Oncogene (2008) 27, 931–941

www.nature.com/onc

ORIGINAL ARTICLE

Functional characterization of TIP60 sumoylation in UV-irradiated DNA damage response

Z Cheng[1,7], Y Ke[1,7], X Ding[2,3], F Wang[1,2], H Wang[1], K Ahmed[1], Z Liu[1], Y Xu[4], F Aikhionbare[2], H Yan[1], J Liu[1], Y Xue[1], M Powell[2], S Liang[5], SE Reddy[2], R Hu[6], H Huang[4], C Jin[1] and X Yao[1,3]

[1]Cellular Dynamics, Hefei National Laboratory, Hefei, China; [2]Cancer Biology Program, Morehouse School of Medicine, Atlanta, GA, USA; [3]Department of Medicine, Beijing University of Chinese Medicine, Beijing, China; [4]Department of Hematology, Zhejiang University, Hongzhou, China; [5]Laboratory of Protein Chemistry, Hunan Normal University, Changsha, China and [6]Department of Endocrinology, Fudan University, Shanghai, China

The histone acetyltransferase TIP60 regulates the DNA damage response following genotoxic stress by acetylating histone and remodeling chromatin. However, the molecular mechanisms underlying the TIP60-dependent response to UV-induced DNA damage remain poorly understood. To systematically analyse proteins that regulate TIP60 activity in response to UV irradiation, we performed a proteomic analysis of proteins selectively bound to TIP60 in response to UV irradiation using mass spectrometry and identified a novel regulatory mechanism by which TIP60 orchestrates transcriptional activation of p53-dependent checkpoint response in UV-irradiated cells. The initial step of this pathway involves UV-induced association of TIP60 with SUMO-conjugation enzymes and site-specific sumoylation of TIP60 at lysines 430 and 451 via Ubc9. This sumoylation initiates the relocation of TIP60 from nucleoplasm to the promyelocytic leukemia body, which is essential for the UV-irradiated DNA damage repair response via a p53-dependent pathway. Significantly, inhibition of TIP60 sumoylation by overexpression of non-sumoylatable mutant abrogates the p53-dependent DNA damage response, demonstrating the importance of TIP60 sumoylation in response to UV irradiation. Our biochemical characterization demonstrated that the sumoylation of TIP60 augments its acetyltransferase activity *in vitro* and *in vivo*. Thus, this study shed new light on the function and regulation of TIP60 activity in UV-irradiated DNA damage response.
Oncogene (2008) **27,** 931–941; doi:10.1038/sj.onc.1210710; published online 20 August 2007

Keywords: UV irradiation; DNA damage; TIP60; sumoylation; p53

Correspondence: Dr X Yao, Laboratory of Cellular Dynamics, University of Science and Technology of China, Hefei, China or Dr C Jin, Hefei National Laboratory for Physical Sciences at Microscale, Hefei, China.
E-mail: yaoxb@ustc.edu.cn or jincj@ustc.edu.cn
[7]These authors contributed equally to this work.
Received 7 May 2007; revised 3 July 2007; accepted 6 July 2007; published online 20 August 2007

Introduction

TIP60 was first identified as a 60-kDa protein associated with the HIV Tat protein (Kamine *et al*., 1996). Sequence analysis indicates that it belongs to the highly conserved MOZ, Ybf2/Sas3, Sas2 and Tip60 acetyltransferase family (MYST) family of histone acetyltransferases (HATs) (Utley and Cote, 2003). TIP60 specifically acetylates lysines on amino-terminal peptides of histones H2A, H3 and H4, but not H2B (Yamamoto and Horikoshi, 1997). The role of chromatin acetylation by HATs in transcriptional regulation is well established (for example, Carrozza *et al*., 2003).

In addition to the HAT activity, purified TIP60 complex possesses ATPase, DNA helicase and structural DNA-binding activities. Cells expressing mutated TIP60 lacking acetylase activity failed to undergo apoptosis in response to DNA damage (for example, Ikura *et al*., 2000). Like p53, TIP60 accumulates in response to DNA damage (for example, Legube *et al*., 2004). Suppression of TIP60 blocks ATM activation and ATM-dependent phosphorylation of p53, suggesting that TIP60 is an upstream molecule in ATM-dependent DNA damage response (Sun *et al*., 2005).

Sumoylation is emerging as an important regulatory mechanism that affects protein function through relocation of modified proteins to specific subcellular compartments (for example, Hay, 2005). In addition, sumoylation alters the biochemical property of its substrates, such as mass and charge. Conjugation of SUMO to the ε-amino groups of certain lysine residues is carried out by a single E1 enzyme (Aos1/Uba2) and a single E2 enzyme (Ubc9). Before conjugation, SUMO isoforms must be cleaved by a SUMO-specific protease to expose a C-terminal di-glycine motif. The ligation of SUMO to its target is conducted by E3 ligases (for example, Hay, 2005). Sumoylation is often augmented in response to genotoxic stress (for example, Stelter and Ulrich, 2003) which can induce an activation of p53-dependent responses via an array of downstream events and lead to cell-cycle arrest or apoptosis (for example, Vogelstein *et al*., 2000). Here, we identify TIP60 as a previously unrecognized substrate for SUMO1 and illustrate the molecular pathway by which TIP60

controls transcriptional activation of the p53-dependent checkpoint response in UV-irradiated cells.

Results

SUMO modifying enzymes form a complex with TIP60 in response to UV irradiation

TIP60 plays an important role in the DNA damage repair (for example, Kusch *et al*., 2004), and the UV irradiation induces TIP60 translocation into punctuate subnuclear structures named nuclear bodies (NBs; also called promyelocyti leukemia (PML) bodies) in 293T cells (for example, Supplementary Figure S1). To further understand the role of TIP60 in the UV irradiation-mediated DNA damage response and identify the proteins that account for the UV-induced translocation of TIP60, we carried out a proteomic search for nuclear proteins that bind to TIP60 in response to UV irradiation. To this end, nuclear extracts from UV-irradiated 293T cells stably expressing FLAG-TIP60 were purified using FLAG antibody-affinity chromatography. 293T cells were chosen because of a high level expression of FLAG-TIP60. The bound proteins were eluted with the FLAG peptide and fractionated on sodium dodeyl sulphate–polyacrylamide gel eletrophoresis (SDS–PAGE) (Figure 1a, lane 4). As a control, we performed a mock affinity-purification using a non-specific IgG (Figure 1a, lanes 1 and 3). In addition, nuclear extracts from non-irradiated cells were empolyed (Figure 1a, lane 2).

As shown in Figure 1a (lane 4), five major protein bands marked with asterisks were found reproducibly enriched on the FLAG antibody-affinity matrix from UV-irradiated sample (lane 4) but not the control (lane 2). These bands were removed, digested in-gel by trypsin, and the resulting peptide fragments were extracted and analysed by mass spectrometry. As listed in the Supplementary Table 1, eight peptides from band P3 (for example, NGLPGSRPGSPER, NQDNE-DEWPLAEILSVK, QGVPMNSLR and IADNHTPK) match tryptic fragments derived from TIP60 and SUMO1.

