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SUMOylation of ATF3 Alters its Transcriptional Activity on Regulation of *TP53* Gene

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ABSTRACT

Cyclic AMP-dependent transcription factor-3 (ATF3), a stress sensor, plays an essential role in cells to maintain homeostasis and has diverse functions in cellular survival and death signal pathways. ATF3 is a novel regulator of p53 protein stability and function. The activities of ATF3 are modulated by post-translational modifications (PTMs), such as ubiquitination, but whether it is modified by small ubiquitin-related modifier (SUMO) remains unknown. The aim of this study was to investigate whether ATF3 is post-translationally modified by SUMO proteins and also to elucidate SUMOylation of ATF3 on *TP53* gene activity. Here we report that ATF3 is clearly defined as a SUMO target protein both in vitro SUMOylation assay using recombinant proteins and at the cellular levels. Furthermore, ATF3 interacted with UBE2I, the only SUMO E2 enzyme found so far. In addition, PIAS3β (a SUMO E3 ligase) enhanced and SENP2 and SENP7 (two SUMOylation proteases) decreased SUMOylation of ATF3, respectively. Finally, we found that ATF3 is selectively SUMOylated at lysine residue 42 but the SUMOylation does not alter subcellular localization of ATF3. We then characterized the functional role of ATF3 SUMOylation on *TP53* gene expression. We found that SUMOylation of ATF3 is required for full repression of *TP53* gene. Overall, we provide the first evidence that ATF3 is post-translationally modified by SUMO and SUMOylation of ATF3 plays a functional role in regulation of *TP53* gene activity. J. Cell. Biochem. 114: 589–598, 2013.

KEY WORDS: ATF3; SUMOYLATION; p53, TRANSCRIPTIONAL ACTIVITY

Cyclic AMP-dependent transcription factor-3 (ATF3), a 22-KDa (181 amino acid residues) nuclear protein, which is ubiquitiously expressed in most human and mouse tissues, belongs to the basic-region leucine zipper (bZIP) transcription factor family of proteins. ATF3 binds the ATF/cAMP response element (CRE) of many viral and cellular promoters to regulate its downstream target genes. Several genes, including *CCNE2* (*Cyclin E2*), *CD82* (*KAI1*), *DDIT3* (*GADD153*), *LDLR* (*LDL receptor*), *SNAI1*, and *TP53* genes [Hagiya et al., 2011; Liu et al., 2011; Lim et al., 2011; Yan et al., 2011], have been identified as ATF3 target genes. Normally, ATF3 expression is maintained at low levels in quiescent cells [Lu et al., 2006]. *ATF3* is a stress-inducible and/or adaptive response gene, as its mRNA and protein levels dramatically increase upon exposure of cells to stress signals including those initiated by cytokines, genotoxic agents, infections, nerve injury, tissue damage, or physiological stress [Hai et al., 1999]. For example, up-regulation of ATF3 is an important marker of neuronal injury. ATF3 also plays a crucial role in the development of cardiac hypertrophy [Zhou et al., 2011]. Emerging evidence has implicated ATF3 in host defense against invading pathogens and many cancers, such as breast and prostate cancers. For example, ATF3 is involved in immune regulation and immune-surveillance by suppressing Toll-like receptor (TLR) signaling pathways [Kawai and Akira, 2006]. Many lines of evidence have characterized ATF3 as an oncogene [Yin et al., 2008]. ATF3 overexpression was found in human breast and prostate cancers, and Hodgkin lymphomas. However, some evidence suggests that ATF3 may be able to inhibit tumorigenesis [Huang et al., 2008]. ATF3 expression is decreased in human colorectal cancer, and ATF3 overexpression results in apoptosis of human prostate cancer cells. Extensive studies have suggested that

Abbreviations: ATF3, cyclic AMP-dependent transcription factor-3/activating transcription factor-3; p53, cellular tumor antigen p53; TP53, gene name for p53 protein; SUMO, small ubiquitin-like modifier; SENP, sentrin-specific protease; PIAS3, E3 SUMO-protein ligase PIAS3; PIASy, E3 SUMO-protein ligase PIASy; CDKN1A, cyclin-dependent kinase inhibitor 1 (p21).

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ATF3 does not function in these processes alone and is known to directly and/or indirectly cooperate with transcription factors and cell cycle regulators such as the tumor suppressor protein p53 which induces growth arrest and/or apoptosis. First, ATF3 is a negative regulator of *TP53* gene expression [Kawauchi et al., 2002; Yoshida et al., 2008]. Second, ATF3 binds to and stabilizes p53 by blocking its ubiquitination [Yan et al., 2005]. Moreover, MDM2 binds to and ubiquitinates ATF3 by accelerating its degradation [Mo et al., 2010]. These results indicate that ATF3 may serve as a p53 activator in stabilizing p53 protein or as a p53 inhibitor in down-regulating *TP53* transcriptional activity. More recently, a study showed that increased ATF3 expression accounts for suppression of p53-dependent senescence and enhances tumorigenic potential in squamous skin cancer [Wu et al., 2010]. These results suggest that ATF3 plays multiple functional roles in host defense, immunity, and cancer development, but the mechanism remains largely unclear.

Post-translational modifications (PTMs) occur in a variety of proteins and are crucial for normal physiological functions in cells. Among them, the small ubiquitin-related modifier (SUMO) family, which is highly conserved from yeast to humans, has been shown to regulate and influence diverse cellular processes and pathways, including cancer development and metastasis [Kubota et al., 2011], cell cycle regulation [Wang et al., 2011], chromosome segregation [Pebernard et al., 2008], DNA repair [Dou et al., 2010], formation of sub-nuclear structures [Sydorskyy et al., 2010], nuclear-cytoplasm transport [Grünwald and Bono, 2011], protein stability [Yan et al., 2010], and regulation of transcription [Yang et al., 2009; Abed et al., 2011]. In mammals, four SUMO paralogs (SUMO1 to -4) are encoded by distinct genes. SUMO1 has approximately 45% identity to either the closely related SUMO2 or SUMO3 [Saitoh and Hinchey, 2000]. In contrast to SUMO1, SUMO2, and SUMO3 contain a clear consensus SUMOylation site in their N-terminal regions, indicating that SUMO2 and SUMO3 are capable to form poly-SUMO chains. Currently, the biological role of SUMO4 remains largely unknown. Despite limited sequence identity, SUMO proteins share with ubiquitin a common three-dimensional structure and use a similar conjugation mechanism, an enzyme-controlled cycle. Newly translated SUMO proteins are processed by specific SUMO proteases (SENPs) to remove C-terminal residues in SUMO and to expose a conserved di-glycine motif [Johnson, 2004]. After this initial cleavage step, SUMO is then activated by the heterodimeric E1-activating enzyme SAE1/SAE2 in an ATP-dependent manner. The thioester-linked SUMO is then transferred to the SUMO-specific E2-conjugating enzyme UBE2I, which in turn recognizes specific substrates and catalyzes the formation of an isopeptide bond between the lysine residue of target protein and the glycine residue of SUMO. This step can be further facilitated by SUMO E3 ligases such as the PIAS family of proteins [Sachdev et al., 2001; Chun et al., 2003]. Covalent modification of proteins by SUMO is short-lived and reversible through action of the SENP family of proteases. Even though the structure and enzymological pathway of conjugation of SUMO are very similar to those of ubiquitin, the biological functions of SUMOylation are much different from ubiquitination [Martin et al., 2007]. SUMOylation of certain proteins prevents their ubiquitin-mediated proteasomal degradation [Martin et al., 2007; Wilkinson and Henley, 2010]. Most importantly, SUMO modification of numerous transcription factors is associated with inhibition of transcription [Gong et al., 2006; Rytinki and Palvimo, 2008; Yang et al., 2009; Abed et al., 2011] or activation of transcription [Duverger et al., 2011]. Overall, the cellular SUMOylation system affects the function of numerous nuclear proteins, oncoproteins, and tumor suppressor proteins such as p53. Thus, understanding the regulation of protein SUMOylation is important for various biological processes such as transcriptional regulation and cancer development.

