ZNF76, a Novel Transcriptional Repressor Targeting TATA-binding Protein, Is Modulated by Sumoylation*

Direct interaction of positive and negative regulators with the general transcription machinery modulates transcription. The TATA-binding protein (TBP) is one target for transcriptional regulators. In this study, we identified ZNF76 as a novel transcriptional repressor that targets TBP. ZNF76 interacts with TBP through both its N and C termini, and both regions are required for ZNF76 to exert its inhibitory function on p53-mediated transactivation. The inhibitory effect of ZNF76 on p53 activity was demonstrated by reporter assays and endogenous target gene expression. We mapped the TBP-interacting region in the C terminus of ZNF76 to a glutamic acid-rich domain, which acts in a dominant negative manner to enhance p53-mediated transactivation in reporter assays. Mutagenesis study for ZNF76 suggests a correlation between interaction with TBP and effect on p53-mediated transactivation, supporting the conclusion that ZNF76 targets TBP for transcriptional repression. Chromatin immunoprecipitation experiments suggest that ZNF76 prevents TBP from occupying the endogenous p21 promoter. ZNF76 is sumoylated by PIAS1 at lysine 411, which is in the minimal TBP-interacting region. Overexpression of PIAS1 and SUMO-1 abolishes the interaction between ZNF76 and TBP and partially relieves the repressive effect of ZNF76. These results suggest that ZNF76 functions as a transcriptional repressor through its interaction with TBP and that sumoylation modulates its transcriptional repression activity.

Gene transcription is regulated by positive and negative acting factors. Negative regulation of transcription is an essential mechanism for precise control of gene expression. So fundamental are such transcriptional repressors that they are necessary for the viability of yeast (1, 2). One of the mechanisms repressors employ is contacting components of the basal transcription machinery. The TATA-binding protein (TBP), an important component of the basal transcription machinery utilized by all three RNA polymerases, is a frequent target for transcriptional regulators. Several general transcriptional repressors that target TBP have been identified, such as E1A (3), p53 (4), Even-skipped (5), NC2 (6), and Mot1 (7). These inhibitors employ different mechanisms of action: Mot1 interferes TATA-TBP complexes by dissociating TBP from DNA in an ATP-dependent manner (7), and NC2 does not interfere with the TBP-DNA interaction but prevents association of TBP with other general transcription factors (6). Most general transcriptional repressors were initially identified in yeast. Despite the wealth of data demonstrating interactions of TBP and transcriptional repressors in vitro, there is less experimental data defining how they work in mammalian cells and how their transcriptional repression functions are modulated.

Sumoylation is one of the important posttranslational modifications that affect protein functions. In mammalian cells, the SUMO protein family consists of three members, SUMO-1, -2, and -3. Sumoylation is biochemically similar to ubiquitination (9). SUMO-1 conjugation utilizes a unique E1-activating enzyme complex termed SAE1/SAE2 (Aos1/Uba2), an E2-conjugating enzyme, Ubc9, and a group of E3 ligases that were characterized recently, including the PIAS family members (10, 11), RanBP2 (12), and PC2 (13). However, the functional consequences of sumoylation are distinct from ubiquitination. Instead of being marked for degradation by ubiquitination, sumoylation has diverse substrate-specific functions. Because both sumoylation and ubiquitination occur at lysine residues, sumoylation has been shown to inhibit protein ubiquitination and enhance the protein stability, as exemplified by sumoylation of cAMP-response element-binding protein (14) and the NF-κB inhibitor IκBa (15). Several transcription factors, including p53 (16, 17), the androgen receptor (18), Sp3 (19, 20), and c-Myb (21), are sumoylation targets, and sumoylation alters their transcriptional activities. Sumoylation also affects protein localization. A transcriptional repressor, CtBP, can be recruited to PcG complexes after being sumoylated (13).

ZNF76 is a human homolog of Staf (selenocysteine tRNA gene transcription-activating factor) (22), which is a Xenopus zinc-finger transcription factor known to regulate genes encoding selenocysteine tRNA (tRNAsec) and small nuclear RNA, such as U1 and U6. Staf shows 84 and 64% amino acid sequence identity with human ZNF143 and ZNF76, respectively. Recently, ZNF76 was also shown to activate transcription of a molecular chaperonin subunit Cct6 gene (23), suggesting multiple roles of ZNF76 for transcriptional regulation. The ZNF76 gene is located at chromosome 6p21 in a region associated with a range of phenotypic abnormalities that affect embryonic development, male fertility, and neoplasia (24, 25).

