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The SUMOylation of Zinc-Fingers and Homeoboxes 1 (ZHX1) by Ubc9 Regulates Its Stability and Transcriptional Repression Activity

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ABSTRACT

Zinc-fingers and homeoboxes protein 1 (ZHX1) belongs to the ZF (zinc-fingers) class of homeodomain transcription factors, and its function remains largely unknown. ZHX1 has been previously found to interact with the activation domain of the nuclear factor Y subunit A (NFYA) and to have a transcriptional repression activity. Here, we report that the SUMO-E2 conjugating enzyme Ubc9 was identified to interact with ZHX1 by an interaction screen using a yeast two-hybrid system. This interaction was confirmed by co-immunoprecipitation and co-localization assays. Further study showed that ZHX1 is SUMOylated by Ubc9 with SUMO1 at the sites K159, K454, and K626. Furthermore, we demonstrated that the SUMOylation of ZHX1 regulated the stability, ubiquitination and transcriptional activity of ZHX1. J. Cell. Biochem. 114: 2323–2333, 2013.

KEY WORDS: ZHX1; SUMO; SUMOYLATION; TRANSCRIPTIONAL ACTIVITY

Zinc-fingers and homeoboxes protein 1 (ZHX1), first discovered in a mouse bone marrow stromal cell line, is a member of the ZF class of homeodomain transcription factors [Barthelemy et al., 1996]. ZHX1 contains two Cys_2–His_2 zinc-fingers and five tandem homeodomains. There are two major ZHX1 transcripts that are 4.5 or 5 kb in length and are expressed in most tissues. The 5 kb ZHX1 transcript is more abundant. ZHX1 mRNA is generally expressed in the adult mouse, and the level of its mRNA is slightly higher in the brain than in the other organs [Barthelemy et al., 1996]. Interleukin-2 (IL-2) specifically induces the expression of ZHX1 mRNA in a mouse cytotoxic T cell line. Furthermore, IL-2 treatment prolongs the stability and half-life of ZHX1 mRNA [Shou et al., 2004]. In addition, PEA3 (polyomavirus enhancer activator 3) and YY1 (Yin and Yang 1) synergistically stimulate the transcription of the ZHX1 gene [Shou et al., 2003]. Human ZHX1 was first identified as a binding protein of the transcription factor NF-Y. The region between amino acids 272 and 564 of ZHX1 interacts with a glutamine-rich region and a serine/threonine-rich region of NF-YA [Yamada et al., 1999b]. The nucleoprotein ZHX1 forms a homodimer or a heterodimer with its homologous protein ZHX3 [Hirano et al., 2002; Yamada et al., 2003]. As a regulatory transcription factor, the DNA binding sequence of ZHX1 has not yet been identified. The transcriptional repressive activity of ZHX1 can be measured using a mammalian one-hybrid system in cells. The region between the amino acids 831 and 873 of ZHX1 is responsible for its repressor activity [Yamada et al., 2002]. ZHX1 also binds with DNA methyltransferase (DNMT) 3B to improve

Abbreviations: Aos1, activation of Smt3p; DNMT, DNA methyltransferase; Mms21, methyl methanesulphonate-sensitivity protein 21; NFY, nuclear factor Y; PC2, polycomb 2 homolog; PEA3, polyomavirus enhancer activator 3; PIAS, protein inhibitors of activated STAT; RanBP2, ran-binding protein 2; SAE1, SUMO-activating enzyme 1; SUMO, small ubiquitin-related modifier; Uba2, ubiquitin-like activating enzyme subunit 2; YY1, yin and yang 1; ZHX1, zinc-fingers and homeoboxes 1; Zip3, zipper protein 3.

The authors declared they have no conflicts of interest.

Shuliang Chen and Xiao Yu contributed equally to this work.

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the DNMT3B-mediated transcriptional repression [Kim et al., 2007]. Despite these studies, the transcriptional regulatory mechanism of ZHX1 remains obscure.