The protein activity level of ATF3 has been shown to be modulated by PTMs, such as ubiquitination [Yan et al., 2005; Mo et al., 2010]. A recent report has shown that MDM2 binds to basic region of ATF3 resulting in the addition of ubiquitin to ATF3 leucine zipper domain [Mo et al., 2010]. Since ATF3 is a nuclear protein and the biological functions of SUMOylation are much different from ubiquitination, we thus investigated whether ATF3 could be SUMOylated and the potential function of ATF3 SUMOylation on transcriptional activity of *TP53*. Here we identify ATF3 as a target for modification by the SUMO machinery and provide evidence demonstrating SUMOylation of ATF3 plays a functional role in regulating *TP53* gene.

MATERIALS AND METHODS

REAGENTS

All cell culture reagents and protein A-agarose were purchased from Invitrogen (Carlsbad, CA). Antibodies against ATF3, p53, UBE2I (also known as UBC9), Tubulin, and Lamin A/C were purchased from Santa Cruz Biotechnology Inc., (Santa Cruz, CA). Antibodies against SUMO1 and SUMO3 were purchased from Active motif (Carsbad, CA). Antibodies against HA and HIS were purchased from Origene (Rockville, MD). Antibodies against FLAG and β-Actin were purchased from Sigma (St. Louis, MO). Luciferase activity was measured using the Dual Luciferase Assay System (Promega, Madison, WI). Ni-NTA agarose was purchased from QIAGEN (Valencia, CA).

DNA CONSTRUCTS

Human ATF3 plasmid (pBP-*ATF3*) was kindly provided by Dr. Tsonwin Hai (Ohio State University, Columbus, OH). We then subcloned human *ATF3* cDNA into pcDNA3.1(+) to create pcDNA3-*ATF3* expression plasmid (with 6× HIS and FLAG tags). K42R *ATF3*, K136R *ATF3*, and 2KR (K42RK136R) *ATF3* plasmids were generated by PCR-based mutagenesis (QuikChange Lightning site-directed mutagenesis kit, Strategene, La Jolla, CA). pcDNA3-*ATF3*-*ΔZIP* plasmid was constructed by removing the C-terminal DNA binding region of ATF3. Human HA-*SUMO1* cDNA, HA-*SUMO2* cDNA, and HA-*SUMO3* cDNA were PCR-amplified and ligated into the HindIII and BamHI sites of pcDNA3(+) to create pcDNA3-HA-*SUMO1*, pcDNA3-HA-*SUMO2*, and pcDNA3 HA-*SUMO3* expression plasmids, respectively. N-terminally HA-tagged *SUMO2*-K42R *ATF3* (for mimicking SUMOylated ATF3) expression plasmid was generated by ligating BamHI fragment from *ATF3* cDNA into the same site of HA-*SUMO2* pcDNA3. Human *UBE2I* cDNA was PCR-amplified by using forward primer 5′-ACGAGGATCCATGTCGGG-

GATCGCC-3′ and reverse primer 5′-TCGTGAATTCTTATGAGGGCG-CAAACTT-3′ and digested with BamHI and EcoRI and then ligated into the BamHI and EcoRI sites of pcDNA3(+) to create pcDNA3-*UBE2I* expression plasmid. FLAG-tagged *PIAS3β* was PCR-amplified by using forward primer 5′-TCGTAAGCTTATGGATTA-CAAGGATGACGACGATAAGGTGATGAGTTTCCGGGTG-3′ and reverse primer 5′-ACGATCTAGATCAGTCCAAGGAAATG-3′ and digested with HindIII and XbaI and then ligated into the HindIII and XbaI sites of pcDNA3.1(+) to create pcDNA3-FLAG-*PIAS3β* expression plasmid. FLAG-tagged *PIAS3y* was PCR-amplified by using forward primer 5'-TCGTGCTAGCATGGATTACAAGGATGAC-GACGATAAGGCGGCGGA GCTGGTGGAG-3' and reverse primer 5'-ACGATCTAGATCAGCAGGCCGGCACC-3' and digested with NheI and XbaI and then ligated into the NheI and XbaI sites of pcDNA3.1(+) to create pcDNA3-FLAG-*PIAS3y* expression plasmid. HA-tagged *SENP2* was PCR-amplified by using forward primer 5'-TCGTGGTACCATGTACCCTTACGACGTTCCTCATTACGCTATGTAC-AGATGGCTG-3' and reverse primer 5'-ACGAGAATTCTCACAG-CAACTGCTGATGAAGGATTTC-3' and digested with KpnI and EcoRI and then ligated into the KpnI and EcoRI sites of pcDNA6(A) to create pcDNA6-HA-*SENP2* expression plasmid. HA-tagged *SENP7* was PCR-amplified by using forward primer 5'-TCGTGGTACCATG-TACCCTTACGACGTTCC TCATTACGCTATGGACAAGAGAAAG-3' and reverse primer 5'-ACGAGCGGCCGCCTAGCTACTGCTGCCC-3' and digested with KpnI and NotI and then ligated into the KpnI and NotI sites of pcDNA6(A) to create pcDNA6-HA-*SENP7* expression plasmid. Both human pCMV6-*TP53* and pCMV6-*ATF3-GFP* expression plasmids were purchased from Origene. We then subcloned human *TP53* cDNA into pcDNA3.1(+) to create the pcDNA3-*TP53* expression plasmid. pCMV6-K42R *ATF3-GFP* and pCMV-SUMO2K42R *ATF3-GFP* expression plasmids were generated by PCR-based mutagenesis from pCMV6-*ATF3-GFP* and pCMV6-SUMO2 *ATF3-GFP* plasmids, respectively. *TP53* promoter luciferase plasmid (2.4Kb upstream the transcription start site) was kindly provided by Dr. Asao Noda (Radiation Effects Research Foundation, Japan) and Dr. Junya Kawauchi (Tokyo Medical and Dental University, Japan). All constructs were verified by nucleotide sequencing.