In this study, we identified ZNF76 as a TBP-interacting protein. Through TBP interaction, ZNF76 inhibits p53-mediated gene expression. We also showed that sumoylation by PIAS1 regulates the transcriptional repression function of ZNF76.
**Sumoylation Modulates Transcriptional Repression**

**MATERIALS AND METHODS**

Reagents and Antibodies—Anti-Myc (9E10), anti-TBP (sc-273), and anti-p21 (H-184) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-PIAS1 (M2) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-HA antibody was from BAbCo (Richmond, CA). Anti-glyceraldehyde-3-phosphate dehydrogenase was from TREVIGEN, Inc. (Gaithersburg, MD). Anti-ZNF76, a rabbit polyclonal antibody against purified GST-ZNF76, was prepared by Bio-Synthesis, Inc. (Lewisville, TX).

**Plasmid Construction**—pGBK7-PIAS1 and subfragments of PIAS1 in pGBK7 vector were constructed by insertion of murine PIAS1 cDNA encoding the full length or the corresponding PIAS1 fragments into EcoRI and BamHI sites of pGBK7 vector. pGEX4T-1-ZNF76 was constructed using EcoRI and BamHI sites of pGEX4T-1. All of the site-directed mutant constructs were generated by a two-step PCR method and were confirmed by sequencing. pCMV5-PIAS1, which encodes FLAG-tagged full-length PIAS1 was a gift from Dr. Ke Shuai (26). The pMT2-T-F-TBP vector was a gift from Dr. Cheng-Ming Chiang (27). The plasmid encoding FLAG-tagged SUMO-1 was a gift from Dr. Hideyo Yasuda (11).

**Cell Culture and Transfection**—HEK293 cells, human osteosarcoma U2OS cells, and HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) 10% fetal calf serum. HeLa cells were transfected with the calcium phosphate precipitation method (28). HeLa cells and U2OS cells were transfected with FuGENE 6 according to the manufacturer’s instructions (Roche Applied Science).

**Transient Transfection, Immunoprecipitation, and Western Blot Analysis**—HEK293 cells were transfected by calcium phosphate precipitation method with various plasmid combinations as indicated. Forty-eight hours later, the cells were washed with phosphate-buffered saline and 1 ml of ice-cold lysis buffer (radioimmune precipitation assay buffer) (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 2 mM Na3VO4, 15 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride) was added. The cells were lysed for 30 min at 4°C with occasional vortexing. To test Zn2+/H9262, the lysates were collected into 1.5-ml tubes and cleared of nuclei by centrifugation for 10 min at 14,000 rpm. The supernatants (whole cell extracts) were incubated with different antibodies for 16 h at 4°C, and protein A-agarose beads were added for the last 75 min. The beads were washed five times in TNEN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40 or 0.5% Brij35 if testing endogenous protein-protein interaction, 10 mM NaVO4, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF; plus 1.3 μM Zn2+ if testing zinc-dependent protein-protein interaction). Bound proteins were extracted with SDS-PAGE sample buffer and analyzed by SDS-PAGE followed by Western blot analysis with the ECL detection system used for protein detection. For sumoylation assays, 48 h after transfection, the cells were lysed either in radioimmune precipitation assay buffer containing 10 μM N-ethylmaleimide or in a denaturing buffer (2% SDS, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl) and analyzed by SDS-PAGE followed by Western blot analysis.

**Luciferase Assay**—The cells were plated and grown overnight before transfection. The total amount of DNA to be transfected was adjusted with pDNA3. Luciferase assay was performed according to the manufacturer’s instructions (Promega). Renilla luciferase internal control plasmid was co-transfected with the plasmids as indicated. The relative luciferase units were corrected based on Renilla luciferase activity. In the assay for p53-mediated gene expression, a reporter containing a synthetic p53-binding site upstream of the luciferase reporter gene (pG13-luc) was used.

**Yeast Two-hybrid Screen**—The MATCHMAKER GAL4 two-hybrid system 3 (Clontech, Inc.) was used in the yeast two-hybrid screen. The bait used was PIAS1(1–390) containing the N terminus and the zinc finger domain. The first reason to use this fragment as the bait was that full-length PIAS1 has high background and is not suitable as a bait in the yeast two-hybrid system. Second, the region we chose contains zinc finger domains and four LXXLL motifs, which are important domains for mediating protein-protein interactions. After screening 106 clones, eight positive clones were identified and sequenced. Two of them are the cDNA sequences for Homo sapiens thioredoxin domain containing 7 (NM_005742); two for H. sapiens translocase of inner mitochondrial membrane 50 (NM_005347); one for Homo sapiens DnaJ (NM_005347); one for H. sapiens ankyrin repeat family A protein 2 (NM_005347); and one for H. sapiens heat shock 70-kDa protein 5 (NM_005347) (protein disulfide isomerase). Among them there is a clone whose sequence is identical to that of human ZNF76.

