBP and P300/CBP-associated factor (PCAF). How these various cofactors are recruited to the promoter regions of NF- κ B target genes and when they associate with NF- κ B transcription factors are not very clear.

TIP60 (HIV Tat-interacting protein, 60 kDa) was identified as a binding partner for the HIV-1 Tat protein, which increases Tat transactivation of the HIV-1 promoter (25). The MYST (MOZ, Ybf2/Sas3, SAS2, and TIP60) domain defines TIP60 as

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² The abbreviations used are: TF, transcription factor; HAT, histone acetyltransferase; MEF, mouse embryonal fibroblast; CBP, cAMP response element-binding protein.

part of the MYST family of HAT proteins that are conserved from yeast to human (26). TIP60 functions as a transcriptional coactivator or corepressor depending upon the cellular context or promoter site (27). As a coactivator, TIP60 associates with transcriptional activators such as HIV-1 Tat (25), amyloid- β precursor protein (28), type I nuclear hormone receptors (29, 30), and MyoD (31). The coactivator function in these instances is mediated by histone acetylation within the promoter region, whereas in other cases TIP60 directly acetylates p53 transcription factor and thus modulates transcriptional activity. It helps to distinguish between the cell cycle arrest and apoptotic functions of p53 (32, 33). In contrast, TIP60 has also been implicated in the negative regulation of gene expression by binding to STAT3 (34), CREB (cAMP response element-binding protein) (35), ZEB (zinc finger E box-binding protein) (36), and p73 (37). Although a growing number of TFs have been identified as transcriptional regulatory targets of TIP60, the precise mechanism by which TFs are involved in TIP60-mediated transcriptional regulation and contribute to the various cellular physiologies remain to be elucidated.

In this study we identified four NF- κ B target genes, *IL-6*, *IL-8*, *C-IAP1*, and *XIAP*, whose expressions are regulated by TIP60. Furthermore, we found that TIP60 is a novel coactivator of NF- κ B RelA/p65 and enhances RelA/p65 transcriptional activity through a protein-protein interaction. We also present the sequential association profiles of TIP60 and RelA/p65 on the NF- κ B target gene promoters with the goal of determining the critical association time point in the TNF- α -induced NF- κ B signaling pathway. Although it is generally accepted that TFs primarily bind to specific promoters and trigger a recruitment cascade of coactivator complexes, interestingly, we found that TIP60 appeared and bound earlier to the NF- κ B target promoters than RelA/p65, and it simultaneously promoted the acetylation of histones. Thus, site-specific early association of TIP60 could serve as the platform for transcription factor RelA/p65 binding sites to promote NF- κ B-mediated gene expression.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HEK 293 and HepG2 cells were obtained from ATCC (American Type Culture Collection) (Manassas, VA). RelA/p65 knock-out mouse embryonal fibroblast (RelA/p65^{-/-} mouse embryonal fibroblast (MEF)) cells were kindly gifted from Professor Jin Won Cho in Yonsei University (Korea), and RelA/p65 wild type MEF (RelA/p65^{+/+} MEF) cells were provided from Professor Sangmyung Rhee in Chung-Ang University (Korea). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen) and penicillin-streptomycin (50 units/ml). Transient transfection was conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Plasmid Construction—The human TIP60 full-length coding region was amplified from hTIP60 cDNA in the human brain library (Clontech) using polymerase chain reaction (PCR) and was introduced to pCRII-TOPO vector (Invitrogen), which was called pCRII-TOPO-TIP60. The TIP60 clone was verified by DNA sequencing. TIP60 full-length was amplified from pCRII-TOPO-TIP60 using a PCR reaction: forward 5'-gaa ttc ATG

GCG GAG GTG GGG-3'; reverse 5'-tct aga TCA CCA CTT CCC CCT-3'. TIP60-chromo, TIP60-Zn, and TIP60-HAT truncated mutants were amplified from TIP60-full-length using a PCR reaction: for TIP60-chromo, forward 5'-gaa ttc ATG GCG GAG GTG GGG-3' and reverse 5'-tct aga GAG GAC AGG CAA TGT-3'; for TIP60-Zn, forward 5'-gaa ttc ACC CCC ACT AAG AAC-3' and reverse 5'-tct aga ATT GTA GTC TTC CGT-3'; for TIP60-HAT, forward 5'-gaa ttc CTA CGA CAT CCT CCA-3' and reverse 5'-tct aga TCA CCA CTT CCC CCT-3'. These PCR products were introduced into the pCRII-TOPO vector (Invitrogen) and subcloned into the pEG-FPC2 vector between EcoRI and XbaI and then verified by DNA sequencing. Oligonucleotide for TIP60 siRNA was introduced into the pBabe-dual vector: forward 5'-aaag AAA CGG AAG GTG GAG GTG-3', reverse 5'-aaaa CAC CTC CAC CTT CCG TTT-3'. FLAG-TIP60 HAT-deficient mutant expression vector was a gift from S. H. Baek in the School of Biological Science at Seoul National University. RelA/p65 full-length cDNA was subcloned into the pFLAG-CMV2 vector (Sigma) and pCDNA3-HA vector. Expression vectors for GST-fused RelA/p65 full-length, RelA/p65 N320, and RelA/p65 C321 were gifts from Professor Cheol O. Joe in KAIST (38).

RNA Preparation and Quantitative Real-time PCR—Total RNA was extracted from HEK 293, HepG2, and MEF cell lines using TRIzol solution (Invitrogen) according to the manufacturer's specifications. Contaminated genomic DNA was removed from 5 μ g of total RNA by incubation with 10 units of RNase-free DNase I (New England Biolabs) and 2 units of RNase inhibitor (New England Biolabs) in DEPC-treated water. The reaction mixture was incubated for 1 h at 37 °C and then for 10 min at 60 °C. RNA concentrations were determined by spectrophotometric analysis. All RNA isolates had an $A_{260}:A_{280}$ between 1.8 and 2.0, indicating that the isolated RNA was suitable for subsequent analyses. Oligo-dT (Intron Biotechnology, Seoul, Korea) was used as the primer in the first step of cDNA synthesis. Total RNA (1 μ g) was combined with 0.5 μ g of oligo-dT, 200 μ M dNTPs, and H₂O and then preheated at 75 °C for 5 min to denature the secondary structures. The mixture was then cooled rapidly to 20 °C, after which 4 μ l of 5 \times reverse-transcriptase buffer, 10 mM DTT, and 200 units of avian myeloblastosis virus reverse transcriptase (Intron Biotechnology, Seoul, Korea) was added to give a total volume of 20 μ l. The reverse-transcriptase mix was incubated at 42 °C for 60 min, after which it was stopped by heating at 95 °C for 5 min. The PCR primers used in this study and their sequences are listed in supplementary information 1 and 2. The primers were designed using the Primer 3 program and cross-checked by a BLAST search of the NCBI data base. The specificity of each of the amplified products generated was confirmed by melting curve analysis. The iQ SYBR Green PCR Supermix (Bio-Rad) and the CFX96 Real-time PCR detection system (Bio-Rad) were used to detect the real-time quantitative PCR products of reverse-transcribed cDNA or chromatin immunoprecipitation (ChIP) samples according to the manufacturer's instructions. The *GAPDH* gene was used for normalization. The relative mRNA expression was calculated by the 2^{-($\Delta\Delta C_t$)} method as previously described (39). PCR was conducted in duplicate for each experimental condition tested.

TIP60 Enhances Transcriptional Activity of RelA/p65

Luciferase Assay—HEK 293 and HepG2 cells were cultured in 60-mm dishes and transfected using Lipofectamine 2000, with the luciferase reporter constructs (0.1 μ g), pCMV- β -galactosidase, RelA/p65, and TIP60 constructs. The cells were lysed in reporter lysis buffer 48 h after transfection (Promega, Madison, WI). Cell extracts were analyzed with the luciferase reporter assay system using a glomax luminometer (Promega). Luciferase activities were normalized based on the β -galactosidase activity of the cotransfected vector. All transfection experiments were repeated independently at least three times.