SUMOylation is a reversible post-translational modification of a substrate, by the covalent conjugation of a small ubiquitin-related modifier (SUMO) protein, found in all eukaryotes. There are four SUMO isoforms (SUMO1–4) in vertebrates, but only SUMO1 is found in lower eukaryotes, such as yeast, worms and flies. SUMO2 shares 97% and 50% sequence identity with SUMO3 and SUMO1, respectively. SUMO2 and SUMO3 are often referred to as SUMO2/3 because the available antibodies cannot distinguish between these two isoforms [Gareau and Lima, 2010]. The human SUMO4 gene is predominantly expressed in the immune system [Kosoy and Concannon, 2005]. A functional variant of SUMO4 has been found to associate with type 1 diabetes [Guo et al., 2004]. However, it is not clear whether SUMO4 conjugates with other proteins [Qu et al., 2005]. The SUMOylation process involves an enzymatic cascade of biochemical reactions [Meulmeester and Melchior, 2008]. In eukaryotic cells, the E1-activating enzyme is a heterodimer containing the SUMO-activating enzyme 1 (SAE1), also known as Aos1 (Activation of Smt3p in yeast), and the SUMO-activating enzyme 2 (SAE2), also known as Uba2 (Ubiquitin-like activating enzyme subunit 2 in yeast). Thus far, only one E2-conjugating enzyme, Ubc9, has been identified. Meanwhile, the E3 ligase can be one of four types of proteins: one type is the SAP and Miz1 domain proteins (SIZ1 and SIZ2); the second type is the Protein Inhibitors of Activated STAT (PIAS), such as methyl methanesulphonate-sensitivity protein 21 (Mms21) and molecular zipper protein 3 (Zip3) in yeast (PIAS1, PIAS3, PIASxα, PIASxβ, and PIASy in human), which, together with type one proteins, constitute the SIZ/PIAS family; the third type are proteins that contain SIMs, such as the nuclear pore complex-associated protein, also known as ran-binding protein 2 (RanBP2); and the last type is the polycomb 2 homologue (PC2) [Gareau and Lima, 2010; Lomeli and Vazquez, 2011]. Interestingly, most of the SUMO targets that have been reported are transcription factors, and their transcriptional activity can be regulated by SUMOylation. For example, both SUMO1 and SUMO2/3 regulate the activity of the transcription factors p53 [Rodriguez et al., 1999; Li et al., 2006; Stindt et al., 2011] and the androgen receptor [Poukka et al., 2000; Zheng et al., 2006].

In the present study, we showed that ZHX1 interacts with Ubc9 and is SUMOylated by SUMO1 at the positions of K159, K454, and K626. The modification of SUMO on ZHX1 regulates its stability, ubiquitination, and transcriptional repressive activity.

MATERIALS AND METHODS

ANTIBODIES

Mouse monoclonal anti-flag (F4045) and anti-HA (H9658) antibodies were purchased from Sigma–Aldrich (St. Louis, MO). Mouse monoclonal anti-Myc (MA1-980) antibody, goat anti-mouse and goat anti-rabbit horseradish peroxidase secondary antibodies (3,2430 and 3,2460) were purchased from Thermo Fisher Scientific (San Jose, CA). Mouse monoclonal anti-Ubc9 (sc-130281) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, inc., CA). Rabbit polyclonal anti-ZHX1 (ab19356) antibody was purchased from Abcam (New Territories, HK). Rabbit polyclonal anti-SUMO1 and anti-SUMO2 were products of NewEast Biosciences (Wuhan, China).

CDNA LIBRARY AND PLASMID CONSTRUCTION

In a previous study [Zhu et al., 2010], 4,676 human genes were fused to the pDBLeu (BD) vector and 4,964 human genes were cloned into the pPC86 (AD) vector. Additionally, a small cDNA library containing 4,964 intact ORFs of human genes was constructed by transforming all of the AD-vectors into *Escherichia coli* DH5α cells and plating the cells on an LB agar medium containing ampicillin. All of the clones on these plates were collected and the plasmids were extracted (the small cDNA library) to perform a yeast two-hybrid screen.