**RESULTS**

**ZNF76 Is a PIAS1-interacting Protein**—We performed a yeast two-hybrid screen to identify novel PIAS1-interacting proteins. The bait used in the screening was PIAS1(1–390) containing the N terminus and the zinc finger domain. The first reason to use this fragment as the bait was that full-length PIAS1 has high background and is not suitable as a bait in the yeast two-hybrid screen. Second, the region we chose contains zinc finger domains and four LXXLL motifs, which are important domains for mediating protein-protein interactions. After screening 106 clones, eight positive clones were identified and sequenced. Two of them are the cDNA sequences for Homo sapiens thioredoxin domain containing 7 (NM_005742); two for H. sapiens translocase of inner mitochondrial membrane 50 (NM_005347); one for Homo sapiens DnaJ (NM_005347); one for H. sapiens ankyrin repeat family A protein 2 (NM_005347); and one for H. sapiens heat shock 70-kDa protein 5 (NM_005347) (protein disulfide isomerase). Among them there is a clone whose sequence is identical to that of human ZNF76.
Fig. 1. ZNF76 is a transcription repressor.  

**a**, ZNF76 represses p53-mediated transactivation in HEK293 cells. HEK293 cells were transfected with 25 ng of pG13-luc reporter, 10 ng of expression plasmid pcDNA-HA-p53, and various amounts of pcDNA-ZNF76-Myc plasmid alone or in combination as indicated.  

**b**, structural integrity is required for ZNF76 to repress p53-mediated transactivation. HEK293 cells were transfected with 25 ng of pG13-luc reporter, 10 ng of pcDNA-HA-p53, and 0.2 μg of pcDNA-ZNF76-Myc or mutant plasmids alone or in combination as indicated.  

**c**, ZNF76 inhibits p53-mediated transactivation in HeLa and U2OS cells. The cells were transfected with 25 ng of pG13-luc, 10 ng of pcDNA-HA-p53, and 0.2 μg of pcDNA-ZNF76-Myc alone or in combination as indicated.  

**d**, ZNF76 inhibits p53-mediated expression of p21. HEK293 cells were transiently transfected with 50 ng of pcDNA-HA-p53 plasmid with or without 0.5 μg of pcDNA-ZNF76-Myc plasmid. After 48 h, the cells were lysed for Western blot analysis with anti-HA, anti-Myc, anti-p21, and anti-glyceraldehyde-3-phosphate dehydrogenase antibody.  

**e**, ZNF76 inhibits Stat1-mediated transactivation. MCF-7 cells were transiently transfected with 0.1 μg of IRF1-Luc and 0.1, 0.3, or 1.0 μg of pcDNA-ZNF76-Myc plasmid. 24 h after transfection, the cells were treated with interferon-γ at the final concentration of 100 unit/ml for 16 h before harvesting.  

**f**, ZNF76 inhibits Smad3-mediated transactivation. HEK-293 cells were transiently transfected with 0.1 μg of SBE4-luc and 0.1 μg of pCMV2-Smad3 plasmid with or without 0.2 μg of pcDNA-ZNF76-Myc.
into HEK293 cells along with plasmids expressing HA-p53 and Myc-ZNF76. p53-mediated reporter gene expression was induced –10-fold by transfecting 10 ng of HA-p53 expressing plasmid. Co-transfection of pcDNA-ZNF76 inhibited p53-induced luciferase expression in a dose-dependent manner. There is a significant inhibitory effect when only 5 ng of ZNF76 expression plasmid was transfected, and protein expression could not be detected by Western blotting. This argues against the notion that the inhibition is an artifact because of gross overexpression of ZNF76. In contrast, as shown in Fig. 1b, overexpression of ZNF76(1–375) or ZNF76(342–570) could not repress the transcriptional activity of p53, suggesting that the structural integrity of ZNF76 is essential for inhibiting the transcriptional activity of p53. Interestingly, transcription of ZNF76(342–570) increased basal and p53-induced activity in the reporter assays. The strong inhibitory effect of ZNF76 on p53 was also observed in various cell lines including HeLa, U2OS (Fig. 1c), MCF-7, and H1299 (data not shown). To test whether ZNF76 also inhibits p53-dependent expression of endogenous genes, we examined whether ZNF76 could inhibit p53-mediated expression of the p21 protein. As shown in Fig. 1d, ectopic expression of ZNF76 abolished p21 expression induced by p53 but had no effect on glyceraldehyde-3-phosphate dehydrogenase expression. These results suggest that ZNF76 is a transcriptional repressor that inhibits p53-mediated gene expression. To address whether the repressive effect of ZNF76 is selective for p53, we tested the effect of ZNF76 on two other transcription activators, Stat1 and Sma3. As shown in Fig. 1e, human MCF-7 cells were transfected with a luciferase reporter construct, IRF1-luc, containing three copies of the Stat1-binding sequence and expression vectors encoding ZNF76-Myc and treated with or without interferon-γ. The Stat1-activated luciferase expression in response to interferon-γ stimulation was significantly inhibited by ZNF76 in a dose-dependent manner. Similarly, ZNF76 also strongly inhibited Sma3-mediated transactivation (Fig. 1f). These results suggest that ZNF76 is a general transcriptional repressor.