In Vivo Binding Assay and Western Blotting—HEK 293 and HepG2 cells were seeded in 100-mm plates at an initial density of 2×10^6 cells and allowed to grow for 12 h. The cells were transfected with the respective plasmids, further incubated for 24 h, and lysed in a buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, and 1 mM PMSF. The cell suspensions were incubated on ice for 20 min and centrifuged at 12,000 rpm at 4 °C for 20 min. For immunoprecipitation assays, the supernatants were precleared with 20 μ l of protein A/G-agarose bead (50% slurry) and then incubated at 4 °C overnight with 40 μ l of fresh protein A/G bead in the presence of appropriate antibodies. The beads were washed 3 times in PBS, resuspended in SDS sample buffer, and boiled for 10 min. The protein samples were electrophoresed on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Whatman, PROTRAN). The membrane was blocked with 5% skim milk in a solution of 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween 20 and incubated with appropriate dilutions of the primary antibody at room temperature for 3 h. Samples were analyzed by Western blotting using the appropriate antibodies to detect protein expression. Polyclonal antibodies against TIP60 (sc-5725), RelA/p65 (sc-109), HA-probe (sc-805), and β -tubulin (sc-397) were purchased from Santa Cruz Biotechnology Inc., and Ac-Lys-310 Rel/p65 was purchased from Abcam. Monoclonal antibodies against green fluorescence protein (GFP-1814 460), FLAG-M2 (F3165), and Ac-lysine (sc-32268) antibodies were purchased from Roche Diagnostics, Sigma, and Santa Cruz Biotechnology, respectively.

ChIP—A ChIP assay was conducted following the protocol provided by Millipore (Temecula, CA). Briefly, the cultured cells were cross-linked with 1% paraformaldehyde (#15710, Electron Microscopy Sciences, Hatfield, PA) in PBS for 15 min at 37 °C. The cells were then washed with ice-cold PBS and resuspended in 200 μ l of SDS-sample buffer containing a protease inhibitor mixture. The suspension was sonicated 3 times for 10 s with a 1-min cooling period on ice, after which it was precleared with 20 μ l of protein A/G-agarose beads blocked with sonicated salmon sperm DNA for 30 min at 4 °C. The beads were then removed, after which the chromatin solution of each experimental group was immunoprecipitated overnight with anti-TIP60 (sc-5725, Santa Cruz Biotechnology), RelA/p65 (sc-109), Ac-Lys-310 RelA/p65 (ab19870, Abcam, Cambridge, MA), Ac-K18 histone H3 (1766-1, Epitomics, Burlingame, CA), Ac-K8 histone H4 (1796-1, Epitomics), and polymerase II (2035-1, Epitomics) antibodies at 4 °C followed by incubation with 50 μ l of protein A-agarose beads (Millipore) for an additional hour at 4 °C. The immune complexes were

eluted with 100 μ l of elution buffer (1% SDS and 0.1 M NaHCO₃), and formaldehyde cross-links were reversed by heating at 65 °C for 4 h. Proteinase K (P2308, Sigma) was added to the reaction mixtures and incubated at 45 °C for 1 h. DNA of the immunoprecipitates and control input DNA were purified using the PCR purification kit (Qiagen, Valencia, CA) and then analyzed by quantitative PCR. The PCR primers used in ChIP assay and their sequences are listed in supplemental data 2. PCR was conducted in duplicate for each experimental condition tested.

Immunofluorescence Staining and Confocal Microscopic Detection—HepG2 cells were grown on a sterile coverslip in 60-mm dishes and transfected with indicated expression vectors using Lipofectamine 2000. Twenty-four hours after transfection, cells were fixed with 4% paraformaldehyde and incubated with mouse anti-FLAG M2 antibody (1:1000) (Sigma) followed by Cy5-conjugated goat anti-mouse (ab97035, Abcam). Plates were washed three times in PBS, and confocal imaging was performed with a Carl Zeiss LSM-510 META laser scanning microscope (Oberkochen, Germany).

Immunohistochemistry and Tissue Array—Formalin-fixed paraffin-embedded tissue microarray slides containing hepatocarcinoma and normal liver tissues were purchased from Super Biochips (Seoul, Korea). Briefly, after deparaffinization in xylene and rehydration in grade ethanol, endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 10 min. Tissue sections were then heated in 100 mM citrate buffer, pH 6.0, for 10 min to retrieve antigens and preincubated with normal horse serum for 20 min at room temperature. Anti-TIP60 (sc-5725) and Ac-Lys-310 RelA/p65 antibodies (ab19870) diluted 1:100 was used as the primary antibody, and the specimens were incubated with appropriate antibodies overnight at 4 °C followed by the addition of biotinylated anti-goat and rabbit secondary antibody (Vectastain Laboratory) and streptavidin-horseradish peroxidase (Zymed Laboratories Inc., South San Francisco, CA). 3,3-Diaminobenzidine was used as a chromogen, and Meyer's hematoxylin was used for counterstaining. For negative control purposes, the same procedure was followed except that the primary antibody was replaced by PBS. The level of TIP60 and Ac-Lys-310 RelA/p65 expressions was calculated by the staining intensity using Quantity One software (Bio-Rad). Staining intensity was rated on a scale of 0–3 as follows: 0, negative (no color); 1, weak (weak yellow); 2, moderate (yellow); 3, strong (brown). The intensity score >2 was considered as positive expression.

Cell Proliferation Assay—For the assay of colony-forming efficiency in soft agar, transfected HepG2 cells were counted using a hemocytometer. 5×10^3 cells in 1 ml of growth medium containing 0.35% Noble agar (Difco) and G418 (800 μ g/ml) were incubated in a 60-mm culture dish overlaid on 1.5 ml of 0.5% base agar medium. Cells were incubated at 37 °C in a moist atmosphere of 95% air and 5% CO₂, and 4 weeks later the number of colonies was counted after 0.005% crystal violet staining.

Statistical Analysis—Statistical analysis of variances between two different experimental groups was conducted with Tukey's post hoc comparison test using SPSS (Version K12). All experiments were repeated at least three times. The levels were considered significant at $p < 0.05$ (shown as a *single asterisk*), very

significant at $p < 0.01$ (shown as *double asterisks*), obviously significant at $p < 0.001$ (shown as *triple asterisks*), or not significant (*n.s.*).

RESULTS

TIP60 Regulates the Expression of NF- κ B Target Genes—TIP60 functions critical roles in development, damage responses, and apoptosis, acting as “Herb genes” (27, 40). However, the genes regulated by TIP60 are not well identified. To screen the TIP60-regulated genes, HEK 293 cells were transfected with FLAG-TIP60 plasmid, and mRNA expression was analyzed by semiquantitative RT-PCR. As shown in the supplemental information 3a, *IL-6*, *IL-8c*, *caspase-7*, *Bad*, *Bax*, *Bcl-2*, *C-IAP1*, and *XIAP* mRNA expressions were increased by TIP60. However, *IL-5*, *p38*, *ERK-2*, *ERK-3*, *ERK-5*, *caspase-2*, and *caspase-10* expressions were down-regulated by TIP60 overexpression. Because TIP60 modulates transcriptional activity of transcription factors as a coactivator and corepressor, we tried to identify co-regulatory transcription factors of TIP60 that might bind to TIP60-responsive gene promoters, such as *IL-6*, *IL-8*, *caspase-7*, *Bad*, *Bax*, *Bcl-2*, *C-IAP1*, and *XIAP*, using TFSEARCH software (Searching Transcription Binding Sites Version 1.3). Several transcription factors overlapped as putative binding factors for these promoter regions including RelA/p65, p53, and AP-1. Interestingly, we found that the RelA/p65 binding site was the most frequent and highly conserved motif among the *IL-6*, *IL-8*, *XIAP*, and *C-IAP1* promoters (supplemental information 3b). To examine whether TIP60 regulates RelA/p65-mediated transcriptional activity, HEK 293 cells were transfected with FLAG-TIP60 plasmids together with an NF- κ B-responsive element luciferase reporter gene with treatment with known activators of NF- κ B such as TNF- α , lipopolysaccharide (LPS), and IL-1 β . TNF- α , LPS, and IL-1 β treatment increases transcriptional activity of the NF- κ B reporter gene by activating endogenous RelA/p65 (Fig. 1a, lanes 2, 6, and 10), and TIP60 most significantly enhances transcriptional activity on NF- κ B reporter gene in a dose-dependent manner within TNF- α -treated groups than other signaling molecules (Fig. 1a, lanes 3 and 4). To confirm TIP60-mediated transcriptional enhancement on NF- κ B target genes, HEK 293 cells were transfected with the FLAG-TIP60 plasmid, and the mRNA expression was analyzed by semiquantitative RT-PCR. Overexpression of TIP60 enhances mRNA expression of *IL-6*, *IL-8*, *C-IAP1*, and *XIAP* in TNF- α -treated cells (Fig. 1b, lane 3). To further examine whether the increased expression of *IL-6*, *IL-8*, *C-IAP1*, and *XIAP* transcripts was specifically modulated by TIP60 through RelA/p65 pathway, we used TIP60 small interfering RNA (siRNA) transfectants of RelA/p65^{+/+} MEF or p65^{-/-} MEF cells in which the expression of TIP60 was ~80% abrogated (supplemental information 3c). As shown in Fig. 1c, transfection of TIP60 siRNA significantly decreased the endogenous RelA/p65-mediated transcription of *IL-6*, *IL-8*, *C-IAP1*, and *XIAP* in TNF- α -treated MEF cells. Taken together, TIP60 increases transcriptional activity of NF- κ B RelA/p65 and enhances the expression of RelA/p65 responsive genes, *IL-6*, *IL-8*, *C-IAP1* and *XIAP*.