CELL CULTURE AND TRANSFECTION

H1299, COS7, HepG2, PC3, MCF7, DU145, HEK293, and Y1 cells were purchased from the American Type Culture Collection. COS7, MCF7, DU145, HEK293, and HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) in the presence of 10% fetal bovine serum and antibiotics (GIBCO) in humidified air containing 5% CO_2, at 37°C. H1299 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics in humidified air containing 5% CO_2, at 37°C. PC3 cells were maintained in F-12K medium supplemented with 10% fetal bovine serum and antibiotics in humidified air containing 5% CO_2, at 37°C. Y1 cells were maintained in DMEM supplemented with 7.5% horse serum and 2.5% fetal bovine serum and antibiotics in humidified air containing 5% CO_2, at 37°C. After incubation, the cells were transfected using Fugene HD Transfection Reagent (Roche). Approximately 45–8 h after transfection, the cells were harvested. Luciferase activity was measured and normalized with Renilla activity. All experiments were performed three times in triplicate.

IMMUNOPRECIPITATION ASSAYS

H1299, MCF7, or PC3 cells (2×10^6) were seeded onto 10-cm plates. Twenty-four hours after transient transfection, cells were harvested and lysed in lysis buffer (40 mM HEPES, 120 mM sodium chloride, 10 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 1 mM EDTA, 50 mM sodium fluoride, 0.5 mM sodium orthovanadate, 1% Triton X-100) containing protease inhibitor cocktail (Sigma), followed by rotation for 1 h at 4°C to solubilize proteins. Soluble protein was collected and immunoprecipitated with the indicated antibody overnight. Protein A agarose beads were added to protein lysates for 2 h at 4°C. Beads were centrifuged and washed at least three times with lysis buffer. For Ni^{2+}-bead pull-down assays, Ni^{2+}-NTA agarose was used to precipitate HIS-tagged ATF3 from cell lysates. Proteins were eluted by boiling in 50 μl of 2× Laemmli sample buffer, resolved by 8% SDS–PAGE, and processed for immunoblotting as described below.

IMMUNOBLOTTING

Protein lysates were allowed to rotate at 4°C for 30 min, and protein contents of the high-speed supernatant were determined using the BCA™ Protein Assay kit assay (Pierce/Thermo Scientific, Rockford, IL). Equivalent quantities of protein (20–45 μg) were resolved on polyacrylamide-SDS gels, transferred to nitrocellulose membrane (Bio-Rad), and immunoblotted with specific antibodies. Results were visualized using the Supersignal West Dura Extended Duration Substrate kit (Pierce Chemical Co., Rockford, IL.). Band intensity was quantified using the ImageJ program.

CELL-FREE SUMOYLATION ASSAYS

The cell-free SUMOylation assays were carried out using a SUMOlink kit according to the manufacturer's instructions (Active motif, Carsbad, CA). Wild-type (WT) ATF3 full-length recombinant proteins with a GST tag (from Abnova, Taiwan) were incubated with WT SUMO1 (or WT SUMO3) or SUMO1 mutant (or SUMO3 mutant) along with SUMO E1 activating and SUMO E2 conjugating enzymes at 30°C for 3 h. The reaction was stopped by adding an equal volume of 2× SDS–PAGE loading buffer and samples were processed to immunoblotting using anti-ATF3 or anti-SUMO1 (or anti-SUMO3) antibodies.

IN VIVO SUMOYLATION ASSAYS

The in vivo SUMOylation assay was carried out as previously described [Yang et al., 2009]. Briefly, COS7, MCF7, HepG2. DU145, HEK293, or Y1 cells (2×10^6) were seeded in 10 cm plates and 24 h later were transfected with indicated HIS-FLAG-*ATF3* and HA-*SUMO1* expression vectors. After 48 h, cells were harvested in 700 μl lysis buffer (500 mM NaCl, 10 mM imidazole, 45 mM Na_2HPO_4, 5 mM Na_2H2PO_4, 8 M urea, pH 8.0) containing complete protease inhibitors without EDTA (1 tablet/10 ml; Roche) and sonicated. Lysates were cleared and incubated with 100 μl of 50% Ni^{2+}-NTA agarose (QIAGEN) at room temperature for 60 min on a rotator. The resin was washed three times in wash buffer 1 (400 mM NaCl, 10 mM imidazole, 17.6 mM Na_2HPO_4, 32.4 mM $Na_2H_2PO_4$, 8 M urea, pH 6.75), washed three times in wash buffer 2 (150 mM NaCl, 10 mM imidazole,

17.6 mM Na_2HPO_4, 32.4 mM $Na_2H_2PO_4$, pH 6.75). Samples were resuspended in 2× EDTA SDS–PAGE sample buffer. Samples (20 μl) were resolved by 8% SDS–PAGE and processed for immunoblotting using anti-ATF3, anti-FLAG, anti-HA, or anti-SUMO1 primary antibody. Images were captured in a Kodak Image Station 440 CF using Super Signal West Femto substrates (Thermo scientific/Pierce, Rockford, IL).

CELLULAR LOCALIZATION STUDY

PC3 cells cultured on six-well plate were transfected with GFP, WT *ATF3-GFP*, or K42R *ATF3-GFP*, or *SUMO2*-K42R *ATF3-GFP* expressing plasmids for 2 days. Cells were counterstained with DAPI and images were obtained with a fluorescence microscope (Olympus DP72) and camera.

STATISTICAL ANALYSIS

Statistical analyses were performed using Student's *t*-test or a one-way ANOVA when more than two groups were compared. After ANOVA analysis, the post-hoc multiple comparisons were performed by using the Tukey honestly significant difference (HSD) test to determine the statistical difference from each other among subgroups. For each test, *P*-values of <0.05 was considered significant.