Reptin, a well-known component of TIP60 complex, was retained on the affinity matrix regardless of UV irradiation (Figure 1b), consistent with previous reports (for example, Ikura *et al*., 2000). In addition, an 18-kDa band was identified as SUMO-conjugation enzyme Ubc9 while a 120-kDa band was identified as

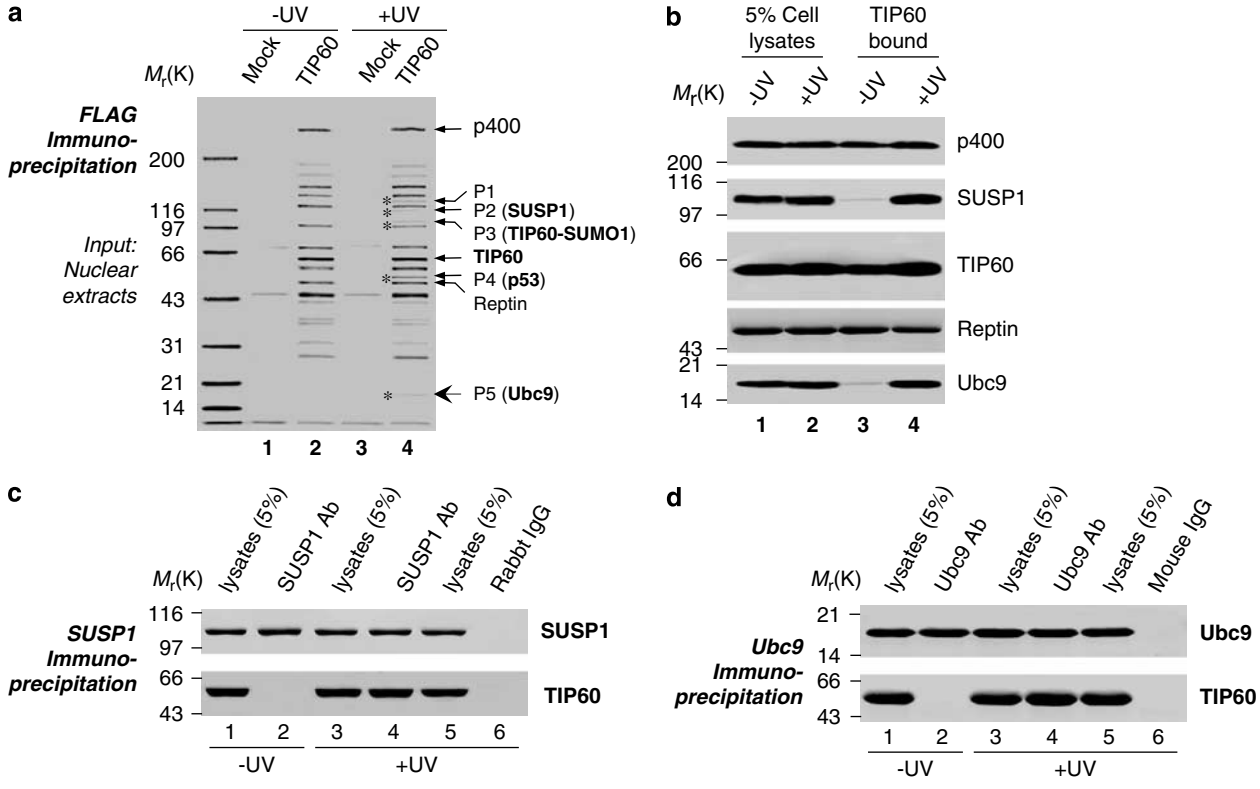


Figure 1 Identification of proteins preferentially associated with TIP60 in response to UV irradiation. (**a**) The TIP60 complex was immunoprecipitated from the nuclear extracts of TIP60-expressing 293T cells using an anti-FLAG antibody-agarose beads, and the bound materials were eluted with FLAG peptide (lanes 2 and 4). A control purification using mouse IgG was performed (lanes 1 and 3). Eluted proteins were resolved by SDS–PAGE and stained with Coomassie brilliant blue. The major polypeptides differentially purified from the UV-irradiated cells are indicated by asterisks. (**b**) SUMO-modifying enzymes Ubc9 and SUSP1 co-precipitated with TIP60 from UV-irradiated cells. The TIP60-precipitating proteins were subject to immunoblotting for p400, SUSP1, TIP60, reptin and Ubc9. (**c**) TIP60 co-precipitates with SUSP1 from nuclear extract of UV-irradiated cells. The SUSP1 was immunoprecipitated from nuclear extracts of 293T cells with and without UV irradiation. (**d**) TIP60 co-precipitates with Ubc9 from nuclear extract of UV-irradiated cells.

SUMO1-specific protease 1 (SUSP1). Of proteins preferentially bound to TIP60, the presence of Ubc9 and SUSP1 was novel and particularly exciting (Figure 1a, lane 4). Western blot analyses confirmed their presence in irradiated samples (Figure 1b, lane 4) but absent from non-irradiated samples (lane 3).

SUSP1-immunoprecipitation (Figure 1c) confirmed the association of SUSP1 with TIP60 in UV-irradiated cells (lane 4). No TIP60 was found in the control preparation (lanes 2 and 6). Ubc9-immunoprecipitation confirmed the SUSP1–TIP60 complex in UV-irradiated cells (Figure 1d, lane 4). Again, no TIP60 was found in control preparations (lanes 2 and 6). Thus, we conclude that Ubc9 and SUSP1 form a complex with TIP60 in response to UV irradiation.

UV irradiation induces the sumoylation of TIP60 at lysines 430 and 451

The existence of SUMO-conjugating and proteolytic enzymes in the TIP60 complex suggests the possible sumoylation of TIP60 in response to UV irradiation. Our *in silico* analysis predicted that TIP60 is a substrate of sumoylation (for example, Zhou *et al*., 2005). Indeed, anti-SUMO1 immunoblotting confirmed that UV-irradiation induced TIP60 sumoylation (Figure 2a, lane 4). This modification was readily apparent when FLAG-TIP60, His-SUMO1 and HA-Ubc9 were co-transfected into 293 cells (Figure 2b, lane 4), although overexpression of SUMO1 and Ubc9 also induced detectable sumoylation of FLAG-TIP60 without UV irradiation (Figure 2a, lane 2). A reconstitution assay using bacterial recombinant proteins confirmed that TIP60 is modified by SUMO1 (data not shown). Thus, we conducted computational analyses and revealed two highest probability sites on TIP60 (K430 and K451; Figure 2c). These two lysine residues were individually mutated to arginine, and double mutation of K430 and K451 abrogated SUMO modification *in vivo* (Figure 2d, lane 3). Therefore, we conclude that TIP60 is sumoylated on the lysine^{430} and lysine^{451} in response to UV irradiation.

As Ubc9 interacts with TIP60 in UV-irradiated cells, we examined its role in TIP60 sumoylation. As shown in Figure 2e, depletion of Ubc9 protein using the siRNA inhibited UV-induced TIP60 sumoylation (lane 4). Thus, we conclude that Ubc9 mediates SUMO1 conjugation to TIP60.

Sumoylation initiates the relocation of TIP60 to the PML body

Previous studies show that protein sumoylation plays an important role in relocation of nuclear proteins such as PML (for example, Fu *et al*., 2005). To evaluate the impact of sumoylation on TIP60 distribution, HeLa cells were transfected with GFP-TIP60 and assessed for its subcellular localization. GFP-TIP60 is diffused throughout the nucleoplasm (Figure 3Aa; green), similar to the endogenous TIP60 distribution (for example, Figure 3Ca; green). However, co-transfection of RFP-SUMO1 with GFP-TIP60 resulted in a redistribution of TIP60 from the nucleoplasm to NB, which is superimposed onto that of SUMO1 (Figure 3Ab; merge).

To test whether the sumoylated TIP60 is indeed located in the NB, the transfected cells expressing GFP-TIP60 and histidine-SUMO1 were stained for PML distribution. As shown in Figure 3A (c; arrows), overexpression of SUMO1 resulted in redistribution of TIP60 to the NB. If SUMO1 specifies TIP60 redistribution, SUMO1-TIP60dm fusion protein would go to the NB. Indeed, GFP-TIP60dm-SUMO1 is co-distributed with PML (Figure 3Ad; arrows).