**ZNF76 Interacts with TBP**—One of the frequent targets for transcriptional repressors is the TBP, and several viral proteins repress p53 transactivation by interacting with TBP (30, 31) or affecting TBP expression (32). To test whether ZNF76 and TBP complex in cells, pCMV2-FLAG-TBP and pcDNA-ZNF76-Myc were co-transfected into HEK293 cells. Lysates of transfected cells were immunoprecipitated with anti-Myc or anti-FLAG antibody. Immunoblots probed with anti-Myc showed that anti-FLAG pulled down Myc-tagged ZNF76 only when pCMV2-FLAG-TBP but not the empty vector was present (Fig. 2a), suggesting TBP complexes with ZNF76 in cells. The endogenous interaction of ZNF76 and TBP was further demonstrated in MCF-7 cells by reciprocal co-immunoprecipitations using anti-ZNF76 and anti-TBP antibodies (Fig. 2b).

To define the regions in ZNF76 essential for interacting with TBP, plasmids encoding wild type or mutant ZNF76 were co-transfected into HEK293 cells together with pCMV2-FLAG-TBP. In contrast to full-length ZNF76, mutant ZNF76(1–375) interacted weakly with TBP. ZNF76(342–570) but not the zinc finger domain ZNF76(150–375) also associated with TBP (Fig. 2c). To confirm these results, we employed the yeast two-hybrid assay to map the interacting regions. Both yeast growth on SD-HTL medium and positive β-galactosidase activity were used as criteria for positive interactions. As shown in Fig. 2d, in agreement with the co-immunoprecipitation results, both the N terminus and the C terminus, but not the zinc finger domain of ZNF76, interacted with TBP. Yeast expression of ZNF76 mutant fusion proteins is shown in Fig. 2e. Because the C terminus of ZNF76 interacts with TBP and can enhance p53-mediated transactivation in the reporter assay (Fig. 1f), we further mapped the interacting regions in the C terminus of ZNF76. After examining the sequence, we found a glutamic acid-rich domain (GARD) 83 amino acids in length (amino acids 382–444) in the C terminus of ZNF76 (labeled as a bar in Fig. 2i and thereafter). We tested whether GARD itself is sufficient for interaction with TBP in the yeast two-hybrid assay. As shown in Fig. 2i, both yeast growth on SD-HTL and β-galactosidase assays support the interaction between GARD and TBP. Fusion protein expression in yeast is shown in Fig. 2g. Consistently, we found that ZNF76(342–570)K400,411R, a C terminus mutant of ZNF76 with two lysine residues in GARD changed to arginine, lost its interaction with TBP (Fig. 2h), suggesting that GARD is essential for the C terminus of ZNF76 to interact with TBP. Fig. 2i is a summary of the mapping results. These data demonstrate that ZNF76 interacts with TBP through both its N terminus and GARD in its C terminus.

The 180-amino acid C-terminal domain of TBP is commonly referred to as the TBP core domain, which is responsible for recognizing the TATA box. The core domain of TBP is highly conserved, whereas the N-terminal region of TBP is more divergent (Fig. 3a). We performed co-immunoprecipitation experiments to map the regions of TBP responsible for interacting with ZNF76. As shown in Fig. 3b, plasmids encoding FLAG-tagged wild type or mutant TBP were co-transfected into HEK293 cells together with pcDNA-ZNF76-Myc. We found that FLAG-tagged TBP(146–339), which includes the core domain of TBP but not FLAG-tagged TBP(1–159), co-immunoprecipitated with Myc-tagged ZNF76. These results suggest that, like other transcriptional repressors that target TBP such as NCo2 (37) and p53 (33), ZNF76 interacts with the core domain of TBP.