RESULTS

ATF3 IS A SUBSTRATE FOR MODIFICATION BY SUMO

ATF3 harbors two evolutionarily conserved sequences that conform to the SUMOylation consensus (Fig. 1A). To examine the SUMO modification of ATF3, we first performed cell-free SUMOylation assays by using the purified GST-ATF3 protein as a substrate. The assay includes the SUMO-activating enzyme (E1), UBE2I (E2), and WT SUMO1 (or WT SUMO3) or mutated SUMO1 (or mutant SUMO3), to which GST-ATF3 protein was added. Western blot analysis of the preparation using anti-ATF3 or anti-SUMO1 (or anti-SUMO3) antibodies (Fig. 1B) revealed that a slowly migrating ATF3-immunoreactive band (SUMOylated ATF3) was detected only in samples coexpressing ATF3 and WT SUMO1 (or SUMO3). Importantly, the SUMOylated ATF3 band was not observed in samples co-expressing ATF3 and mutant SUMO1 (or mutant SUMO3). Notably, and as is the case for most SUMOylated proteins, the content of ATF3 modification appeared to be relatively low ($<10\%$). We next tested whether ATF3 can be SUMOylated by

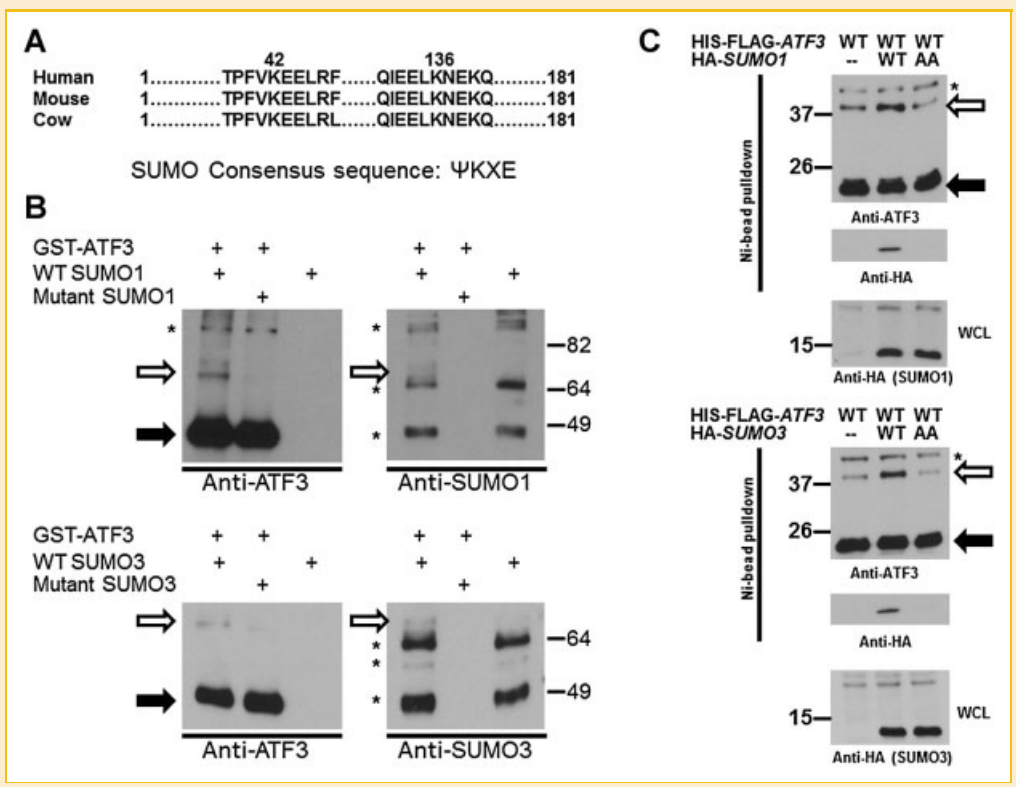

Fig. 1. ATF3 can be SUMOylated. A: Sequence alignment of the human, mouse, and cow ATF3 proteins showing the regions that contain the two potential SUMO sites (K42 and K136). B: In vitro SUMOylation of ATF3 with SUMO1 WT (or SUMO3 WT) and mutated forms. GST-ATF3 protein was incubated with E1 and E2 enzymes and either WT or mutated SUMO1 (or SUMO3) at 30°C for 3 h. Western blot analysis was performed by immunoblotting with ATF3 antibody (left) or SUMO1 (or SUMO3) antibody (right). (*) indicates non-specific band. C: MCF7 cells were transiently transfected with 3 μg HIS-FLAG-tagged WT ATF3 and 2 μg HA-SUMO1 or HA-SUMO3 (WT or AA mutant) expression vectors as indicated. After 48 h, cells were harvested and the cell lysates were subjected to Ni^{2+} bead pulldown, followed by anti-ATF3 or HA immunoblotting. Whole cell lysates (WCL) were subjected to anti-HA immunoblotting for SUMO1 or SUMO3 expression. The empty arrows indicate SUMOylated ATF3. The solid arrows indicate non-SUMOylated ATF3. (*) indicates non-specific band.

SUMO1 and SUMO3 (which is closely-related to SUMO2) in mammalian cells. MCF7 breast cancer cells were transiently transfected with HIS-FLAG tagged *ATF3* expression plasmids with or without HA-tagged *SUMO1* or *SUMO3* (WT or AA mutant) expression plasmids. Western blot analysis (Fig. 1C) of the ATF3 preparations by Ni^{2+} chelate chromatography under denaturing condition revealed that a slowly migrating species (about 40 KDa) was detected in cells expressing WT ATF3 alone. Overexpression of WT SUMO1 or WT SUMO3 significantly enhanced ATF3 SUMOylation. However, AA mutant SUMO1 and AA mutant SUMO3, which the di-glycine residues of SUMO1 and SUMO3 were mutated to di-alanine residues, respectively, could not increase ATF3 SUMOylation. Together, these results indicate that ATF3 can be SUMOylated in cell-free system and in mammalian cells by SUMO1 and SUMO3 (closely-related to SUMO2).