TIP60 Interacts and Co-localizes with RelA/p65—To examine whether TIP60 could affect RelA/p65-dependent transcrip-

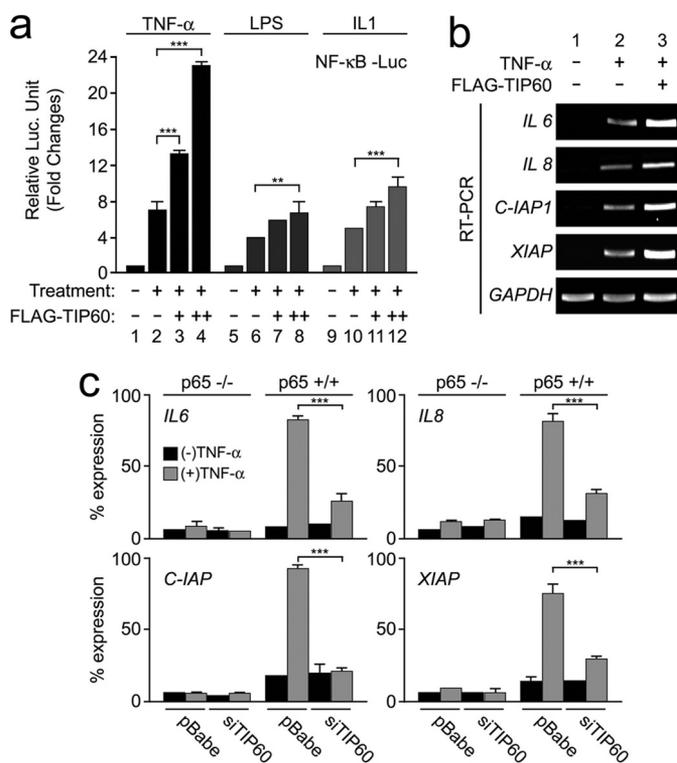


FIGURE 1. Regulation of NF- κ B target gene expressions by TIP60. a, HepG2 cells were co-transfected with expression vector FLAG-TIP60 (0.4 and 0.8 μ g) and luciferase (*Luc*) reporter plasmid (0.1 μ g). Twenty-four hours after transfection, HepG2 cells were treated with 50 ng/ml TNF- α or 500 ng/ml LPS or 10 ng/ml interleukin-1 β (IL1) for 1 h, and then luciferase activity was measured. The data were normalized to β -galactosidase activity. All data are representative of three independent experiments, and statistical significance is represented by Tukey's post hoc test (**, $p < 0.01$; ***, $p < 0.001$). b, HEK 293 cells were transfected with the plasmid expressing FLAG-TIP60 and treated with 50 ng/ml TNF- α for 6 h to activate NF- κ B signaling pathway. RNA was extracted, and semi-quantitative reverse transcriptase PCR analysis was conducted using specific primers for *IL-6*, *IL-8*, *C-IAP1*, and *XIAP* genes. The *GAPDH* gene was used as an internal control. c, RelA/p65^{-/-} or p65^{+/+} MEF cells were transfected with pBabe-TIP60 siRNA plasmid or its control empty vector and treated with 50 ng/ml TNF- α for 6 h to activate NF- κ B pathway. Then RNA was extracted, and quantitative reverse transcriptase PCR analysis was conducted using the primers for *IL-6*, *IL-8*, *C-IAP1*, and *XIAP* genes. The *GAPDH* gene was used as an internal control. The statistical significance is represented by Tukey's post hoc test.

tional activity through protein-protein interactions, we exogenously expressed HA-RelA/p65 along with FLAG-TIP60 with TNF- α treatment in HEK 293 cells. The whole cell lysates were co-immunoprecipitated with anti-FLAG antibodies, and we performed Western blotting using specific anti-HA and FLAG antibodies to determine RelA/p65 and TIP60 expression levels. FLAG-TIP60 co-immunoprecipitated with HA-RelA/p65 (Fig. 2a, lane 2) but did not coprecipitate with control FLAG-empty vector (Fig. 2a, lane 1). Conversely, exogenous HA-RelA/p65 also co-immunoprecipitated with FLAG-TIP60 (Fig. 2b, lane 2) but not with the control HA empty vector (Fig. 2b, lane 1). These results suggest that TIP60 physically interacts with RelA/p65 in HEK 293 cells. To further investigate whether RelA/p65 interacts with TIP60 endogenously *in vivo*, HepG2 cells were treated with 50 ng/ml TNF- α for 60 min; untreated cells served as negative controls. Whole cell lysates were immunoprecipitated with anti-TIP60 antibodies together with their control rabbit sera, and Western blotting was performed using anti-RelA/p65 or anti-TIP60 antibodies. We clearly observed that

TIP60 Enhances Transcriptional Activity of RelA/p65

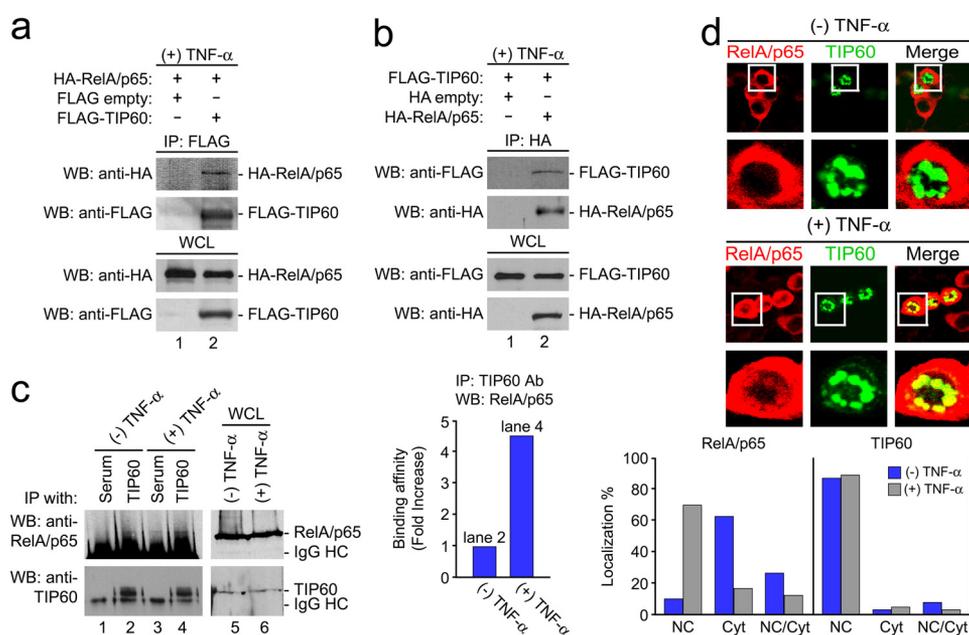


FIGURE 2. Interaction of TIP60 with RelA/p65. *a* and *b*, HEK 293 cells were co-transfected with expression vector for HA-RelA/p65 and FLAG-TIP60 plasmids. 24 h after transfection, whole cell lysates (WCL) were prepared and immunoprecipitated (IP) with anti-FLAG or anti-HA antibodies. Proteins were detected by Western blotting (WB) using anti-HA or anti-FLAG antibodies as indicated. *c*, HepG2 cells were treated with 50 ng/ml TNF- α for 30 min. Whole cell lysates were immunoprecipitated with anti-TIP60 antibodies and were analyzed by Western blotting using antibodies against TIP60 and RelA/p65. *d*, HepG2 cells were transfected with a plasmid expressing GFP-TIP60, and FLAG-RelA/p65 was subjected to immunofluorescence with anti-FLAG antibody. HepG2 cells were treated with 50 ng/ml TNF- α for 30 min. GFP-TIP60 and FLAG-RelA/p65 were immunostained and visualized (green for TIP60 and magenta for RelA/p65). NC, nucleus; Cyt, cytoplasm.