**ZNF76 Targets TBP to Inhibit p53 Activity**—Because ZNF76 interacts with TBP, we tested whether ZNF76 inhibits p53-mediated gene expression through interaction with TBP. As shown in Fig. 4a, ectopic expression of TBP reversed the inhibitory effect of ZNF76 on p53-mediated transactivation. Although ectopic expression of TBP modestly increased both basal and p53-activated reporter activity, the increase was less significant than the increase in the p53 reporter activity in the presence of ZNF76. This argues against the possibility that TBP reversed the inhibitory effect through a nonspecific increase in general transcription. We further tested whether a correlation exists between the ability of ZNF76 to interact with TBP and repression of p53-mediated transactivation. As shown in Fig. 4b, transfection of a construct encoding GARD strongly enhanced p53 activity in reporter assays, which is similar to the effect of ZNF76(342–570) shown in Fig. 1b. We speculate that this GARD-containing peptide acts in a dominant negative manner to abolish the strong association between endogenous ZNF76 and TBP. Because we previously showed that ZNF76(342–570)K400,411R lost its interaction with TBP, we tested the effect of double mutation (K400R, K411R) on p53-mediated transactivation. We found that ZNF76(342–570)K400,411R, unlike ZNF76(342–570), lost its ability to enhance p53 activity (Fig. 4c). Likewise, ZNF76(342–570)K400,411R lost its inhibitory function on p53-mediated transactivation, which is in agreement with the observation that C terminus of ZNF76 is required for its transcriptional repression function (Fig. 1b). The strict correlation between interaction with TBP and an effect on p53-mediated transactivation strongly suggests that ZNF76 targets TBP to mediate its transcriptional repression function.

To test whether ZNF76 is present in the promoter of p53 target genes, we performed chromatin immunoprecipitation. As shown in Fig. 4d, HEK293 cells transfected with only pcDNA-HA-p53 showed binding of p53 and TBP to the p21
**Fig. 2. ZNF76 interacts with TBP.**

**a.** co-immunoprecipitation (IP) of ZNF76 and TBP in HEK293 cells. 1 μg of plasmids pFLAG-CMV2-TBP and pCDNA-ZNF76-Myc was co-transfected or transfected alone into HEK293 cells, and the cell lysates were immunoprecipitated with anti-FLAG (2 μl) or anti-Myc (10 μl) and immunoblotted with anti-FLAG or anti-Myc.

**b.** endogenous association of ZNF76 with TBP in MCF-7 cells. Whole cell lysates (WCL) were immunoprecipitated with anti-ZNF76, normal rabbit IgG, or anti-TBP antibodies, and the immunoprecipitates were

**c.** TBP interaction in yeast. Growth on SD-HTL medium

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promoter. However, when FLAG-ZNF76 was overexpressed, only p53, but not TBP or ZNF76, was present in the p21 promoter. We also tested whether endogenous ZNF76 occupies the p21 promoter using chromatin immunoprecipitation experiments. As shown in Fig. 4e, endogenous p53, but not ZNF76, occupied p21 promoter after MCF-7 cells were treated with 200 nM adriamycin, a DNA damage agent, for 4 h. These results suggest that ZNF76 modulates the binding of TBP with associated transcription factors, but itself does not occupy the target promoter.

PIAS1 Is an E3 Ligase for ZNF76 Sumoylation—To study the molecular basis for the interaction of PIAS1 and ZNF76, the yeast two-hybrid assay was performed to identify interacting domains. Full-length ZNF76 and various deletion mutants of ZNF76 were tested for their ability to interact with the bait: PIAS1(1–390). As shown in Fig. 5a, ZNF76(160–570), but not ZNF76(1–375), interacted with the bait, suggesting that both the zinc finger domain and the C terminus of ZNF76 are necessary for interaction with PIAS1. Similarly, a series of PIAS1 mutants were tested for their interaction with ZNF76(Fig. 5b).