ATF3 IS SUMOYLATED AT LYSINE 42

To facilitate the analysis of ATF3 SUMOylation, we next created HIS-FLAG-tagged mutant forms of ATF3 in which the acceptor lysines within the two SUMOylation motifs were replaced with arginines (Fig. 2A). Importantly, these mutant forms of ATF3 can be readily isolated and distinguished by virtue of the associated HIS and FLAG tags. To determine which lysine residues in ATF3 are modified by endogenous SUMO, we probed ATF3 preparations isolated from COS7 cells by Ni^{2+} chelate chromatography under denaturing condition. As can be seen in Figure 2B, a slowly migrating species (about 40 KDa) was detected in cells expressing WT ATF3. We interpret this form as being ATF3 modified by endogenous SUMO. Moreover, this species is not observed in cells expressing an ATF3 form with mutations in both SUMOylation motifs (2KR). Interestingly, disruption of the second motif (K136R) produced no significant reduction in ATF3 SUMOylation. In contrast, mutation of K42 led to a complete loss of detectable SUMOylation. We observed similar result by using MCF7 breast cancer cells (Fig. 2B bottom). To further confirm K42 is the major SUMO site for ATF3, we coexpressed ATF3 with HA-tagged SUMO1 in MCF7 breast cancer cells. As can be seen in Figure 2C, exogenous SUMO1 enhanced SUMOylation on WT ATF3 but not on K42R ATF3. We also observed similar results from four different cell lines, including HepG2, Y1, DU145, and HEK293 cells (see supplementary Fig. S1A–D). Taken together, these results indicate that ATF3 can be SUMOylated in vivo in cell-based system and K42 is the major SUMO site for ATF3.

ATF3 INTERACTS WITH UBE2I

Since ATF3 can be SUMOylated and UBE2I is the sole E2 enzyme involved in the SUMOylation pathway, we next investigated whether ATF3 physically interacts with UBE2I in cells. HIS-tagged *ATF3* expression plasmid or pcDNA3 backbone vector was co-

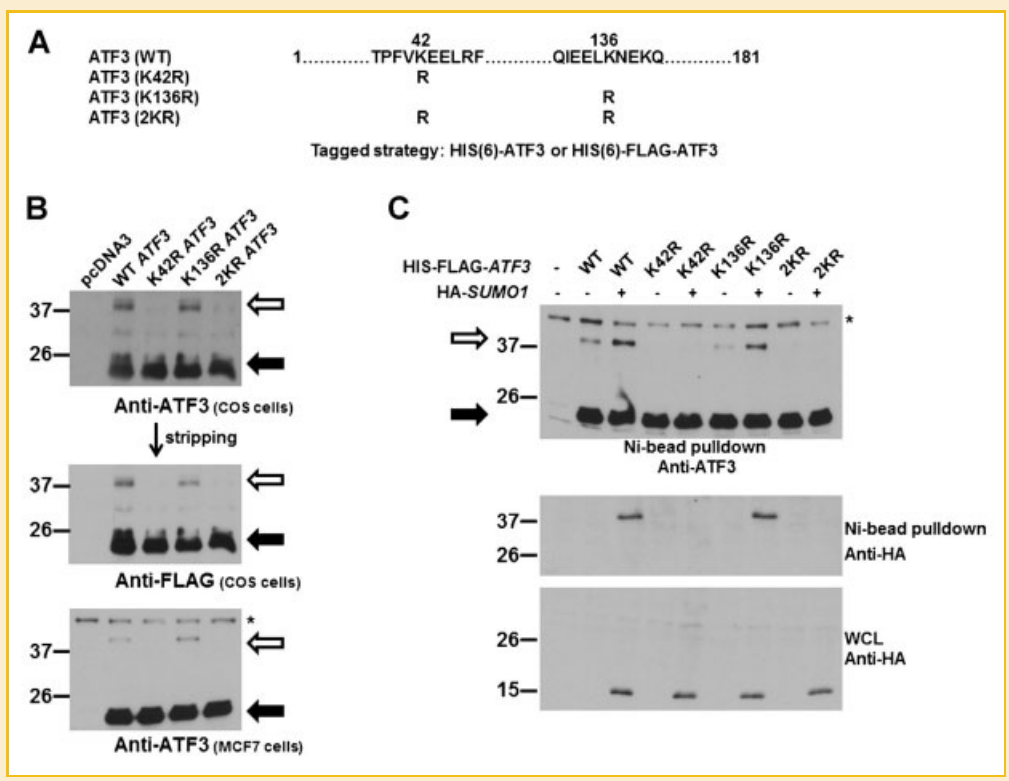

Fig. 2. Lysine 42 is the major SUMO site in ATF3. A: Schematic representation of the human ATF3 protein with the lysine-to-arginine ATF3 mutants generated in this study to determine potential SUMOylation sites on ATF3. B: Lysates of COS7 or MCF7 cells transiently transfected with 3 μg HIS-FLAG-tagged WT *ATF3* or *ATF3* in which lysine 42 (K42R), lysine 136 (K136R), or both lysines (2KR) were mutated to arginine were subjected to Ni^{2+} bead pulldown, followed by anti-ATF3 immunoblotting. For COS cell experiments, the membrane was then stripped and subjected to anti-FLAG immunoblotting. C: MCF7 cells were transiently transfected with 3 μg HIS-FLAG-tagged *ATF3* (WT or K42R) and 1 μg HA-*SUMO1* expression vectors as indicated. After 48 h, cells were harvested and the cell lysates were subjected to Ni^{2+} bead pulldown, followed by anti-ATF3 or anti-HA immunoblotting. The empty arrows indicate SUMOylated ATF3. The solid arrows indicate non-SUMOylated ATF3. WCL indicates whole cell lysates. (*) indicates non-specific band.

transfected with *UBE2I* plasmid in PC3 prostate cancer cells for 48 h, and the HIS-tagged ATF3 was precipitated by Ni^{2+} bead. As can be seen in Figure 3A, HIS-tagged ATF3 was expressed in PC3 cells and was precipitated (WCL and Ni-bead pulldown). UBE2I was also present in the cell lysate (WCL), and was co-precipitated with HIS-tagged ATF3 (Ni-bead pulldown). This result indicates that UBE2I binds to ATF3 and subsequently facilitates SUMOylation of ATF3 in vivo.

SUMOYLATION OF ATF3 IS ALTERED BY PIAS AND SENP PROTEINS

Generally, SENPs are responsible for activating and de-conjugating SUMO from target proteins. In particular, SUMO is de-conjugated from substrates by SENP1 and SENP2 in mammals [Mukhopadhyay and Dasso, 2007]. Therefore, we next examined whether SENP2 and/or SENP7 deSUMOylate ATF3. First, we confirmed lysine 42 of ATF3 is modified by endogenous SUMO machinery in PC3 cells. HIS-tagged WT *ATF3* or K42R *ATF3* plasmid was transfected in PC3 cells for 48 h. The cell lysate was then precipitated by Ni-bead pulldown. As shown in Figure 3B, a SUMOylated ATF3 band was observed in cells expressing WT ATF3 but not in cells expressing K42R ATF3. Next, as shown in Figure 3C, both SENP2 and SENP7 reduced ATF3-SUMO modification (40 and 30% reduction, respectively). These findings clearly indicate that SENP2 and SENP7 are involved, but not the only SENP proteins, in mediating the deSUMOylation of ATF3.