Since UV irradiation induced TIP60 sumoylation (Figure 2), we reasoned that UV irradiation would trigger TIP60 relocation to the NB. To test this, real-time imaging was performed on GFP-TIP60-expressing HeLa cells. Indeed, UV irradiation causes a time-dependent accumulation of TIP60 in the NB (Figure 3B).

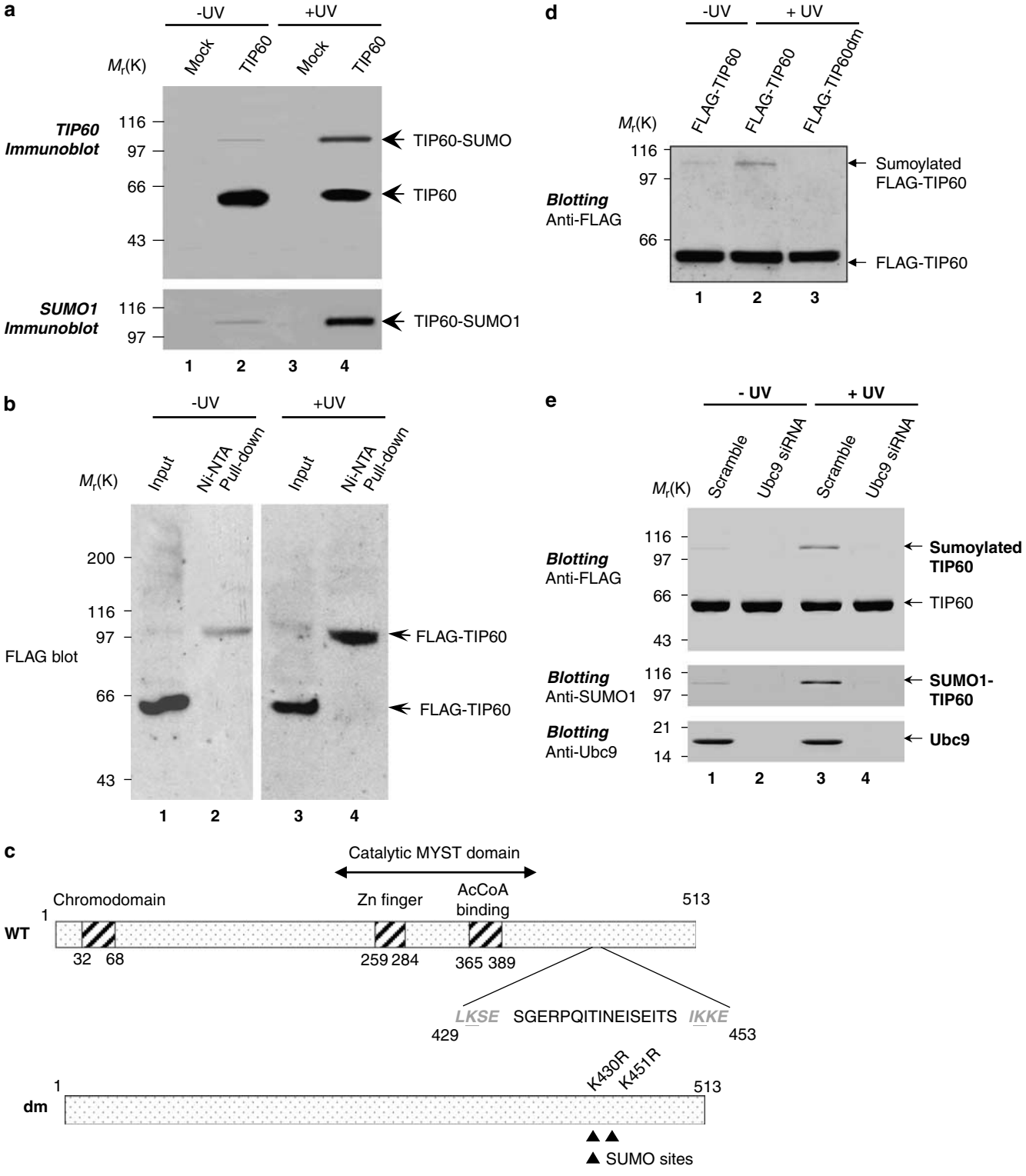
UV irradiation triggered the relocation of both endogenous (Figure 3Ca–b) and exogenous GFP-TIP60 (Figure 3Da) to NB. This translocation is a function of sumoylation as suppression of Ubc9-abrogated sumoylation (Figure 2e) and TIP60 relocation to the NB (Figure 3Cc). While this UV-elicited relocation does not require addition of exogenous SUMO1 (Figure 3Da), overexpression of SUMO1 induced a greater accumulation of TIP60 in the NB (Figure 3Db). To examine whether the sumoylation accounts for TIP60 redistribution in response to UV irradiation, a constitutively sumoylated form of TIP60 was generated and its expression resulted in exclusive NB localization (Figure 3Dc; green). Conversely, co-expression of non-sumoylatable mutant TIP60 with SUMO1 failed to localize in the NB (Figure 3Dd). However, TIP60dm-SUMO1 fusion protein enables the localization of non-sumoylatable TIP60 to the NB (Figure 3De). Together, these data indicate that sumoylation is an active mechanism responsible for recruiting TIP60 to the NB.

Sumoylation of TIP60 is necessary in the UV-induced DNA damage response

Cells expressing catalytically inactive TIP60 harbor double strand breaks, suggesting that TIP60 is important for DNA damage repair (for example, Ikura *et al*., 2000). To assess the function of TIP60 in UV-induced DNA damage response, we introduced siRNA oligonucleotides to suppress TIP60 protein level. As reported previously (for example, Legube *et al*., 2004), the siRNA duplexes suppress TIP60 protein accumulation (Figure 4A).

Since UV-irradiation induces TIP60 sumoylation, we hypothesized that sumoylation may be required for TIP60-dependent DNA damage response. To this end, U2OS cells were transfected with GFP-TIP60, GFP-TIP60dm, GFP-TIP60dm-SUMO1 and TIP60 siRNA duplex, respectively. U2OS cells were selected as they harbor wild-type p53. Thirty-six hours post-transfection, cells were irradiated with UV ($100\,J/m^2$) and allowed to recover and repair UV lesions for 4 h before harvesting for cell-cycle distribution analysis. As shown in Figure 4B(b), UV-induced DNA damage led to a

G1/S block, and this block is augmented when GFP-TIP60 is added (c). Even more cells accumulated in G1 phase when GFP-TIP60dm-SUMO1 is overexpressed (d), suggesting that TIP60 sumoylation participates in the DNA damage response. If TIP60 sumoylation is essential for the UV-induced G1 arrest, suppression of such modification would impair the G1 checkpoint. Indeed, overexpression of TIP60dm abrogated G1 arrest and induced a G2/M block instead (f). Cell-cycle distribution analysis supports the notion that TIP60 is



essential for UV-induced damage repair (g and h). Thus, we conclude that TIP60 sumoylation is necessary in the UV-induced DNA damage response.