TIP60 endogenously interacts with RelA/p65 in TNF- α -treated HepG2 cells (Fig. 2c, lane 4). These results demonstrate that the physical interaction between TIP60 and RelA/p65 was increased in TNF- α -treated HepG2 cells *in vivo*.

To determine the subcellular localization of the TIP60-RelA/p65 complex in the nucleus, HepG2 cells were co-transfected with GFP-TIP60 and FLAG-RelA/p65 expression plasmids, and the cells were immunostained using anti-FLAG antibodies (Fig. 2d). Inactivated RelA/p65 (magenta) mostly sequestered in the cytoplasm (~60%), whereas TIP60 (green) intrinsically located in the nucleus (~90%). TNF- α treatment resulted in RelA/p65 accumulation into TIP60 condensed sites (~70%), which mostly co-localized with chromatin (supplemental information 4a). To further elucidate the distribution patterns within these formations, we subjected our confocal image stack to *z* axis analysis (supplemental information 4b), which revealed an apparent overlap of localization in the nucleus between TIP60 and RelA/p65 upon TNF- α treatment.

TIP60 HAT Domain Interacts with RelA/p65 N-terminal Rel Homolog Domain Region—To define the region of TIP60 that is required for interactions with RelA/p65, HepG2 cells were transfected with TIP60 encoding GFP fused to full-length TIP60, the TIP60 chromo domain (TIP60-Ch), the TIP60 zinc-finger domain (TIP60-Zn), or the TIP60 histone acetyltransferase domain (TIP60-HAT) together with the FLAG-RelA/p65 expression plasmid (Fig. 3a, upper panel). After whole cell lysates were immunoprecipitated with anti-GFP antibodies, Western blotting was performed with anti-FLAG and anti-GFP antibodies. Although chromo (amino acids 1–260) and zinc finger (amino acids 77–366) domain-containing regions of TIP60 did not appear to interact with FLAG-RelA/p65, the HAT domain-containing region (amino acids 285–513)

strongly interacted with FLAG-RelA/p65 (Fig. 3a, lower panel), showing that RelA/p65 interacts with histone acetyltransferase domain of TIP60.

To determine the binding region of RelA/p65 necessary for interaction with TIP60, HepG2 cells were transfected with the RelA/p65 encoding GST fused to the RelA/p65 full-length, N-terminal 320 amino acid-containing region (N320) and the C-terminal transactivation domain containing region (C321) together with FLAG-TIP60 expression plasmid (Fig. 3b, upper panel). Whole cell lysates were immunoprecipitated with anti-GST antibodies, and then Western blotting was performed with anti-FLAG and anti-GST antibodies. As shown in the lower panel of Fig. 3b, TIP60 strongly interacts with the N-terminal Rel homolog region (RHR) (amino acids 1–320) of RelA/p65 but not with the C-terminal region (amino acids 321–551). All together, these results demonstrate that the HAT domain of TIP60 interacts with the N-terminal Rel homolog-containing region of RelA/p65 in HepG2 cells.

TIP60 Increases Transcriptional Activity of RelA/p65 through Physical Interactions—To demonstrate whether TIP60 could affect RelA/p65-dependent transcriptional activation through a protein-protein interaction, HepG2 cells were co-transfected with TIP60-truncated mutants (described in Fig. 3a, upper panel) together with the FLAG-RelA/p65 expression plasmid plus a luciferase reporter gene-containing NF- κ B response element. The transcriptional activity of RelA/p65 was significantly enhanced by the full-length TIP60 and the HAT domain-containing region of TIP60 (Fig. 4a, third and sixth lanes) but not by the chromo and the zinc finger domains of TIP60 (fourth and fifth lanes), indicating that a physical association with the HAT domain of TIP60 is necessary for enhancement of RelA/p65 transactivation. To further examine whether the enhanced

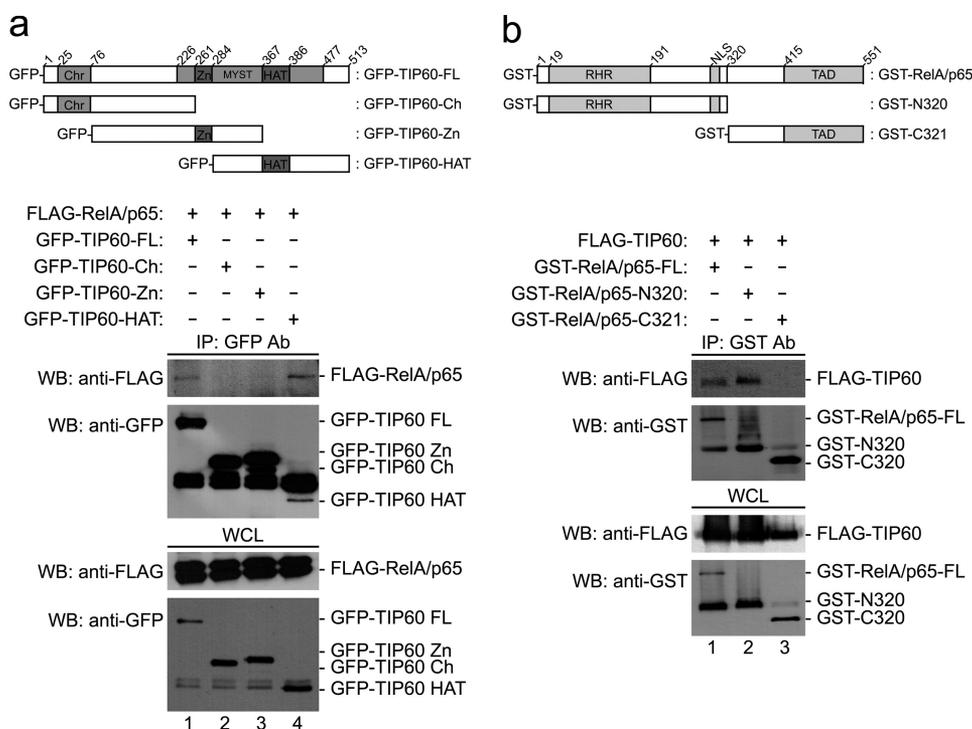


FIGURE 3. Physical interaction of TIP60 and RelA/p65 using their HAT domain and N-terminal Rel-homolog region. *a* and *b*, HEK 293 cells were co-transfected with expression plasmids of FLAG-RelA/p65 and GFP-TIP60 full-length (FL) or each domains of TIP60 or RelA/p65. After the whole cell lysates were immunoprecipitated (IP) with anti-GFP or GST antibodies, the expression of proteins was determined by Western blot (WB) analysis using anti-FLAG, anti-GFP, and anti-GST antibodies. As controls, whole cell lysates (WCL) were also analyzed by Western blot using anti-FLAG, anti-GFP, and anti-GST antibodies, respectively. *Ch*, chromo.

transcriptional activity of RelA/p65 was specifically modulated by TIP60, we used siTIP60 transfectants of RelA/p65^{+/+} MEF or p65^{-/-} MEF cells. As shown in Fig. 4*b*, transfection of TIP60 siRNA significantly decreased the endogenous RelA/p65-mediated transcriptional activation on NF- κ B promoter (*sixth* and *eighth* lanes). Indeed, a knockdown of TIP60 also specifically eliminated TIP60-mediated enhancement of transcriptional activity of overexpressed RelA/p65 (Fig. 4*c*, *fourth* lane). Because RelA/p65 interacted with the HAT domain of TIP60 and their interaction was required for full transcriptional activation of RelA/p65, we used TIP60 HAT mutant to demonstrate whether TIP60 enhances the transcriptional activity of RelA/p65 through its HAT activity. HepG2 cells were co-transfected with HA-RelA/p65 together with the FLAG-TIP60 wild type or HAT mutant expression plasmid plus a luciferase reporter gene containing NF- κ B-response element. As shown in Fig. 4*d*, TIP60 enhanced the transcriptional activity of RelA/p65 in a dose-dependent manner (*third* and *fourth* lanes). However, TIP60 HAT mutant lacks the ability of transcriptional activation of RelA/p65, although it increases expression of TIP60 HAT mutant (*fifth* and *sixth* lanes). Taken together, our results show that TIP60 HAT activity is required for RelA/p65-mediated transcriptional activation through a physical interaction.