A PIAS1 mutant with only the N-terminal 1–277 region can interact with ZNF76. Interestingly, deletion of the first 9 amino acids of PIAS1 abolished this interaction. The expression of the fusion proteins in yeast is shown in Fig. 5 (c and d). These results demonstrate that the zinc finger domain and the C terminus of ZNF76 interact with the N terminus of PIAS1. Co-immunoprecipitation experiments were next performed to confirm the interaction between ZNF76 and PIAS1. We co-transfected Myc-tagged pCDNA3-ZNF76 and FLAG-tagged pCMV5-PIAS1 into HEK293 cells. After 48 h, the cells were lysed and immunoprecipitated with anti-Myc. The immunoprecipitates were then washed and analyzed by Western blots with anti-FLAG antibody. Surprisingly, PIAS1 was not in the immunoprecipitates (Fig. 5e, left panel). Because we mapped the zinc finger domain of ZNF76 to be necessary for the interaction in yeast, we tested whether the interaction of ZNF76 with PIAS1 is zinc-dependent by including 1.3 μM zinc chloride in radioimmune precipitation assay buffer and TNEN washing buffer. As shown in Fig. 5e (right panel), PIAS1 was present in the anti-Myc immunoprecipitates only when Zn²⁺ was added, indicating that zinc is necessary for the interaction between PIAS1 and ZNF76.

Because ZNF76 interacts with PIAS1, a well characterized E3 ligase for sumoylation, we tested whether ZNF76 can be
sumoylated by PIAS1. HEK293 cells were transfected with pcDNA-ZNF76-Myc, a FLAG-SUMO-1 expression plasmid, with or without the pCMV5-FLAG-PIAS1 plasmid, and lysed after 48 h. The cell lysates were separated by SDS-PAGE, and a Western blot was probed with anti-Myc to detect unmodified and sumoylated forms of ZNF76. As shown in Fig. 6a, overexpression of FLAG-SUMO-1 led to the appearance of multiple weak bands that represent different forms of SUMO-1 conjugated ZNF76. Co-transfection of pCMV5-FLAG-PIAS1 strongly enhanced the intensity of these bands. To map the regions of ZNF76 that can be sumoylated, we co-transfected plasmids expressing different deletion mutants of ZNF76 with PIAS1 and SUMO-1. As shown in Fig. 6b, all of the mutants except ZNF76(1–165) can be sumoylated. Based on the mapping study, both the zinc finger domain and the C terminus of ZNF76 have at least one lysine residue that can be sumoylated. In the C terminus of ZNF76, lysine 411 and the surrounding sequences are consistent with a conserved sumoylation motif (42), ΦKXE. Notably, Lys411 is within GARD that interacts with TBP. We therefore mutated lysine 411 to arginine (ZNF76K411R) and tested sumoylation of ZNF76K411R. As shown in Fig. 6c, mutation of Lys411 diminished sumoylation of ZNF76. On the contrary, mutation of another residue, lysine 30, which is also in the conserved motif, had no effect on the sumoylation of ZNF76 (data not shown). To map the rest of lysine residues that can be sumoylated, we made single mutants ZNF76K156R, ZNF76K226R, ZNF76K237R, ZNF76K364R, and ZNF76K404R and double mutants ZNF7K411R, ZNF76K411415R, ZNF76K411R415R, and ZNF76K404R415R to test their sumoylation by PIAS1. No difference was detected in the sumoylation pattern of the single mutants and wild type ZNF76 or the double mutants and ZNF7K411R (data not shown). It is possible that other lysine residues are sumoylated or multiple lysines are responsible for the remaining sumoylation bands. These results suggest that lysine 411 is one of the major sumoylation target residues of ZNF76.

Sumoylation Regulates the Transcriptional Repression Activity of ZNF76—Because one of the sumoylation target residues, Lys411, lies in the minimal TBP interaction region, GARD, we tested whether that sumoylation by ZNF76 may regulate its interaction with TBP and thus modulate its transcriptional repression function. As shown in Fig. 7a, overexpression of PIAS1 and SUMO-1 significantly inhibited the association between ZNF76 and TBP in co-immunoprecipitation experiments. Consistently, we found that sumoylation of ZNF76 suppressed its transcriptional repression activity in reporter assays. As shown in Fig. 7b, ZNF76 completely abolished p53 activity in the absence of PIAS1 and SUMO-1 overexpression, but its inhibitory effect was reversed when PIAS1 and SUMO-1 were overexpressed. These results suggest that sumoylation of ZNF76 negatively modulates its interaction with TBP and its repression effect.