TIP60 sumoylation regulates DNA damage response through p53 pathway

TIP60 is essential for p53 protein stabilization and DNA damage repair (for example, Ikura *et al*., 2000) while p53 regulates CDK inhibitor p21 protein expression, resulting in cell-cycle arrest in G1 phase (Vogelstein *et al*., 2000). Since suppression of TIP60 sumoylation affected G1 block (Figure 4B and E), we reasoned that TIP60 sumoylation may be essential for p53-p21 signaling cascade. To validate this hypothesis, we analysed p21 protein levels in UV-irradiated U2OS cells-expressing GFP-TIP60, TIP60dm, TIP60dm-SUMO1 and TIP60 siRNA duplexes, respectively. Expression of both TIP60 and TIP60dm-SUMO1 elevated p21 protein levels in response to UV irradiation (Figure 4C, lanes 2 and 4) and suppression of TIP60 by siRNA markedly reduced p21 protein level (Figure 4C, lane 5). Since UV-irradiation activates the ATR/ChK1 kinase cascade that triggers the phosphorylation of Ser^{20} on p53 important for the stabilization of p53 (for example, Shieh *et al*., 2000), we assessed the phosphorylation of Ser^{20} on p53 using a phospho-antibody. As shown in Figure 4D, TIP60dm-SUMO1 fusion protein enhanced the phosphorylation of p53 at Ser^{20} (lane 5). Suppression of TIP60 by siRNA markedly reduced Ser^{20} phosphorylation (Figure 4D, lane 6), suggesting that TIP60 sumoylation is a function of UV-induced checkpoint response. UV-induced TIP60 sumoylation activates ATR and subsequent Chk1 as judged by Ser^{428} phosphorylation on ATR and Ser^{345} phosphorylation on Chk1 (Figure 4D). The activated Chk1 is responsible for Ser^{20} phosphorylation on p53 protein. Thus, we conclude that TIP60 sumoylation stabilizes p53 in response to UV irradiation via ATR/Chk1 cascade, which then transactivates for p21 protein synthesis.

TIP60 sumoylation recruits p53 to the NB

Since UV-irradiation triggers TIP60 sumoylation and accumulation in the NB, we assessed whether TIP60 sumoylation governs the nuclear distribution of p53. To this end, we carried out an immunofluorescence study of TIP60 and p53 in U2OS cells (Figure 5Aa). Remarkably, UV irradiation resulted in accumulation of p53 to nuclear dots in a majority of cells (Figure 5Ab; red), which is superimposed onto that of TIP60 (Figure 5Ab; merge). If TIP60 sumoylation is essential for its recruitment of p53 to the NB, TIP60dm would block the translocation of p53 to the NB. Indeed, overexpression of TIP60dm retained p53 protein diffused throughout the nucleoplasm regardless of UV irradiation (Figure 5Ad). In contrast, TIP60dm-SUMO1 restored the NB localization of p53 in U2OS cells without UV irradiation (Figure 5Ac), indicating the TIP60 sumoylation is essential for the localization of p53 in NB. Both TIP60 and TIP60dm-SUMO1 retained their NB localization in p53-deficient Saos-2 cells regardless of UV irradiation (Supplementary Figure 2), confirming that TIP60 localization to the NB is independent of p53. Thus, we conclude that TIP60 sumoylation specifies the NB localization of p53.

The association and co-localization of TIP60 and p53 in UV-irradiated cells led us to hypothesize that sumoylation may be essential for the interaction between TIP60 and p53. To this end, we probed for p53 in GFP-immunoprecipitates from lysates of UV-irradiated 293T cells transiently transfected to express GFP-TIP60, GFP-TIP60dm or GFP-TIP60-SUMO1, respectively. Our immunoblotting confirmed that p53 was pull down by wild type but not non-sumoylatable TIP60 (Figure 5B, lower panel, lane 5), suggesting that p53 associates with sumoylated TIP60 in response to UV irradiation. Furthermore, a reciprocal immunoprecipitation validated that only sumoylated TIP60 proteins (wild type and TIP60dm) were found in p53 immunoprecipitates (Figure 5C, lanes 2 and 6). Non-sumoylatable TIP60 failed to interact with p53 (Figure 5C, lane 4). Thus, we conclude that UV-irradiation induces co-distribution of p53 with TIP60 to the NB in a TIP60-sumoylation-dependent manner.

Sumoylation promotes TIP60 HAT activity in response to UV irradiation

Since TIP60 is sumoylated in response to UV irradiation (Figure 2) and its HAT activity is activated by DNA damage (Sun *et al*., 2005), we tested whether the sumoylation modulates its HAT activity using histone-2A acetylation as a reporter (Yamamoto and Horikoshi, 1997). As shown in Figure 6a, the level of histone-2A acetylation is increased in cells expressing GFP-TIP60 with the highest level observed in cells expressing SUMO-TIP60dm. A lower level of acetylation was seen in cells expressing non-sumoylatable TIP60dm while the lowest level was seen in cells depleted of TIP60. Because neither exogenously expressed TIP60 levels nor histone-2A levels were altered by DNA damage, increased histone-2A acetylation in cells

Figure 2 TIP60 is sumoylated in response to UV irradiation. (**a**) TIP60 complexes precipitated from nuclear extracts of UV-irradiated and non-irradiated cells were probed for TIP60 and SUMO1. Western blot of TIP60 revealed a higher molecular weight (~100 kDa) band enriched in UV-irradiated sample, which also contains SUMO1 (lower panel, lane 4). (**b**) UV-irradiation induced TIP60 sumoylation. 293T cells were transfected with FLAG-TIP60, His-SUMO1 and HA-Ubc9 followed by UV irradiation (100 J/m^2). The lysates were prepared under denaturing condition and incubated with nickel-coupled Sepharose beads. The bound proteins were probed for TIP60 and SUMO1 (lane 4, upper arrow). (**c**) Schematic diagram of SUMO-modification sites predicted by SUMOsp. (**d**) Lys^{430} and Lys^{451} are substrates of SUMO1 in response to UV irradiation. 293T cells transfected with FLAG-TIP60 (wild type and non-sumoylatable TIP60dm) were exposed to UV-irradiation (100 J/m^2) 24 h after transfection. The lysates were prepared and equivalent amounts of proteins were subject to immunoblotting. Mutation of Lys^{430} and Lys^{451} abolishes SUMO1 conjugation (lane 3, upper arrow). (**e**) Ubc9 is essential for TIP60 sumoylation. 293T cells transfected with Ubc9 siRNA and scramble control were exposed to UV irradiation as described above. The lysates were prepared and equivalent amounts of proteins were applied for immunoblotting of TIP60, SUMO1 and Ubc9.