TIP60 Is Recruited to Subset of NF- κ B Target Gene Promoters and Is Required for Full Transcriptional Activation of RelA/p65—Having identified TIP60 as a coactivator of RelA/p65 through a physical interaction, we next conducted a ChIP assay to examine whether TIP60 is recruited to the promoter of NF- κ B target genes *in vivo*, an essential step for the function of

a coactivator. ChIP patterns were obtained from chromatin prepared from HepG2 cells treated with 50 ng/ml TNF- α ; correlations were found between TIP60 and RelA/p65 on target gene promoters (*supplemental information 5a*). To further dissect the sequential protein association to NF- κ B target promoters, each promoter was examined by quantitative PCR of ChIP samples using the indicated appropriate primers (Fig. 5). Also, IgG and water negative controls for antibodies were presented in *supplemental information 5b*. We stimulated HepG2 cells with TNF- α for 0, 15, 30, 45, and 60 min and examined the binding of TIP60, acetylated histones, RelA/p65, acetylated lysine 310 RelA/p65, and RNAP II to the promoters of *IL-6*, *C-IAP*, *IL-8*, and *XIAP*, the expression of which is regulated by TIP60. In the absence of TNF- α stimulation, the binding of TIP60, RelA/p65, Ac-Lys-310 RelA/p65, and RNAP II to the each promoter was barely detected (time point 0 min at each experimental groups). After TNF- α stimulation (during 0–30 min), RelA/p65, Ac-Lys-310 RelA/p65, acetylated histones, and RNAP II were recruited to the promoters of *IL-6*, *C-IAP*, *IL-8*, and *XIAP*. Importantly, TIP60 was first recruited to each promoter during TNF- α treatment until 15 min (Fig. 5; *a1–a4*), and then histone acetylation occurred on NF- κ B target gene promoters (Fig. 5, *b* and *c*). Accompanied by TIP60 association, total RelA/p65 and Ac-Lys-310 RelA/p65 were cooperatively recruited to *IL-6*, *C-IAP*, *IL-8*, and *XIAP* promoters (Fig. 5, *d* and *e*). However, when we transfected HepG2 cells with TIP60 siRNA, recruitment of RelA/p65, Ac-Lys-310 RelA/p65, TIP60, and RNAP II was significantly reduced, demonstrating the specificity of the recruitment by TIP60 association. These quantitative ChIP data suggest that TIP60 is recruited to the

TIP60 Enhances Transcriptional Activity of RelA/p65

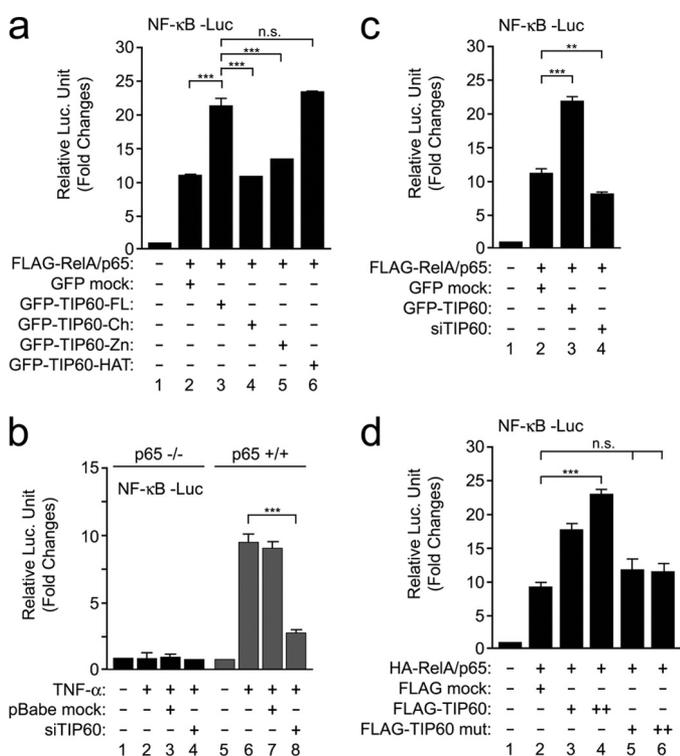


FIGURE 4. Enhancement of the transcriptional activity of RelA/p65 by the protein interaction with TIP60. *a*, HepG2 cells were co-transfected with combined expression plasmids of FLAG-RelA/p65 and GFP-fused full-length (FL), chromo (Ch), zinc finger, and HAT domains of TIP60 together with a luciferase reporter plasmid containing the NF- κ B promoter. Luciferase activities (Luc) were measured 24 h after the transfection as described under "Experimental Procedures." *b*, RelA/p65^{-/-} or p65^{+/+} MEF cells were transfected with pBabe-TIP60 siRNA plasmid or its control empty vector and treated with 50 ng/ml TNF- α for 1 h to activate NF- κ B pathway. Luciferase activities were measured 24 h after the transfection. *c*, HepG2 cells were co-transfected with expression vector FLAG-RelA/p65, GFP-TIP60, siRNA for TIP60, and luciferase reporter plasmid (0.1 μ g). Luciferase activities were measured 24 h after the transfection. *d*, HepG2 cells were transfected with HA-RelA/p65 and FLAG-TIP60 or FLAG-TIP60 HAT mutant-expressing plasmids together with luciferase reporter plasmid. Luciferase activities were measured 24 h after transfection. The data were normalized to β -galactosidase activity and are expressed in relative -fold increase of luciferase units. All data are representative of three independent experiments, and statistical significance is represented by Tukey's post hoc test (**, $p < 0.01$; ***, $p < 0.001$; n.s., not significant).

promoters of a subset of NF- κ B target genes, and TIP60 association is critical step for subsequent recruiting events on target gene promoters during the activation of the NF- κ B pathway.

TIP60 Maintains Ac-Lys-310 RelA/p65 Levels through Their Protein Interaction—In previous reports it was shown that TIP60 enhances transcriptional activity of the transcription factor p53 through the acetylation of the Lys-121 residue (32, 33). Therefore, we hypothesized that TIP60 may increase the transcriptional activity of RelA/p65 through direct acetylation of RelA/p65 by a physical interaction with the TIP60-HAT domain. To test this hypothesis, we first investigated the acetylation levels of RelA/p65 affected by TIP60 overexpression. HepG2 cells were transiently transfected with either an empty FLAG vector and FLAG-TIP60 wild type or FLAG-TIP60 HAT mutant together with the HA-RelA/p65 expression plasmid. The transfected cells were treated with trichostatin A to inhibit deacetylation of RelA/p65 by histone deacetylase. After the whole cell lysates were immunoprecipitated with anti-HA anti-

bodies, Western blotting was performed with anti-acetylated lysine, anti-Ac-Lys-310 RelA/p65, anti-HA, and anti-FLAG antibodies. Total acetylated RelA/p65 levels were clearly increased in the presence of TIP60 expression (Fig. 6a, lane 2), in contrast to the control group (lane 1). However, although TIP60 HAT mutant effectively associated with RelA/p65, both total acetylated RelA/p65 and Ac-Lys-310 RelA/p65 were not reduced by TIP60 HAT mutant overexpression (lane 3). To further verify the acetylation of RelA/p65 by TIP60, we performed an *in vitro* acetylation assay using affinity-purified TIP60 and RelA/p65. Consistent with overexpression data in Fig. 6a, we did not observe the acetylated RelA/p65 by TIP60, although TIP60 did acetylate itself (supplemental information 6, TIP60 autoacetylation was previously reported; Ref. 41).