DISCUSSION

TBP is universally required for eukaryotic transcription, which can be positively or negatively modulated by its interacting proteins. Our data support the conclusion that ZNF76 is a novel TBP associated factor that interacts with TBP through its N and C termini, leading to repression of p53-mediated transactivation and down-regulation of endogenous p53 target genes. The inhibitory effect of ZNF76 on p53-mediated gene expression can be relieved by ectopic expression of TBP. We also identified sumoylation as one of the regulatory mechanisms for the transcriptional repression function of ZNF76. To our knowledge this is the first demonstration of a transcriptional repressor that targets TBP, and the repression activity is regulated by sumoylation. ZNF76 interacts with the N-terminal region of PIAS1, which sumoylates ZNF76 at Lys411. Lysine 411 lies in an essential region of ZNF76 for TBP function. Consistent with a model in which sumoylation negatively regulates interaction between ZNF76 and TBP, overexpression of PIAS1 and SUMO-1 reverse the repressive effect of ZNF76 on p53-mediated transactivation in the reporter assay.

Our conclusion that ZNF76 targets TBP to exert its transcriptional repression function is based on several lines of independent evidence: (i) In reporter assays, we observed a significant repressive effect on p53-mediated transactivation. (ii) TBP is a target through which ZNF76 exerts its transcriptional repression effect on p53-mediated gene expression. (iii) Overexpression of ZNF76(1–375), which lacks the C terminus of ZNF76, or ZNF76K440R, whose C terminus loses the interaction with TBP, failed to repress p53 transactivation. (iv) We identified an 83-amino acid region in ZNF76 that activates rather than inhibits p53 transactivation. The dominant negative effect of the construct suggests that endogenous ZNF76 may contribute to the transcriptional repression effect.

Consistent with our data, several viral proteins inhibit p53 transactivation by targeting TBP. Hepatitis C virus NS5A and...
FIG. 4. ZNF76 targets TBP to repress p53-mediated transactivation. a, ectopic expression of TBP relieves the repressive effect of ZNF76. HEK293 cells were transfected with 25 ng of pG13-luc reporter, 10 ng of pcDNA-HA-p53, 0.2 μg of pcDNA-ZNF76, and 0.5 μg of pMT2-F-TBP alone or in combination as indicated. b, GARD domain enhances p53-mediated transactivation. HEK293 cells were transfected with 25 ng of pG13-luc reporter, 10 ng of pcDNA-HA-p53, 0.2 μg of pcDNA-ZNF76, or pcDNA-ZNF76(362–444) plasmid alone or in combination as indicated. c, ZNF76K400-K411R loses its inhibitory function on p53-mediated transactivation. HEK293 cells were transfected with 25 ng of pG13-luc reporter, 10 ng of pcDNA-HA-p53, 0.2 μg of pcDNA-ZNF76-Myc, or mutant plasmids alone or in combination as indicated. d, ZNF76 interferes with the TBP occupancy on the p21 promoter. HEK293 cells (one 150-mm plate) were transfected with pcDNA-HA-p53 (0.2 μg) with or without pCMV2-FLAG-ZNF76(5 μg). 48 h after transfection, the cells were harvested, and chromatin immunoprecipitation was performed as described under “Materials and Methods.” e, endogenous ZNF76 is not recruited to the p21 promoter. MCF-7 cells were treated with 200 nM adriamycin for 24 h, and the cells then were harvested for chromatin immunoprecipitation experiments.
human papilloma virus E7 repress p53 transactivation by binding to and possibly sequestering TBP (30, 31). Epstein-Barr virus immediate-early protein BZLF1 regulates p53 function in part by suppressing TBP expression (32). Because p53 and TBP can synergistically activate transcription when p53 is bound to DNA (38), it is not surprising that TBP can be targeted to affect p53 transactivation. Several general transcriptional repressors function by interaction with TBP. For example, NC2, a well conserved heterodimer, binds to and stabilizes TBP/TATA complexes (39). Interaction of NC2 with TBP precludes association of TBP with other general transcription factors (6), such as TFIIGA and TFIIB. Proteins such as E1A and Mot1 function as transcriptional repressors by different mechanisms. E1A represses transcription by disrupting the interaction between TBP and the TATA box (3). In the in vitro experiments, the transcriptional repression effect of E1A can be reversed by the addition of recombinant TBP (3), which is similar to our observation that overexpression of TBP relieves the inhibitory effect of ZNF76 on p53-mediated transactivation (Fig. 4a).