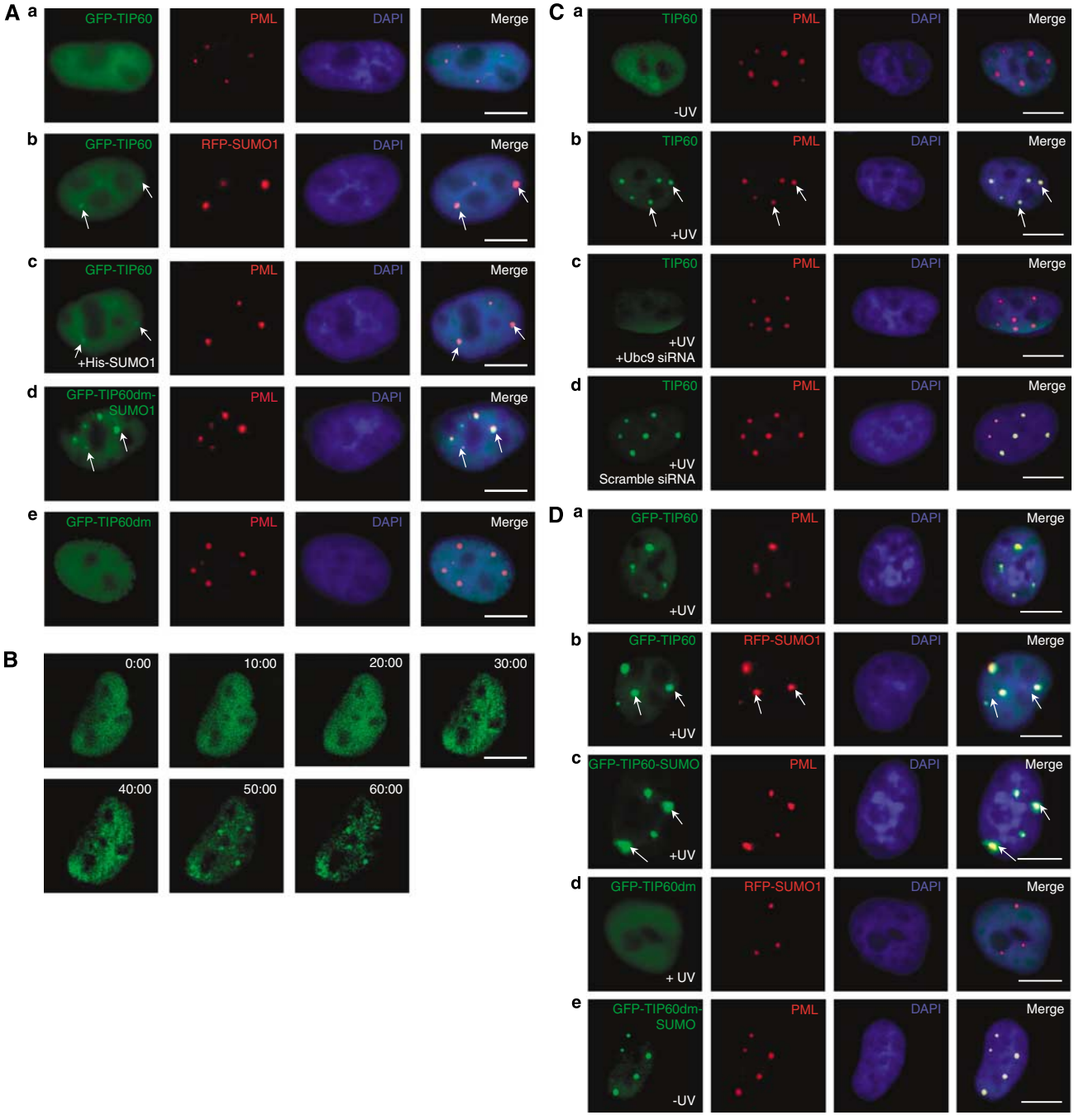


Figure 3 UV irradiation induces the sumoylation of TIP60 and its translocation to NB. (**a**) TIP60 sumoylation promotes its translocation to NB. HeLa cells were transfected with various GFP-TIP60 constructs. Thirty-six hours after transfection, cells were fixed, permeabilized and stained for PML or SUMO1. Co-transfection of SUMO1 with TIP60 resulted in accumulation of GFP-TIP60 in the NB (b and c, merges; arrows). Bars: 10 μm. (**b**) Real-time imaging UV-induced TIP60 accumulation in NB. HeLa cells transfected with GFP-TIP60 for 36 h before exposed to UV irradiation. Bars: 10 μm. (**c**) Suppression of Ubc9 by siRNA eliminates the accumulation of TIP60 at the NB. Bars: 10 μm. (**d**) Sumoylation is essential for UV-induced translocation of TIP60 to NB. HeLa cells were transfected with various constructs. Thirty-six hours after transfection, cells were exposed to UV irradiation, fixed, permeabilized and stained for TIP60, PML or SUMO1. Co-transfection of SUMO1 with TIP60 resulted in greater accumulation of GFP-TIP60 in the NB in response to UV (b). This SUMO1-dependent accumulation of TIP60 in NB was confirmed in the NB (c) regardless of UV irradiation (e). Non-sumoylatable TIP60 failed to accumulate in the NB (d). Bars: 10 μm. (**e**) Quantification of SUMO1-mediated translocation of TIP60 to the NB.

expressing wild-type TIP60 or TIP60dm-SUMO1 probably reflects an elevated HAT activity induced by UV-elicited sumoylation.

To test this hypothesis, we carried out an enzymatic assay using a synthetic histone peptide as a substrate and immuno-isolated FLAG-TIP60 from UV-irradiated cells. Sumoylation of TIP60 does indeed contribute to the augmentation of HAT activity since: UV-irradiation increased HAT activity about fivefolds, HAT activities were minimal in HAT-defective TIP60mt (TIP60$^{Q377E/G380E}$)

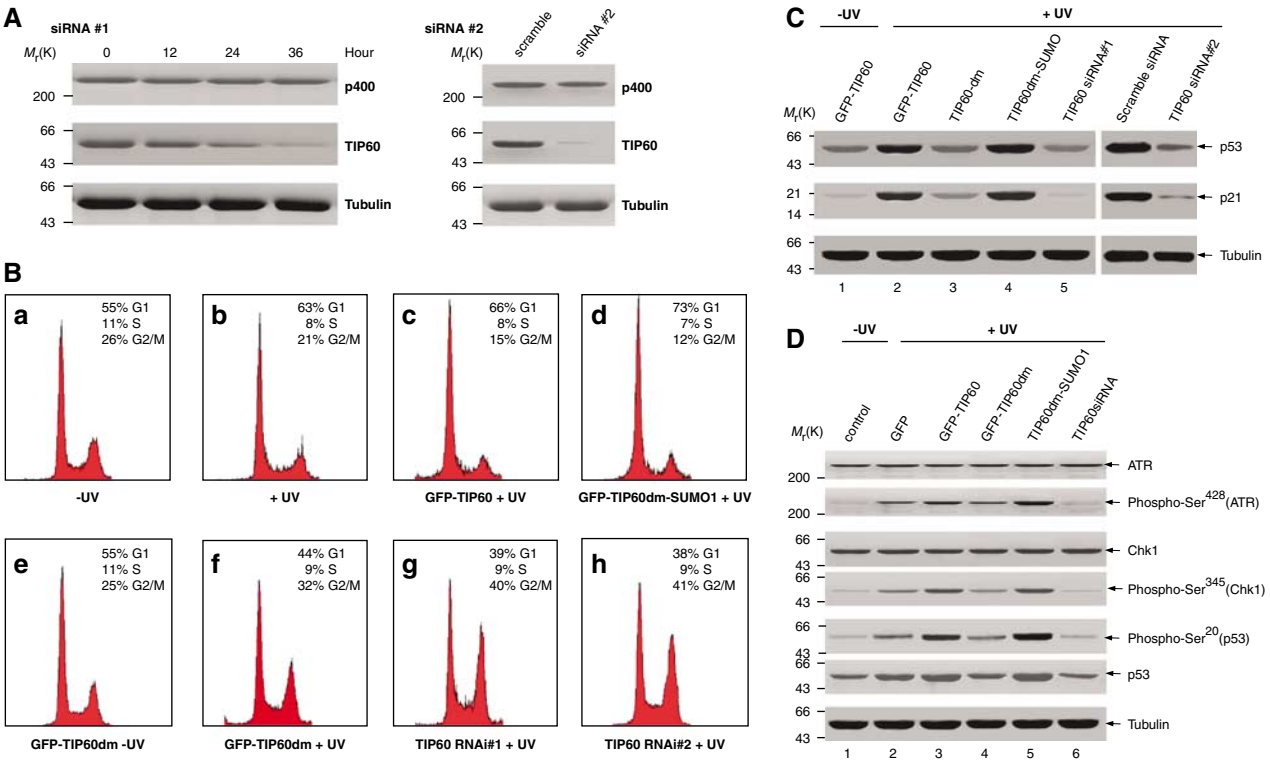


Figure 4 TIP60 sumoylation is essential for DNA damage checkpoint signaling. (**a**) Left; suppression of TIP60 protein level by siRNA. U2OS cells were transfected with the TIP60 siRNA oligonucleotide (siRNA #1) for different intervals (0, 12, 24 and 36 h). Right; Another siRNA oligonucleotide (siRNA #2) targeted to a different sequence also suppressed TIP60 protein accumulation. (**b**) Cell-cycle analyses of HeLa cells transiently transfected to express various TIP60 constructs or treated with TIP60 siRNA. Thirty-six hours after the transfection, these cells were exposed to UV irradiation and allowed 4 h for recovery. The cells were then stained with PI and assessed for cell-cycle distribution. (**c**) p21 protein expression level as a function of TIP60-sumoylation. U2OS cells were transiently transfected to express various TIP60 constructs or treated with TIP60 siRNA (siRNA#1 and siRNA#2). Thirty-six hours after the transfection, these cells were exposed to UV irradiation and allowed 4 h for recovery. The cells were then subjected to immunoblotting of p53 and p21. (**d**) p53 protein phosphorylation at Ser^{20} as a function of TIP60-sumoylation. U2OS cells were transiently transfected to express TIP60, non-sumoylatable-TIP60 and SUMO-TIP60 fusion proteins. The cells were then treated as above and collected for immunoblotting after recovery from irradiation. Top two panels, ATR and phospho-ATR (p-Ser^{428}); the third and fourth panels: Chk1 and phospho-Chk1 (p-Ser^{345}); the fifth and sixth panels: phospho-p53 (pSer^{20}) and p53, lower panel, tubulin.

and both non-sumoylatable TIP60 and depletion of Ubc9 yielded a minimal level of HAT activity (Figure 6b).