The pDBLeu-ZHX1 (BD-ZHX1) and pPC86-Ubc9 (AD-Ubc9) expression vectors were constructed previously [Zhu et al., 2010]. All of the primers and PCR conditions used to make these constructs are shown in Table S1. The ZHX1 gene was amplified from the pDBLeu-ZHX1 expression vector and then inserted into the *Sal*I and *Not*I sites of the pRK-Flag vector (from Dr. Hongbing Shu, Wuhan University) to generate the Flag-ZHX1 expression vector. The site-directed mutations (K159R, K454R, and K626R) of ZHX1 were constructed by blunt end ligation of PCR products amplified from the Flag-ZHX1 vector with specific primers (F-K159R, R-K159R, F-K454R, R-K454R, or F-K626R, R-K626R). K159R/K454R, K159R/K626R, and K454R/K626R mutants were generated by blunt end ligation of PCR products amplified from the K159R or K454R expression vectors using specific primers (F-K454R, R-K454R, or F-K626R, R-K626R, respectively). The mutant 3KR was generated by blunt end ligation of PCR products amplified from the K159R/K454R expression vector using specific primers (F-K626R, R-K626R). The human Ubc9 cDNA was cloned into the *EcoR*I and *Xho*I sites of the pCMV-Myc vector (Clontech) to generate a Myc-Ubc9 expression vector. The human SUMO1 and SUMO2 cDNAs were amplified by RT-PCR from the total RNA of HEK293T cells and cloned into the *Sal*I and *Not*I sites of the pRK-HA vector (from Dr. Hongbing Shu, Wuhan University). SUMO1 was inserted into the *Xho*I and *Bam*HI sites of the DsRed-C1 vector (Clontech) to form the Red-SUMO1 expression vector. ZHX1 (WT) and ZHX1 (3KR) were cloned into the *Xho*I and *Bam*HI sites of the EGFP-C1 vector (Clontech) to generate EGFP-ZHX1 (WT) and EGFP-ZHX1 (3KR) expression vectors, respectively. The plasmids pSG424, 5xGal 4-luc and Gal 3-control were kindly provided by Dr. Kazuya Yamada (Matsumoto University). HA-UB was kindly provided by Dr. Hongbing Shu (Wuhan University). Myc-tagged ZHX1 deletion mutants were kindly provided by Professor Tae-You Kim (Seoul National University). The Gal4 (DNA binding domain) fused wild type and mutant ZHX1 expression vectors were generated by inserting cDNA products amplified from the indicated Flag-tagged plasmids into the *Sal*I and *Sac*I sites of the pSG424 vector. The ZHX1-SUMO1 mutant was amplified by two-stage PCR, using primers F1 and R2 and F2 and R1, to obtain two segments (primers F2 and R2 contained 24 bp complementary nucleotides); the annealed DNA was then used as a template and the primers F1 and R1 were used to obtain a ZHX1-SUMO1 fused gene. The fragment was inserted into the *Sal*I and *Not*I sites of the pRK-Flag vector. All of the constructs were confirmed by DNA sequencing.

YEAST TWO-HYBRID SCREENING

Yeast two-hybrid screening and co-transformation assays were performed using the Proquest two-hybrid system (Gibco BRL, Cat. series 10,835), following the manufacturer's instructions. First, BD-ZHX1 was chosen as the bait and was tested for self-activation on SC-Leu agar plates containing a series of increasing concentrations of 3-Amino-1, 2, 4-Triazole (3AT, Sigma). The BD-ZHX1 plasmid was first transformed into the yeast strain *MaV203* to generate stable BD-ZHX1 transformed yeast cells, and these cells were then transformed with the small cDNA library constructed in the pPC86 plasmid. Approximately 2×10^5 clones were screened on selective agar plates lacking leucine (-Leu), tryptophan (-Trp) and histidine (-His) and supplemented with 25 mM 3AT (SC-Leu-Trp-His + 25 mM 3AT). Positive clones were verified by using the X-gal assay. The plasmid DNAs from X-gal positive colonies were isolated, propagated in *E. coli* DH5α cells, extracted and re-transformed into yeast *MaV203* cells together with either BD-ZHX1 or other control plasmids to verify the specific interaction. Finally, the plasmids with positive results were sequenced and blasted using the GenBank database provided by NCBI.