Because TIP60 increased the levels of Ac-RelA/p65 without direct RelA/p65 acetylation *in vitro* and *in vivo*, we next examined the TNF- α -induced endogenous Ac-RelA/p65 protein levels in the absence or presence of TIP60. HepG2 cells were transfected with or without the FLAG-TIP60 plasmid together with HA-RelA/p65, and the cells were treated with TNF- α for the indicated time points. After the whole cell lysates were immunoprecipitated with anti-HA antibodies, Western blotting was performed with anti-Ac-Lys-310 RelA/p65 and anti-HA antibodies. As shown in Fig. 6b, TIP60 stabilized Ac-Lys-310 RelA/p65 contrast to the TIP60 absent group. These data showed that Ac-Lys-310 RelA/p65 status was increased by the presence of TIP60 from the 30- to 120-min time points after TNF- α treatment. To further confirm the TIP60-mediated stabilization of RelA/p65 *in vivo*, we used TIP60 siRNA transfectants of HepG2 cells. As shown in Fig. 6c, knockdown of TIP60 using its specific siRNA markedly reduced the Lys-310-acetylated RelA/p65 contrast to control vector-transfected groups. Taken together, our results show that TIP60 stabilizes Ac-Lys-310 RelA/p65 through a physical interaction.

Increased Expression of Ac-Lys-310 RelA/p65 and TIP60 in Hepatocarcinoma Tissues—To examine whether TIP60 regulates the hepatocarcinoma cell growth, we examined colony-forming abilities in the HepG2 cells. The cells were transfected with FLAG-TIP60 or TIP60 siRNA and maintained for 4 weeks in G418 containing soft agar medium, and drug-resistant colonies were stained with crystal violet. As shown in Fig. 7a, whereas large numbers of colonies were observed in the empty vector-transfected control group, only a few colonies were observed in the TIP60 siRNA-transfected group (upper right panel). Conversely, TIP60 overexpression induced the increase of colony-forming abilities in HepG2 cells (lower right panel). These data demonstrate that TIP60 regulated the HepG2 liver cancer cell proliferation under physiological conditions.

To identify the expression levels of TIP60, which is involved the enhancement of transcriptional activity of NF- κ B in hepatocarcinoma, we performed immunohistochemical analysis for Ac-Lys-310 RelA/p65 and TIP60 on a paraffin-embedded human hepatocarcinoma tissue microarray (Fig. 7b). This tissue microarray contained a total of 59 tissue spots consisting of 38 hepatocarcinoma, 2 cholangiocarcinoma, 10 metastatic hepatocarcinoma, and 9 non-neoplastic liver tissue samples, which are adjacent to cancer tissues. In the normal hepatocytes, the positive rate of TIP60 expression was 22.2% (2/9) (upper,

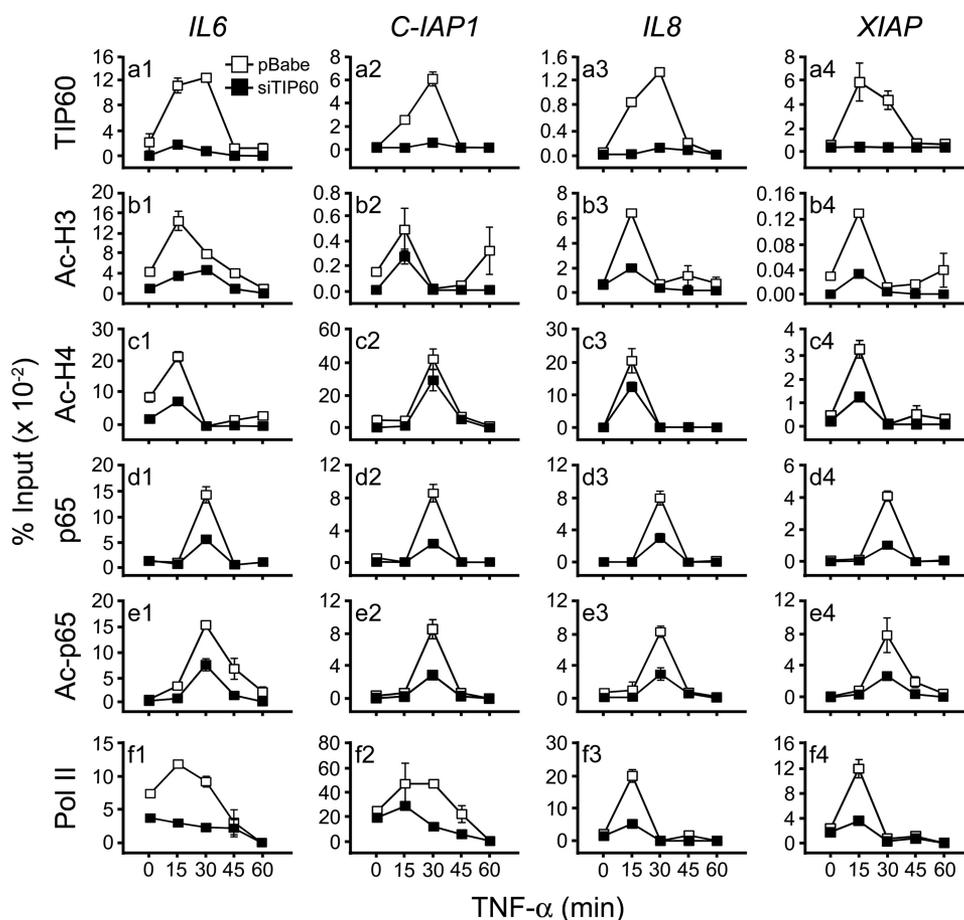


FIGURE 5. **Sequential recruitments of TIP60 and RelA/p65 on the subset of NF- κ B target gene promoters in TNF- α stimulation.** HepG2 cells were transfected with TIP60 siRNA (black box) or empty vector (white box). Twenty-four hours after transfection, HepG2 cells were treated with 50 ng/ml TNF- α for indicated time points (0, 15, 30, 45, and 60 min) and processed for ChIP assay using anti-p65 or anti-TIP60, anti-Ac-H3, anti-Ac-H4, and anti-RNA polymerase II antibodies. Immunoprecipitated chromatin was analyzed by quantitative PCR using the specific primers for *IL6*, *C-IAP1*, *IL8*, and *XIAP* promoters. All values are shown as the mean \pm S.E. of three independent experiments.

left panel). However, positive expression of TIP60 was significantly increased ($p < 0.01$) in hepatocarcinoma (34.2%, 13/38) (upper, right panel). We also confirmed the expression level of Ac-Lys-310 RelA/p65 by tissue microarray. As shown in previous reports (42, 43), we detected the up-regulated positive expression of Ac-Lys-310 RelA/p65 in hepatocarcinoma cells (71%, 27/38) (bottom, right panel). Among TIP60 up-regulated tissues (13/38), 11 tissues (84.6%, 11/13) overlapped with Ac-Lys-310 RelA/p65-enhanced groups. TIP60 expressions were significantly up-regulated in TNM (tumor, node, metastasis) stage II hepatocarcinoma, and its histological properties were well or moderately differentiated hepatocytes (Table 1). These tissue microarray data showed that up-regulated TIP60 expression was significantly correlated with Ac-Lys-310 RelA/p65 expressions in hepatocarcinoma tissues.

DISCUSSION

Histone acetylation by HATs plays important roles in the regulation of gene expression. HATs are generally thought to activate transcription by loosening compacted chromatin and by opening binding sites for the chromatin proteins or TFs, which facilitate the access of the transcriptional machinery to the DNA (44). There are five families of HATs: Gcn5-related HATs, p300/CBP HATs, general transcription factor HATs,

nuclear hormone receptor-related HATs, and the MYST family of HATs. The MYST family is named for its founding members *MOZ*, *Ybf2/Sas3*, *SAS2*, and *TIP60* (26). MYST family members function in a broad range of biological processes, such as gene regulation, dosage compensation, repair of DNA damage, and tumorigenesis (26, 45).