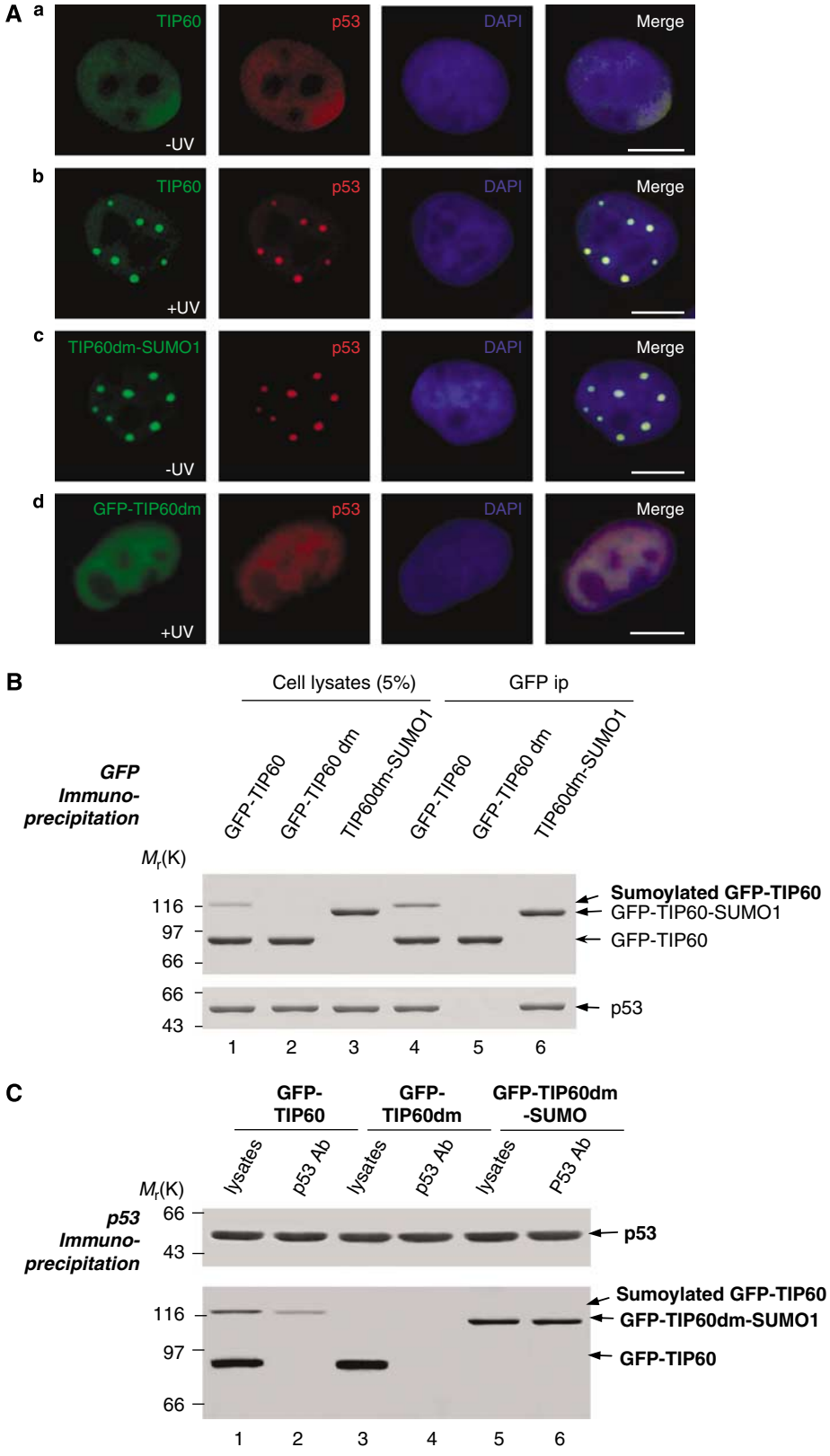
Having demonstrated that UV irradiation is able to induce TIP60 sumoylation, phosphorylation of Ser^{20} on p53, p21 protein expression and an elevation of HAT activity, we sought to determine the temporal order of these events and their relationship in the orchestration of DNA damage repair. Strikingly, sumoylation of TIP60 is readily apparent within 5 min following UV irradiation (Figure 6c). Phosphorylation of p53 at Ser^{20} peaks at 15–20 min while protein levels of p21 peak by 20 min. These experiments support a model in which TIP60 sumoylation triggers a p53-dependent G1 checkpoint activation cascade and induces histone acetylation essential for DNA damage repair (Figure 6d).

Discussion

Genetic and biochemical studies have revealed that the process of UV-irradiated DNA damage repair in eukaryotic cells is extremely complicated. In the present study, we validate the critical role for TIP60 in the UV-irradiation response and show its involvement in both p53-dependent cell-cycle checkpoint signaling and histone acetylation at DNA lesions. We identified and characterized Ubc9 as a SUMO E2-ligase catalysing the conjugation of SUMO to TIP60 in response to UV irradiation. Significantly, we mapped the sumoylation sites on TIP60 and revealed the role of such sumoylation in linking cell-cycle signaling to UV-irradiated DNA lesion repair.

Protein sumoylation is an important regulatory mechanism in orchestrating cellular dynamics. SUMO is attached to most substrates at the lysine in a ΨKXE sequence (Johnson, 2004). Our early computational analysis predicted that TIP60 is a potential substrate for SUMO modification as it contains two consensus ΨKXE motifs, $LK^{430}SE$ and $IK^{451}KE$ (Zhou *et al*., 2005). Our present studies validated that Lys^{430} and Lys^{451} are indeed the substrates for SUMO1 and essential for DNA damage response. It has been shown that TIP60 regulates the ATM/ATR activation caused by ionizing radiation (Sun *et al*., 2005) and participates in UV-induced DNA damage repair (Murr *et al*., 2006). Identification of TIP60 sumoylation provides great

insight into the molecular mechanisms underlying DNA damage responses to UV irradiation vs ionizing radiation. The comparative proteomics established here will help to delineate pathways and networks involved in the aforementioned damage responses. Future work will be directed to illustrate how TIP60 sumoylation is