It is well known that E3 ligases, such as PIAS family of proteins, in the SUMO conjugation cycle function as adaptors that stabilize the interaction between the acceptor substrates and the UBE2I-SUMO thioester. Therefore, we next investigated whether the stability of the SUMO-UBE2I thioester and ATF3 is increased by PIASy and/or PIAS3β, two of PIAS family of proteins, to facilitate ATF3 SUMOylation. SUMO conjugation to ATF3 was analyzed in PC3 cells transiently expressing FLAG-tagged PIASy and PIAS3β (Fig. 3D). The intensities of the SUMOylated ATF3 bands were increased 4.7-fold when PIAS3β was co-expressed but not PIASy. These results indicate that PIAS3β, at least in part, enhances the SUMOylation of ATF3.

THE K42R MUTATION DOES NOT AFFECT ATF3 CELLULAR LOCALIZATION

Given that the regulatory effects of SUMOylation can be accompanied by alterations in the subcellular localization of the modified protein [Guo et al., 2007], we examined whether SUMO conjugation to ATF3 is associated with modulation of its subcellular localization. As can be seen in Figure 4A, subcellular fractionation revealed that WT ATF3 is observable only in the nuclear fractions of

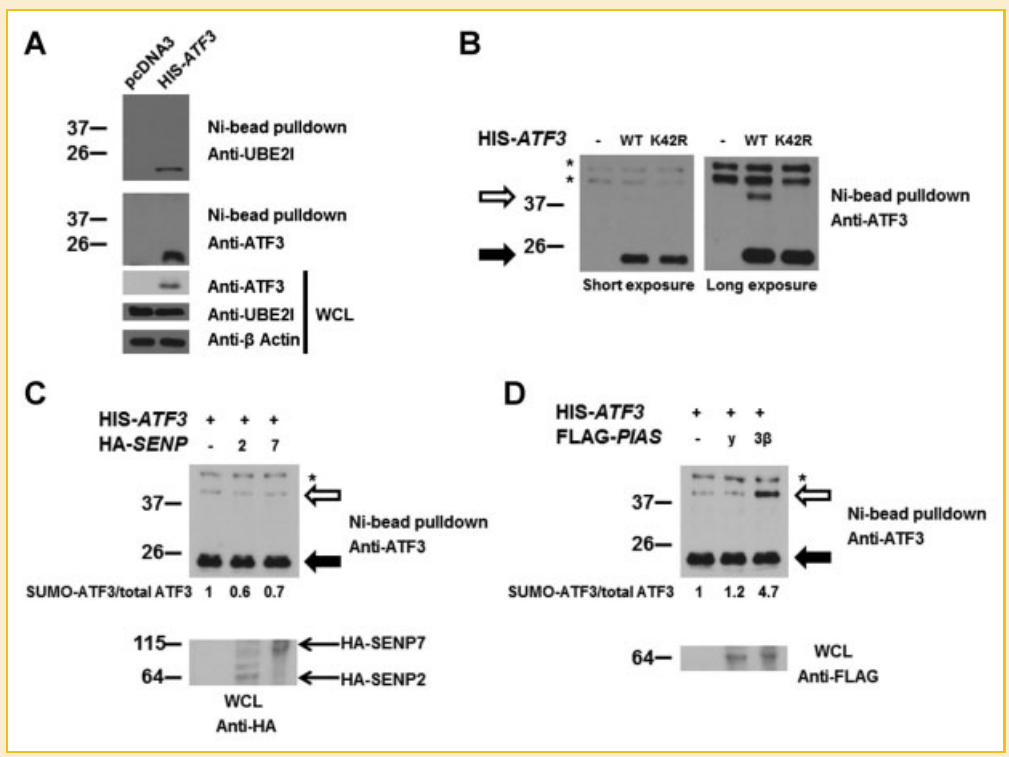

Fig. 3. SUMO machinery is involved in ATF3 SUMOylation. A: ATF3 interacts with UBE2I in PC3 cells. PC3 cells were transfected with different combinations of pcDNA3 vector or HIS-tagged *ATF3* and *UBE2I* expression plasmids. The cell lysates were subjected to Ni^{2+} bead pulldown, followed by anti-ATF3 or anti-UBE2I immunoblotting. WCL indicates whole cell lysates. B: ATF3 SUMOylation in vivo in PC3 cells. Lysates of PC3 cells transfected with 3 μg HIS-tagged WT or K42R *ATF3* were subjected to Ni^{2+} bead pulldown, followed by anti-ATF3 immunoblotting. C: SENPs reduce ATF3 SUMOylation. PC3 cells were co-transfected with or without HA-tagged *SENP2* or HA-tagged *SENP7* and HIS-tagged *ATF3* plasmids. The cell lysates were subjected to Ni^{2+} bead pulldown, followed by anti-ATF3 immunoblotting. WCL indicates whole cell lysates. D: PIAS3β enhances ATF3 SUMOylation. PC3 cells were co-transfected with or without FLAG-tagged PIASy or FLAG-tagged *PIAS3β* and HIS-tagged *ATF3* plasmids. The cell lysates were subjected to Ni^{2+} bead pulldown, followed by anti-ATF3 immunoblotting. WCL indicates whole cell lysates. The empty arrows indicate SUMOylated ATF3. The solid arrows indicate non-SUMOylated ATF3. (*) indicates non-specific bands.