**FIG. 5.** PIA1 interacts with ZNF76. a, the zinc finger domain and the C terminus of ZNF76 are required for interaction with PIA1. Plasmid pGADT7-ZNF76 was transformed into yeast strain Y187. PIA1 deletion mutants in pGBK7 vector were transformed into yeast strain AH109. After mating, both yeast growth on SD-HTL and positive \( \beta \) galactosidase activity were indicative of positive interaction. b, N terminus of PIA1 interacts with ZNF76. c, expression of Gal4AD-HA-ZNF76 and its mutants in yeast. d, expression of Gal4DBD-Myc-PIA1(1–390) and various mutants in yeast. e, the interaction between PIA1 and ZNF76 is zinc-dependent. 5 \( \mu \)g of plasmids pFLAG-CMV5-PIA1 and pcDNA-ZNF76-Myc was co-transfected or transfected alone into HEK293 cells, and the cell lysates were immunoprecipitated (IP) with anti-Myc (10 \( \mu \)l) and immunoblotted with anti-FLAG. To detect zinc dependent interaction, 1.3 \( \mu \)M of ZnCl\(_2\) was included in both radioimmune precipitation assay buffer and TNEN buffer as described under “Materials and Methods.”
What impedes TBP from binding TATA DNA in vivo is unclear but could be due to dimerization of TBP (40) or interaction with other inhibitory factors. In chromatin immunoprecipitation analysis (Fig. 4, d and e), we found that ZNF76 was not present in the promoter of p53 target genes, suggesting that ZNF76 acts differently from NC2, which stably binds to TBP/TATA complexes to repress transcription. Instead, ZNF76 prevents the binding of TBP to the promoter in a way similar to the action of TBP dimmers.

There are DNA-binding proteins that repress transcription by interacting with TBP. These include p53 (4), unliganded thyroid receptor (41), and Msx1 protein (42). Among them the most extensively studied is p53. Besides acting as a sequence-specific transcriptional activator, p53 can repress the expression of many genes that lack p53 response elements. The transcriptional repression effect of p53 is at least in part due to its interaction with TBP (4, 43). p53 interacts with TBP in vitro and in vivo (4, 43, 38), and the interaction has been mapped to the N and C termini of p53 (33, 34). Both the N- and C-terminal domains are necessary for p53 to exert its repression function on RNA polymerase II directed transcription. It was suggested that the interaction of the N-terminal domain of p53 with TBP contributes to the transcription activation when bound to promoters (34) However, when p53 is not bound to DNA, its N- and C-terminal domains are needed to interact with TBP to achieve transcriptional repression. Recently, it was shown that by targeting TBP, p53 represses RNA polymerase I- and III-mediated transcription (35, 36). Notably, interaction of ZNF76 with TBP...
is very similar to that of p53; the N-terminal activation region and C-terminal region of ZNF76 interact with TBP, although the interactions are much weaker compared with the full-length ZNF76. Like p53, both N- and C-terminal regions are required for ZNF76 to repress transcription. Apparently, the interaction of either the N or C terminus of ZNF76 alone with TBP is not sufficient to achieve the repression effect. ZNF143 shows high sequence similarity with ZNF76 in the N terminus and the zinc finger domains but has low similarity in the C terminus (22). Consistently, we did not detect significant transcription repression effect for ZNF143 (data not shown). We speculate that during evolution, the sequence divergence in the C-terminal region renders ZNF76 a novel transcriptional repressor through the interaction of its C terminus with TBP.

Our studies raise the possibility that the repression effects of sumoylation observed on several transcription factors are due to the loss of interaction with the general transcription machinery. Sumoylation of transcription factors has varied effects. Although sumoylation was shown to be a mechanism that inhibits the transcriptional activity of Sp3 (19, 20), c-Myb (21), and the androgen receptor (18), the exact mechanism that mediates repression by sumoylation is unclear. Sumoylation of p53 has been shown to alter its transcriptional activity (16, 17); however, the data is controversial in part because of the use of different cell types and/or different reporter constructs by different investigators. In our system, p53 was weakly sumoylated, but no significant change in p53-mediated transactivation was detected when PIAS1 and SUMO-1 were overexpressed (Fig. 7b). Nonetheless, in this study, sumoylation was identified as a regulatory mechanism for the interaction between ZNF76 and a crucial component of general transcription machinery, TBP. A major site of sumoylation, Lys+111, is located within a region essential for TBP interaction. Our study indicates that one possible mechanism to achieve the repression by sumoylation is to regulate the interaction between transcription factors and general transcription machinery.

In summary, we have identified ZNF76 as a novel transcriptional repressor that targets TBP. Sumoylation of ZNF76 by PIAS1 occurs in its TBP interaction region and negatively regulates the transcriptional repression function of ZNF76. Because TBP is required for expression of nearly all eukaryotic genes, study of the function of ZNF76 as a TBP inhibitor will provide fundamental knowledge about normal cellular processes and how alteration in these processes can contribute to developmental abnormalities and various disease states.

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