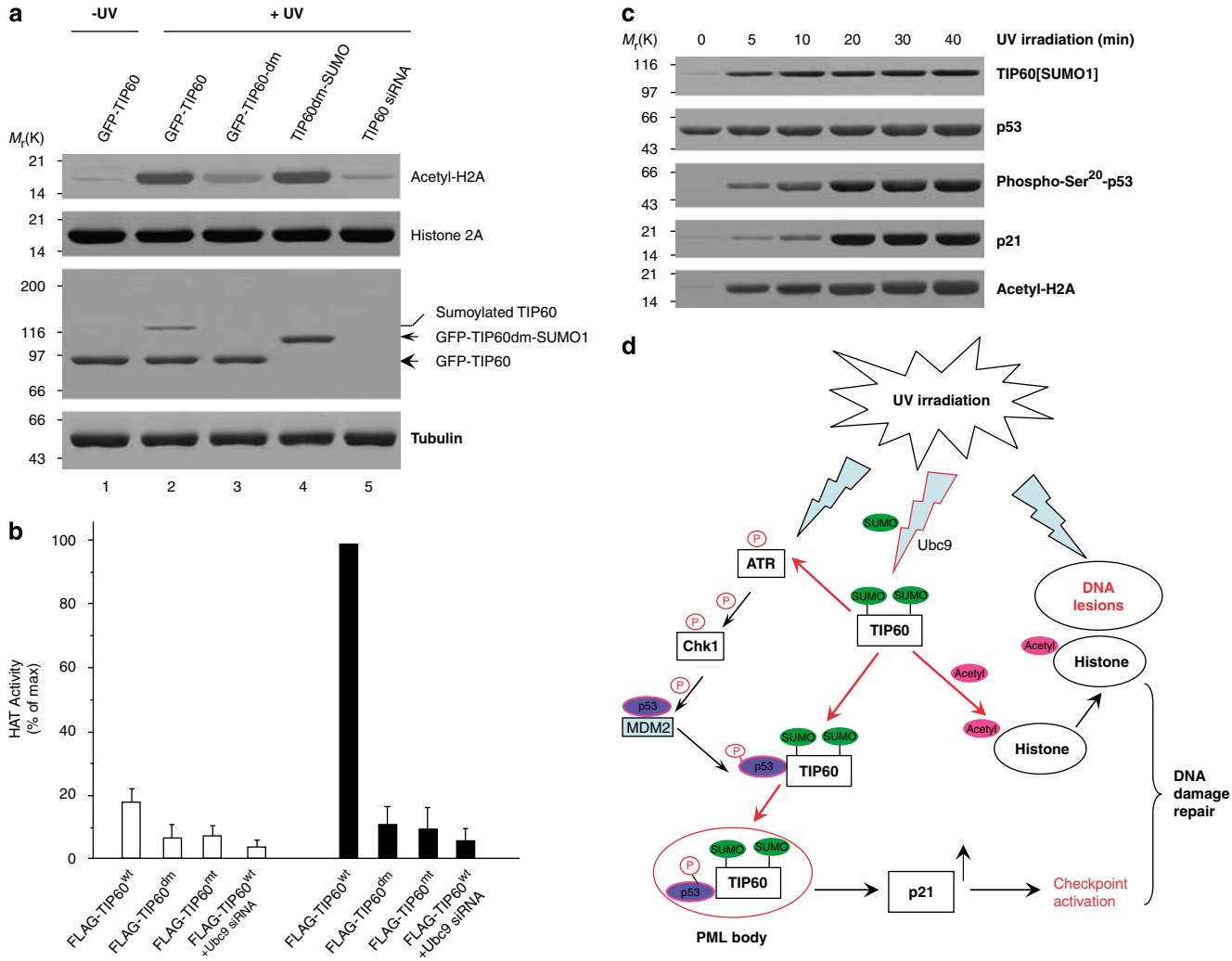


Figure 6 Sumoylation of TIP60 augments its HAT activity. (**a**) HeLa cells were treated as above and collected after recovery from irradiation for immunoblotting of acetyl-histone 2A (upper panel), GFP (middle panel) and tubulin (lower panel). (**b**) Sumoylation of TIP60 promotes its HAT activity *in vitro*. 293T cells were transiently transfected to express various FLAG-TIP60 proteins. An aliquot of 293T cells was co-transfected with FLAG-TIP60 and Ubc9 siRNA (100 nM). Cells were then treated as above and collected for anti-FLAG immunoprecipitation after recovery from irradiation. FLAG immunoprecipitates were normalized and added into HAT assay containing acetyl-CoA and histone H4 peptide. The error bars represent s.e.; $n = 3$ preparations. (**c**) Molecular dynamics in response to UV irradiation. Aliquots of U2OS cells were exposed to UV (100 J/m^2) and an aliquot of cells was collected at intervals of 10 min. The cell lysates were then collected for immunoblotting of SUMO1-TIP60 (upper panel), p53 protein (second panel), phospho-Ser^{20} of p53 (third panel), p21 (fourth panel) and acetyl-histone 2A (lowest panel). (**d**) Working model of TIP60 sumoylation in UV-induced damage repair. UV irradiation triggers TIP60 sumoylation which augment its HAT to acetylate proteins such as histone 2A. Sumoylated TIP60 also facilitates the activation of ATR-Chk1 signaling cascade which leads to the phosphorylation of p53 at Ser^{20}. Phosphorylation of p53 at Ser^{20} disrupts p53-MDM2 association which allows the association of p53 with sumoylated TIP60 and subsequent translocation to PML nuclear body. The p53-mediated transactivation allows p21 protein synthesis and subsequent G1 checkpoint activation. This enables sufficient time to allow DNA damage to repair.

Figure 5 Sumoylated TIP60 interacts with and recruits p53 into the NB. (**a**) TIP60-sumoylation promotes the translocation of p53 to NB. U2OS cells were transiently transfected to express various TIP60 fusion proteins. The cells were then treated as above and collected for immunofluorescence after recovery from irradiation. The fixed and permeabilized cells were then stained for TIP60 and p53. Both endogenous TIP60 and p53 proteins are diffused throughout the nucleoplasm (a; merge). UV-irradiation induced accumulation of p53 to NB which is superimposed onto that of TIP60 (b; merge). While SUMO-TIP60 enhanced the accumulation of p53 in the NB (c), non-sumoylatable TIP60 failed to localize and recruit p53 to the NB (d). Bars: 10 μm. (**b**) TIP60 sumoylation promotes its association with p53. 293T cells were transiently transfected to express various TIP60-GFP fusion proteins. Thirty-six hours after the transfection, cells were exposed to UV irradiation followed by GFP-immunoprecipitation. The immunoprecipitates were probed for TIP60 and p53. (**c**) p53 preferentially binds to sumoylated TIP60. 293T cells were transiently transfected to express various TIP60-GFP fusion proteins. The cells were then treated as above and collected for anti-p53 immunoprecipitation. The immunoprecipitates were probed with antibodies to p53 (upper) and GFP (lower).

regulated in response to UV irradiation. In addition, it would be of great interest to ascertain whether the mechanisms described herein for transformed cells would be applicable to primary cells.

Mounting evidence implicates TIP60 in DNA damage response (for example, Kastan and Bartek, 2004). Our studies show that UV-induced TIP60 sumoylation is correlated with p53 accumulation in the NB, raising the possibility that TIP60-p53 orchestrates the activity in NB. It has been suggested that the NB is involved in chromatin remodeling (Seeler and Dejean, 2003). TIP60, as a HAT, is an important chromatin modifier to regulate chromatin transcription (Kimura and Horikoshi, 1998). Our studies show that UV-irradiation induced translocation of sumoylated TIP60 to the NB is correlated with increased HAT activity and provide mechanistic link between UV irradiation and p53 translocation to the NB. Since accumulation of p53 in the NB turns on p53 transcriptional activity (for example, Fogal *et al*., 2000), we propose that TIP60 sumoylation is essential for the assembly of p53 transcription complexes in response to UV irradiation. The activation of DNA damage checkpoint in G1 through increased p21 protein expression provides a threshold for DNA lesion repair and maintaining genomic stability.