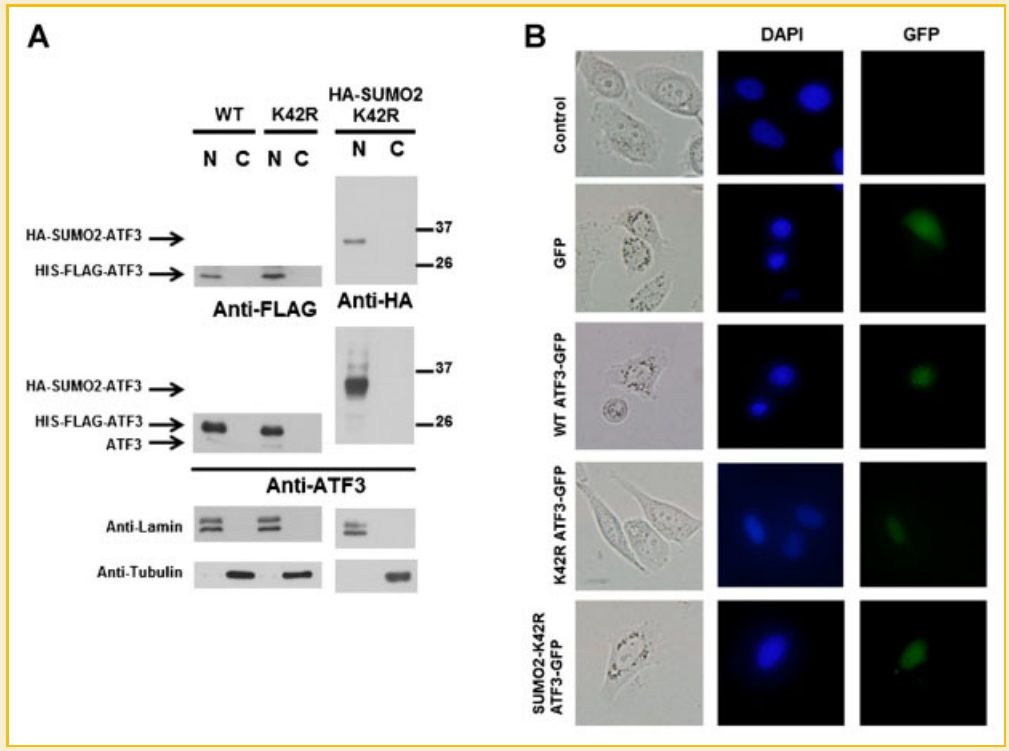

Fig. 4. The K42R mutation does not alter ATF3 subcellular localization. A: Nuclear (N) and cytoplasmic (C) fractions of PC3 cells expressing WT HIS-FLAG-ATF3, K42R ATF3, or HA-SUMO2K42R ATF3 were subjected to anti-ATF3, anti-HA, anti-Lamin, and anti-Tubulin immunoblotting. B: PC3 cells were transfected with *GFP*, *WT ATF3-GFP*, or *K42R ATF3-GFP*, or *SUMO2K42R ATF3-GFP* expressing plasmids for 2 days. Cells were then counterstained with DAPI and observed under a fluorescence microscope.

PC3 cells. Moreover, this distribution was not visibly altered in cells expressing the K42R SUMOylation-deficient ATF3 or in the case of the N-terminal SUMO2 fusion to K42R ATF3, which mimics SUMOylated ATF3 (similar method was demonstrated previously [Yang et al., 2009]). We next transfected PC3 cells with expression vectors bearing *GFP*, WT *ATF3-GFP*, K42R *ATF3-GFP*, or *SUMO2*-K42R *ATF3-GFP*, and used fluorescence to localize the ATF3 proteins. As expected, while GFP alone was localized both in the cytoplasm and the nucleus, WT ATF3-GFP, K42R ATF3-GFP, and SUMO2K42R ATF3-GFP proteins were localized in the nucleus only (Fig. 4B). These results indicate that SUMOylation of ATF3 does not alter subcellular localization of ATF3 and is in favor of an intranuclear action for this modification.

THE K42R MUTATION IN ATF3 RELIEVES THE REPRESSION OF TP53 GENE PROMOTER BY ATF3

Because ATF3 is a negative regulator of *TP53* gene expression [Kawauchi et al., 2002; Yoshida et al., 2008] and to gain insight into the role of SUMO modification of ATF3, we assessed the effect of this modification on ATF3-dependent transcription using a natural *TP53* promoter. As can be seen in Figure 5A (H1299 cells) and 5B (HepG2 cells), expression of WT ATF3 leads to a robust reduction in the activity of a *TP53* promoter-driven luciferase reporter. Notably, while expression of the SUMO2-K42R-ATF3 (for mimicking SUMOylated ATF3) further repressed the activity of *TP53* promoter-driven luciferase reporter, expression of the SUMOylation-deficient K42R mutant relieved the reduction by 15–5%. The similar result was observed in p53-positive MCF7 cells (Fig. 5C), while ATF3 without DNA binding motif (ΔZIP) was not able to repress *TP53* promoter. The expression levels of WT ATF3 and ATF3 mutants in H1299 and MCF7 cells from the reporter assays were validated in Figure 5D. These findings indicate that recruitment of SUMO to ATF3 plays a role, at least in part, for its full inhibitory effect on natural *TP53* transcription.

DISCUSSION

Post-translational protein modifications by the SUMO family of proteins have been established as critical events in cell cycle regulation, cell death, genomic instability, transcriptional regulation, tumorigensis, and their interaction with other proteins. The PTMs of ATF3 such as ubiquitination [Yan et al., 2005; Mo et al., 2010] play an important role in the regulation of ATF3 activity. Here, we have demonstrated for the first time that ATF3 can be SUMOylated and lysine 42 is the major SUMO site. We further demonstrated that the full repressive activity of *TP53* gene activity by ATF3 requires SUMOylation in ATF3, suggesting that ATF3 plays a functional role in regulating *TP53* gene promoter activity.

Several lines of evidence have demonstrated that many UBE2I-interacting proteins are SUMOylated [Melchior, 2000; Suda et al., 2011]. In the present study, we demonstrated that ATF3 interacts with the E2-conjugating enzyme, UBE2I, in a mammalian cell