Although growing evidence suggests that TIP60 plays an important role in the transcriptional regulation of transcription factors acting as coactivator or corepressor, TIP60-mediated gene expression regulation is not well determined. Because TIP60 is not a transcription factor that recognizes specific DNA sequences, it requires the selective function of transcription factors to regulate the expression of specific target genes. In this regard it is necessary to identify TIP60-regulated transcription factors to elucidate the TIP60-modulated genes. In this study we found that the gene expressions of *IL-6*, *IL-8*, caspase-7, *Bad*, *Bax*, *Bcl-2*, *cIAP*, and *XIAP* are increased by TIP60 overexpression (supplemental information 3a). Among these genes, the *in silico* method showed that *IL-6*, *IL-8*, *cIAP*, and *XIAP* have conserved NF- κ B binding sites in their promoter regions (supplemental information 3b). Previous studies revealed that *IL6*, *IL8*, *XIAP*, and *cIAP* were modulated by NF- κ B RelA/p65 as target genes (46–50). We demonstrated that TIP60 is involved in NF- κ B pathway through activating the transcrip-

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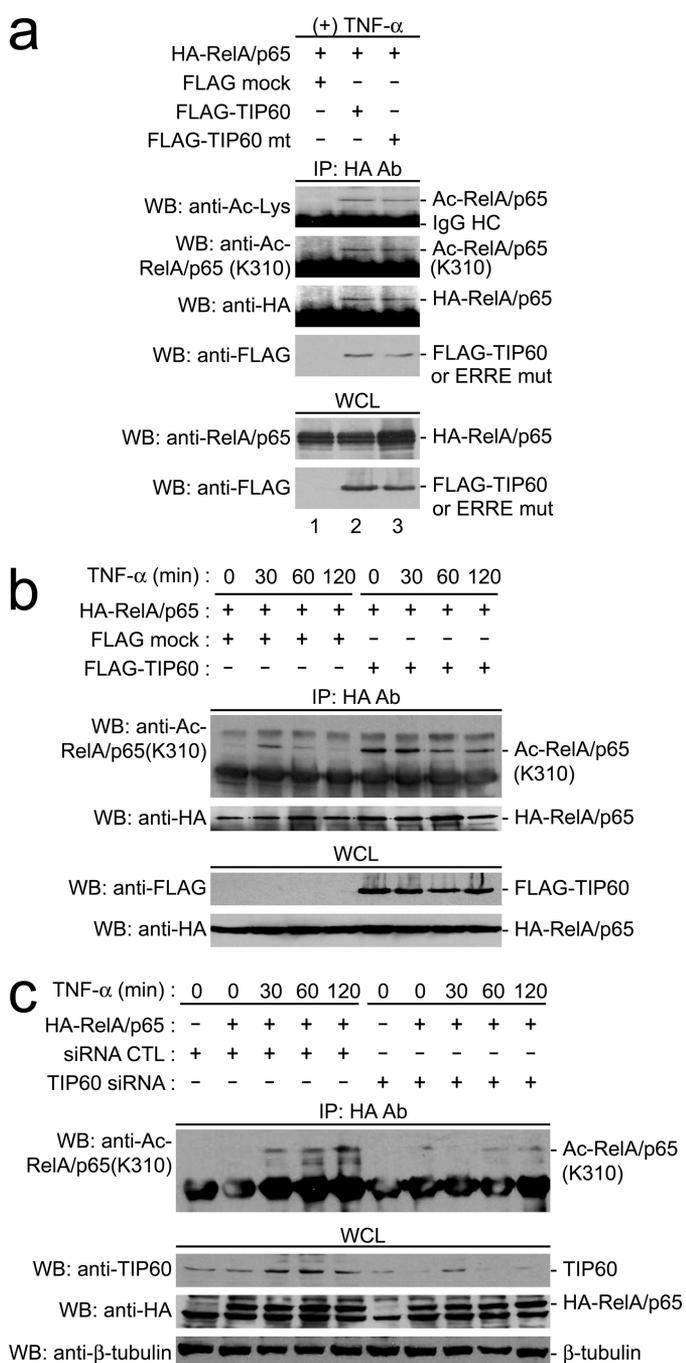


FIGURE 6. Stabilization of the acetylated-Lys-310 RelA/p65 by TIP60. *a*, HepG2 cells were transfected with either an empty FLAG vector, FLAG-TIP60, or FLAG-TIP60 HAT mutant (*mt*) together with the HA-RelA/p65 expression plasmid. Twenty-four hours after transfection, cells were treated with 10 nM trichostatin A for 12 h and 50 ng/ml TNF- α for 30 min. Then the whole cell lysates (WCL) were immunoprecipitated (IP) with anti-HA antibodies, and Western blotting (WB) was performed with anti-acetylated lysine, anti-FLAG, and anti-HA antibodies. *b*, HepG2 cells were transfected with or without the FLAG-TIP60 plasmid together with HA-RelA/p65 plasmid, and then the cells were treated with TNF- α for the indicated time points (0, 30, 60, and 120 min). After the whole cell lysates were immunoprecipitated with anti-HA antibodies, Western blotting was performed with anti-Ac-Lys-310 RelA/p65 and anti-RelA/p65 antibodies. *c*, HepG2 cells were transfected with or without the pBabe-TIP60 siRNA plasmid together with HA-RelA/p65 plasmid, and then the cells were treated with TNF- α for the indicated time points (0, 30, 60, and 120 min). After the whole cell lysates were immunoprecipitated with anti-HA antibodies, Western blotting was performed with anti-Ac-Lys-310 RelA/p65 antibody.

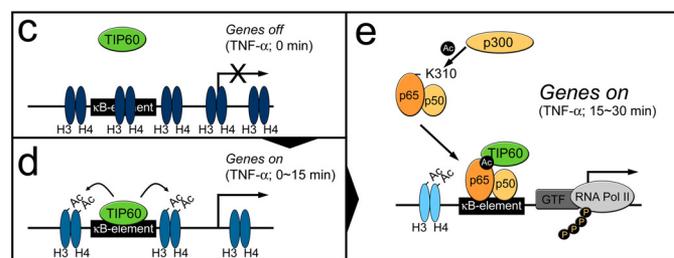
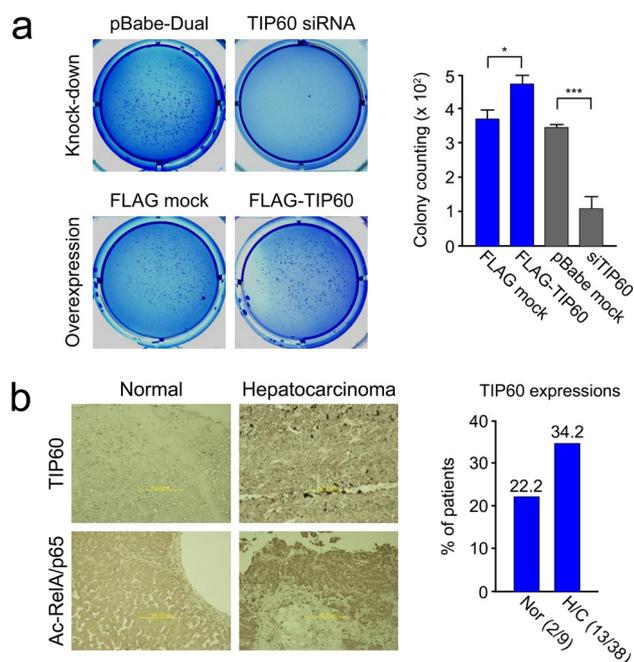


FIGURE 7. Enhancement of hepatocellular proliferation by TIP60 and protein expression of TIP60 and Ac-Lys-310 RelA/p65 in human hepatocarcinoma tissues. *a*, HepG2 transfectants of TIP60 or TIP60 siRNA were plated in 0.35% agar on 6-well culture plates, and the rate of colony forming at 4 weeks after incubation was analyzed. All data are representative of three independent experiments, and statistical significance is represented by Tukey's post hoc test (*, $p < 0.05$; ***, $p < 0.001$). *b*, formalin-fixed tissue microarray slides containing hepatocarcinoma and normal liver tissues were performed immunohistochemistry for TIP60 or acetyl-Lys-310 RelA/p65 and 3,3'-diaminobenzidine (DAB) staining as described under "Experimental Procedures" (at a magnification of 100 \times). *c–e*, shown is a schematic model for the subset of the NF- κ B target gene promoters in TNF- α stimulation. In a TNF- α induced NF- κ B activation, phosphorylation of RelA/p65 subunit by the PKA or PKC kinases facilitates the recruitment of p300/CBP to acetylate the RelA/p65, and TIP60 independently opens chromatin structure to allow the protrusion of NF- κ B recognition sites. Finally, acetylated RelA/p65 positioned on the target promoter recruits other cofactors to perform the transcription initiation involving basal transcription factor recruitment.

tional activity of RelA/p65 in response to TNF- α stimulation (Fig. 1*a*). TIP60-mediated enhancement of transcriptional activation of RelA/p65 was also confirmed by knockdown of TIP60 (Figs. 1*c* and 4, *b* and *c*). Thus, we propose that TIP60 has a coactivator function in the NF- κ B signaling pathway like other cofactors, such as p300/CBP, PCAF, and Brd4 (22, 51).