Although TIP60 was initially identified as HAT, pure TIP60 displays no detectable HAT activity on chromatin (Yamamoto and Horikoshi, 1997). However, the TIP60 complex bears HAT activity toward chromatin (Ikura *et al*., 2000), suggesting that the conformational change regulates TIP60 activity. Our studies demonstrate that UV-irradiation induces TIP60 complex remodeling with enrichment of SUMO-conjugation enzyme Ubc9 and TIP60 sumoylation. This sumoylation greatly promotes HAT activity *in vivo* and *in vitro*. Thus, activation of TIP60 HAT by sumoylation may provide a mechanistic link between UV irradiation-elicited signaling and DNA lesion repair.

Several studies show that ATR-Chk1 signaling cascade plays a role in UV-induced DNA damage response (for example, Helt *et al*., 2005). ATR/Chk2 phosphorylates p53 at Ser^{20} and stabilize p53 protein levels (for example, Legube *et al*., 2004). Sun *et al*. (2005) demonstrated that TIP60 HAT activates ATM autophosphorylation in response to DNA damage. Our findings that UV-induced sumoylation of TIP60 is concomitant with an elevated HAT activity and activation of ATR/Chk1 demonstrate an essential role of TIP60 in the orchestration of cellular dynamics in response to DNA damage. Based on the temporal order of p53 phosphorylation related to TIP60 sumoylation, we propose that UV-irradiation triggers DNA damage response by initiating sumoylation of TIP60 and subsequent activation of ATR/Chk1 signaling cascade via an activation of TIP60 HAT activity (Figure 6d). Therefore, TIP60 sumoylation provides an essential link between local DNA lesion repair process and cell-cycle clock reset.

In conclusion, our studies identify a new signaling pathway, sumoylation of TIP60, in the regulation of UV-induced DNA damage response. Our results demonstrate that TIP60 HAT is a key component of the signal-transduction pathway that links the detection of DNA lesion to the activation of the p53-dependent cell-cycle checkpoint. Thus, the sumoylated TIP60 complex, through the combined action of the ATR/Chk1 and the p53-p21-cyclin A-Cdk2 pathways, allows repair of the damaged DNA before replication is initiated in order to ensure genetic integrity of the replicated DNA.

Materials and methods

Cell culture and transfection
HeLa, U2OS, Saos-2 and 293T cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) and transfected as described previously (Fang *et al*., 2006).

Affinity purification and proteomics analyses
The TIP60 complex was immunoprecipitated from UV-irradiated 293T cells and fractionated on a SDS–PAGE gel. The protein bands were removed for mass spectrometric identification essentially as described (Fang *et al*., 2006).

Histone acetyltransferase (HAT) activity assay
TIP60 immunoprecipitates were prepared and HAT assay was performed according to Sun *et al*. (2005).

Live cell imaging
HeLa cells grown on coverslips were transfected with GFP-TIP60. Thirty-six hours post-transfection, cells were exposed to UV ($100\,J/m^2$) and real-time images were acquiring using a confocal microscope (LSM510, Carl Zeiss, Germany).

Abbreviations

HAT, histone acetyltransferase; MYST, MOZ, Ybf21/Sas3, Sas2 and Tip60 acetyltransferase family; NB, nuclear body.

Acknowledgements

This work was supported by grants from Chinese 973 project (2002CB713700 and 2006CBOF0503), Chinese Academy of Sciences (KSCX1-YW-R65), Chinese Natural Science Foundation (39925018, 30270293, 90508002) and National Institutes of Health (DK56292). SER and XY are GCC Eminent Scholars. The facilities used were partially supported by NIH/NCRR/RCM1 grant G-12-RR03034.

References

Carrozza MJ, Utley RT, Workman JL, Cote J. (2003). The diverse functions of histone acetyltransferase complexes. *Trends Genet* **19**: 321–329.

Fang Z, Miao Y, Ding X, Deng H, Liu S, Wang F *et al*. (2006). Proteomic identification and functional characterization of a novel ARF6 GTPase-activating protein ACAP4. *Mol Cell Proteomics* **4**: 711–724.

Fogal V, Gostissa M, Sandy P, Zacchi P, Sternsdorf T, Jensen K *et al*. (2000). Regulation of p53 activity in nuclear bodies by a specific PML isoform. *EMBO J* **19**: 6185–6195.

Fu C, Ahmed K, Ding F, Ding X, Lan J, Yang Z *et al*. (2005). Stabilization of PML nuclear localization by conjugation and oligomerization of SUMO-3. *Oncogene* **24**: 5401–5413.

Hay RT. (2005). SUMO: a history of modification. *Mol Cell* **18**: 1–12.

Helt CE, Clioy WA, Keng PC, Bambara RA, O'Reilly MA. (2005). Ataxia telangiectasia mutated (ATM) and ATM and Rad3-related protein exhibit selective target specificities in response to different forms of DNA damage. *J Biol Chem* **280**: 1186–1192.

Ikura T, Ogryzko VV, Grigoriev M, Groisman R, Wang J, Horikoshi M *et al*. (2000). Involvement of the Tip60 histone acetylase complex in DNA repair and apoptosis. *Cell* **102**: 463–473.

Johnson ES. (2004). Protein modification by SUMO. *Ann Rev Biochem* **73**: 355–382.

Kamine J, Elangovan B, Subramanian T, Coleman D, Chinnadurai G. (1996). Identification of a cellular protein that specifically interacts with the essential cysteine region of the HIV-1 Tat transactivator. *Virology* **216**: 357–366.

Kastan MB, Bartek J. (2004). Cell-cycle checkpoints and cancer. *Nature* **432**: 316–323.

Kimura A, Horikoshi M. (1998). Tip60 acetylates six lysines of a specific class in core histones *in vitro*. *Genes Cells* **3**: 789–800.

Kusch T, Florens L, Macdonald WH, Swanson SK, Glaser RL, Yates JR *et al*. (2004). Acetylation by Tip60 is required for selective histone variant exchange at DNA lesions. *Science* **306**: 2084–2087.

Legube G, Linares LK, Tyteea S, Caron C, Scheffner M, Chevillard-Briet M *et al*. (2004). Role of the histone acetyl transferase Tip60 in the p53 pathway. *J Biol Chem* **279**: 44825–44833.

Murr R, Loizou JI, Yang YG, Cuenin C, Li H, Wang ZQ *et al*. (2006). Histone acetylation by Traap-Tip60 modulates loading of repair proteins and repair of DNA double-strand breaks. *Nat Cell Biol* **8**: 91–99.

Seeler JS, Dejean A. (2003). Nuclear and unclear functions of SUMO. *Nat Rev Mol Cell Biol* **4**: 690–699.

Shieh SY, Ahn J, Tamai K, Taya Y, Prives C. (2000). The human homologs of checkpoint kinase Chk1 and Cds1 (Chk2) phosphorylates p53 at multiple DNA damage-inducible sites. *Gene Deve* **14**: 289–300.

Stelter P, Ulrich HD. (2003). Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation. *Nature* **425**: 188–191.

Sun Y, Jiang X, Chen S, Fernandes N, Price BD. (2005). A role for the Tip60 histone acetyltransferase in the acetylation and activation of ATM. *Proc Natl Acad Sci USA* **102**: 13182–13187.

Utley RT, Cote J. (2003). The MYST family of histone acetyltransferases. *Curr Top Microbiol Immunol* **274**: 203–236.

Vogelstein B, Lane D, Levine AJ. (2000). Surfing the p53 network. *Nature* **408**: 307–310.

Yamamoto T, Horikoshi M. (1997). Novel substrate specificity of the histone acetyltransferase activity of HIV-1-Tat interactive protein Tip60. *J Biol Chem* **272**: 30595–30598.

Zhou F, Xue Y, Lu H, Chen G, Yao X. (2005). A genome-wide analysis of sumoylation-related biological processes and functions in human nucleus. *FEBS Letters* **579**: 3369–3375.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).