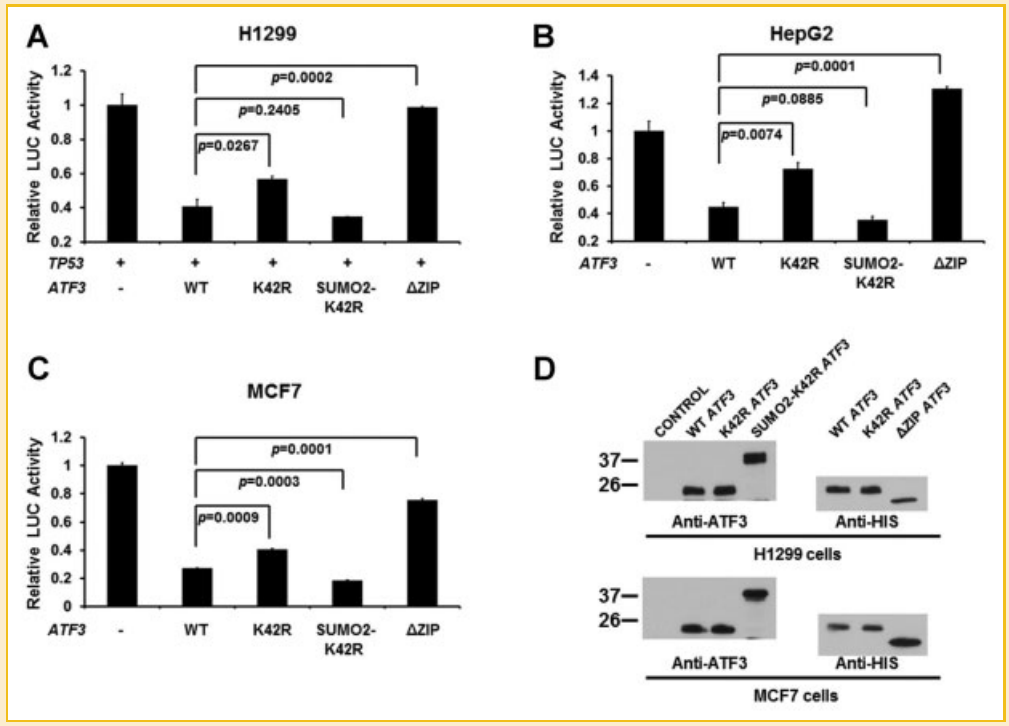

Fig. 5. Loss of SUMOylation relieves the repression of *TP53* promoter by ATF3. A: H1299 cells were transfected, where indicated, with *TP53* expression plasmid (0.02 μg), HIS-tagged WT *ATF3*, HIS-tagged K42R *ATF3*, HA-tagged SUMO2-K42R *ATF3* or HIS-tagged ΔZIP *ATF3* expression plasmid (0.2 μg), and a reporter plasmid with *TP53* natural promoter (0.1 μg). B: HepG2 cells were transfected with WT *ATF3*, K42R *ATF3*, SUMO2-K42R *ATF3*, or ΔZIP *ATF3* expression plasmid (0.2 μg), and a reporter plasmid with *TP53* natural promoter (0.1 μg). C: MCF7 cells were transfected with WT *ATF3*, K42R *ATF3*, SUMO2-K42R *ATF3*, or ΔZIP *ATF3* expression plasmid (0.2 μg), and a reporter plasmid with *TP53* natural promoter (0.1 μg). Luciferase activities were measured 48 h after transfection. Luciferase activity was measured and normalized with Renilla activity. Relative LUC activity (fold activation) was calculated and plotted. Experiments were performed three times in triplicate. Error bars indicate standard errors. D: The expression levels of WT ATF3 and ATF3 mutants in H1299 and MCF7 cells from the reporter assays were validated using anti-ATF3 and anti-HIS immunoblotting. Due to lack of C-terminal domain in ΔZIP ATF3, the levels of ΔZIP ATF3 was determined by anti-HIS immunoblotting.

system. Furthermore, the E3 enzyme PIAS3β facilitates ATF3 SUMOylation and SENP2 and SENP7, two of the SUMO proteases, reduce ATF3 SUMO modification. These findings suggest that the typical SUMO machinery anticipates on ATF3 modification. The potential SUMOylation sites in ATF3 are located at lysine 42 and lysine 136 based on the core SUMOylation motif (PsiKXE). Our data showed that lysine 42 was efficiently conjugated by SUMO in vitro and in vivo and that lysine 136 could not be conjugated by SUMO even though its sequence matches the SUMOylation consensus well. This is important to physiological condition and function because lysine 136 is located in the leucine-zipper domain of ATF3. Thus, SUMO conjugation on lysine 136 of ATF3 could disrupt its binding ability and affinity to DNA. These observations reveal that lysine 42 of ATF3 could be important in interactions among transcriptional regulators. Among ATFs, current and previous studies have shown that ATF3 (current study) and ATF7 [Hamard et al., 2007] could be SUMO modified and the SUMO acceptor sites are both located outside the leucine-zipper and basic domains, suggesting the importance of SUMO modification in regulating transcriptional functions of ATFs.

In the current study, we observed that loss of SUMOylation on ATF3 reduces its full repression on the *TP53* gene, suggesting that SUMOylation of ATF3 is required for regulating *TP53* gene activity. This observation is supported by many lines of evidence that SUMO modification of transcription factors play a general role in transcriptional repression, such as SUMOylation of NR5A1 [Campbell et al., 2008; Yang et al., 2009]. However, there are now a growing number of cases which demonstrate SUMOylation plays a significant role in transcriptional activation. For instance, SUMOylation of p53 enhances the ability of p53 to activate target genes, perhaps by competing against MDM2-mediated ubiquitination, which targets p53 for proteasomal degradation [Gostissa et al., 1999; Rodriguez et al., 1999]. Another example is that SUMOylation of TCF4 by SUMO E3 enzyme enhances β-catenin-dependent transcriptional activity of *TCF4* [Yamamoto et al., 2003]. Recently, SUMO modification of DNMT3A, a DNA cytosine methyltransferase, has been shown to disrupt its interaction with HDACs and decreases its ability to repress target genes [Ling et al., 2004]. Overall, the cellular SUMOylation system affects the transcriptional function of numerous nuclear and cytosolic proteins.

It has been suggested that SUMO modification could alter subcellular localization of proteins [Gill, 2005; Castillo-Lluva et al., 2010]. For instance, SUMOylation of numerous nuclear proteins keep them away from the DNA and thus takes them out of *trans*-activation or *trans*-repression action [Zhong et al., 2000; Takahashi et al., 2004; Nagai et al., 2011]. Furthermore, the status of SUMO modification can influence the transport of proteins between the nucleus and the cytoplasm [Finkbeiner et al., 2011]. In the current

study, SUMO modification does not alter subcellular localization of ATF3, suggesting the transcriptional effects of ATF3 SUMOylation are unlikely to be due to alteration in subcellular localization and argue in favor of an intranuclear action for this modification.

During cancer progression, successful cancer cells often manage to foil many stress signals to avoid cell death. ATF3 is a highly conserved transcriptional factor that is ubiquitously up-regulated in the mammalian response to damage signals, such as DNA damage and stress. Recent studies have shown that ATF3 is significantly up-regulated in many human tumors such as breast and prostate tumors. Moreover, ATF3 has been identified as having a dichotomous role: Pro-apoptotic in non-transformed breast cells but promoting metastasis in malignant cells [Yin et al., 2008]. Therefore, ATF3 acts as metastasis-promoting factor and may be a target molecule for the development of new treatment modalities that can overcome resistance to currently available chemotherapeutics. The current study, focusing on the regulation of ATF3 function, might bring the field one step closer to developing new treatment modalities.

In summary, this investigation has demonstrated that ATF3 is post-translationally modified by specific enzymes mediating the SUMO cycle of ATF3 and SUMOylation of ATF3 influences *TP53* gene activity. This PTM by SUMO might be important for regulating overall ATF3 function as well as the function of transcription factors and cell cycle regulators in various biological processes that cooperate with ATF3. Since ATF3 has been shown to be modulated by ubiquitination [Yan et al., 2005; Mo et al., 2010] and SUMOylation (the current study), further studies are indeed necessary to determine the interplay between ubiquitination and SUMOylation on ATF3 activity. Together our study adds a new layer to the previous understanding of how ATF3 functions to regulate stress response, host defense, immunity, and cancer development.

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