TIP60 involvement in NF- κ B transcriptional activation was previously described by Baek *et al.* (52). In unstimulated cells, the IL-1 β /NF- κ B-regulated *KAI1/CD82* gene promoter is repressed by a corepressor complex that contains NCoR, histone deacetylase 3, and TAK1-binding protein 2 (TAB2) (52). In response to IL-1 β cytokines, the NCoR complex is phosphorylated and exported from the nucleus, thus allowing a TIP60-coactivating complex to bind and derepress the *KAI1* promoter

TABLE 1
TIP60 expression patterns in hepatocarcinoma

Tissue types	n	Positive rate of TIP60 expression
		%
Groups		
Non-neoplastic liver	9	22.2 (2/9)
Hepatocarcinoma	38	34.2 (13/38)
Cholangiocarcinoma	2	0 (0/2)
Metastatic carcinoma	10	10 (1/10)
Total tissues	59	
Clinicopathologic factors		
Histological differentiation		
High and moderate	25	36 (9/25)
Poor	5	20 (1/5)
TNM stage		
I	2	15.4 (2/13)
II	8	61.5 (8/13)
III	2	15.4 (2/13)
IV	1	7.7 (1/13)

(52). This TIP60 complex contains TRRAP and is recruited to NF- κ B p50 homodimer complexes bound to the promoter via interactions with the NF- κ B adaptor protein B-cell leukemia/lymphoma 3 (Bcl3), which is accompanied by acetylation of histones H3 and H4 in a promoter-specific fashion (52). They provided a possible role of TIP60 in NF- κ B-regulated gene expression acting as a coactivator through distinct mechanisms (gene activation with NF- κ B p50/p50 homodimer). Our findings indicate that the association of TIP60 with NF- κ B RelA/p65 occurs through a protein-protein interaction between the HAT domain of TIP60 and the N-terminal Rel homolog domain of RelA/p65 (Fig. 3). The importance of this protein interaction in TIP60-mediated transcriptional activation of RelA/p65 was further revealed by performing a luciferase assay using TIP60 truncation mutants designated by chromo, Zn²⁺, and HAT domains. Only the HAT domain of TIP60 that interacts with RelA/p65 possesses transcriptional activation properties (Fig. 4a). However, how other cofactors differentially cooperate with the NF- κ B RelA/p65 and each NF- κ B target gene promoter in response to TNF- α stimulation is not clear; this subject should be explored further.

To further understand the role of transcription initiation on NF- κ B target gene promoters, we identified the sequential order of protein recruitment on the promoters in TNF- α -stimulated hepatocarcinoma HepG2 cells using a chromatin immunoprecipitation assay (Fig. 5). The data presented demonstrate that the protein association of TIP60 on NF- κ B target genes occurred first and that acetylated histone H3/H4 was increased by TIP60 recruitment. Acetylated K310-RelA/p65 finally appeared on the target gene promoters, preceding TIP60-mediated chromatin remodeling. How does TIP60 prioritize the recognition of target gene promoter regions without specific assistance of transcription factors for cellular signaling? It is possible that TIP60 rapidly responds in some signaling pathways (in our data TIP60 prefers TNF- α signaling through NF- κ B pathway than other IL-1 and LPS signaling) using its chromodomain, which recognizes specifically methylated histones for early responsive genes before transcription factor appearance on their target promoter regions. The binding of chromodomain containing proteins to methylated histones helps to interpret the "histone code" that is embedded within chromatin (53, 54). A recent report supported the possibility

that direct interactions between TIP60 chromodomain and trimethylated histone H3 on lysine 9 (H3K9me3) at the DNA double strand break activates TIP60 acetyltransferase activity (55). Furthermore, gene regulatory regions display distinct histone modifications and variants, and these marks modulate protein recognition. In large scale chromatin immunoprecipitation screening of the human genome, high levels of histone H3K4/79 methylation and H3 acetylation were found to be strict prerequisites for binding of the Myc transcription factor (17). Frank *et al.* (56) also demonstrated that Myc associates with TIP60 and recruits it to chromatin with four other components of the TIP60 complex: TRRAP, p400, TIP48, and TIP49. Overexpression of enzymatically inactive TIP60 delays Myc-induced acetylation of histone H4 and reduces the level of Myc binding to chromatin (56). These reports imply that chromatin modifications can actually regulate TF binding in appropriate signaling pathways. The "code translator proteins" containing chromodomain could serve as bridges between the epigenetic histone code and executive transcription factors. It will be interesting to determine whether other transcription factors are similarly regulated by TIP60 to activate RNAPII-dependent transcription and to identify a histone code that is specifically recognized by the chromodomain of TIP60.

Although acetylation of lysine 310 of RelA/p65 is responsible for the optimized transcriptional potential of NF- κ B, the precise mechanism underlying the specific effect of this single lysine acetylation remains unclear (57). In this study we found that TIP60 enhanced acetylated RelA/p65 in TNF- α stimulation (Fig. 6a). To further identify the TIP60-mediated direct acetylation of RelA/p65, we performed *in vitro* acetylation assays because TIP60 interacts with RelA/p65 using its HAT domain (Fig. 3a). We could not detect acetylation of RelA/p65 by TIP60 but confirm autoacetylated TIP60 in same experimental group (supplemental information 6). Although TIP60 could not directly acetylate RelA/p65, we found that acetyl-Lys-310 RelA/p65 was maintained by TIP60 overexpression with TNF- α stimulation (Fig. 6b). It is possible that TIP60 interacts with the N-terminal region of RelA/p65, which contains a Lys-310 residue (Fig. 3b) and inhibits histone deacetylase recruitment on their complex to turn off the NF- κ B signaling pathway. Although we did not confirm that acetylation of residue RelA/p65 is critical for interaction with TIP60 in this study, it seems that p300-mediated RelA/p65 acetylation could be modulated by TIP60 through a protein interaction.

Previous studies reveal that the activation of NF- κ B, a hallmark of inflammatory responses that is frequently detected in tumors, might constitute a link between inflammation and cancer (58–60). The inflammatory process triggers hepatocyte NF- κ B through up-regulation of TNF- α in adjacent endothelial and inflammatory cells (42, 61). We also confirmed that the expression of Ac-Lys-310 RelA/p65 was up-regulated in hepatocarcinoma tissues in contrast to normal liver tissues (Fig. 7b). Furthermore, TIP60 expression was increased in TNM stage II of a moderately differentiated hepatocarcinoma (Table 1). Our findings here showed that TIP60 expression might be correlated with RelA/p65 in the early stage of hepatocarcinoma generation. We also suggest the possibility that sustainable high expression of TIP60 delays the "turn-off" signal of the NF- κ B

pathway by maintaining acetylated K310-RelA/p65 through a protein interaction that correlated with activated NF- κ B signaling in hepatocarcinomas (Fig. 7).

The identification of TIP60 as a coactivator of NF- κ B RelA/p65 suggests a novel mechanism for the enhanced transcriptional activity of activated RelA/p65. Unlike other coactivators that recognize the acetylation code of transcription factors, the occupancies of TIP60 on the subset of NF- κ B target gene promoters were preceded by independent of transcription factor binding. Moreover, a knockdown of TIP60 significantly reduced acetylated histones H3/H4 and recruitment of RelA/p65 on the promoter regions. Therefore, in TNF- α -induced NF- κ B activation, phosphorylation of RelA/p65 facilitates the recruitment of p300/CBP to acetylate RelA/p65, and TIP60 opens the chromatin structure to allow protrusion of NF- κ B recognition sites. Acetylated RelA/p65 positioned on the promoter recruits other cofactors to execute transcription initiation involving basal transcription factor recruitment (Fig. 7, *c–e*). Our study will contribute to a better understanding of the regulation of transcriptional activation of NF- κ B in a view of chromatin remodeling, which is mediated by a protein-protein interaction and a protein-DNA interaction. Furthermore, regulation of the interaction between TIP60 and NF- κ B might be a potential target for the systemic inhibition of NF- κ B pathway and for therapeutic approaches of NF- κ B-mediated carcinogenesis.

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