CELL CULTURE, TRANSFECTION, AND FLUORESCENCE IMAGING

HEK293T, HeLa and COS7 cells (China Center for Type Culture Collection, Wuhan) were maintained in Dulbecco's modified Eagle's medium (Gibco), supplemented with 10% fetal bovine serum (Gibco), 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen), in a 37°C incubator with 5% CO_2. All plasmids were transfected using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. For cell imaging, HeLa cells (5×10^4) were seeded onto cover slips in 12-well plates. Twenty-four hours after transfection, the cells were washed twice with PBS, fixed with 4% paraformaldehyde for 20 min, permeabilized in PBS with 0.1% Triton X-100 for 10 min, blocked in 5% fat-free milk, and then incubated with a rabbit anti-flag antibody (1:100, Sigma) and a mouse anti-Myc antibody (1:100, Roche) for 2 h. Next, the cells were washed with PBS three times and then incubated with a Cy3-tagged goat anti rabbit IgG antibody (1:100, Roche) and a FITC-tagged goat anti mouse IgG antibody (1:100, Roche) for another 2 h. The nuclei were stained with Hoechst33258 (Sigma, Buchs, Switzerland). Finally, the cells were observed using a laser scanning confocal microscope (FV1000, Olympus, Tokyo, Japan). For the imaging of COS7 cells, a mouse anti-flag antibody and a FITC-tagged goat anti mouse IgG antibody were used to detect Flag-tagged proteins.

IMMUNOPRECIPITATION, SUMOYLATION, UBIQUITINATION, WESTERN BLOT ANALYSIS, AND CHX CHASE ASSAY

HEK293T cells (7.5×10^5) were transiently transfected with the indicated plasmids. The cells were lysed in lysis buffer [(20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1 mM Na_2EDTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1% NP-40 and a protease inhibitor cocktail (Roche)] 36 h after transfection. For SUMOylation and ubiquitination assay, the cells were lysed with lysis buffer containing 20 mM of the deSUMOylation inhibitor *N*-ethylmaleimide (NEM, Sigma). The lysates were centrifuged at 14,000 rpm for 20 min at 4°C, and 10% of the supernatants were reserved for the Western blotting analysis of protein expression. The remaining supernatants were incubated with 0.5 μg of the indicated antibodies and 25 μl (1:1) protein A/G beads (Santa Cruz Biotechnology) at 4°C overnight. Next, the samples were centrifuged at 3,000 rpm for 3 min at 4°C and the immunoprecipitates were washed three times with lysis buffer. The proteins were removed from the protein A/G beads by boiling for 10 min in SDS sample buffer and were separated on a 6–12% SDS–PAGE, as needed, and then electro-transferred onto a nitrocellulose membrane (Millipore). Western blotting was performed using specific antibodies, and the blots were developed by Immobilon Western chemiluminescent HRP substrate (Millipore). For CHX chase assay, HEK293T cells transfected with wild type or the 3KR mutant of ZHX1 were treated with CHX1 (100 μg/ml) alone or together with MG132 (10 μM) for the indicated times. Then, the cell lysates were collected and analyzed by Western blot analysis with specific antibodies.

REPORTER ASSAYS AND STATISTICAL ANALYSES

HEK293T (1×10^5) cells were seeded into 24-well plates and transfected with 0.1 μg of the reporter plasmid 5xGal4-Luc by using the standard calcium phosphate precipitation method. In the same experiment, where necessary, the corresponding empty vector was used to maintain an equal amount of total DNA for each condition. To normalize the transfection efficiency, 0.02 μg of the pRL-TK (*Renilla luciferase*) reporter plasmid was added to each transfection. Luciferase assays were performed using a dual-specific luciferase assay kit (Promega) and measured by a GloMax 20/20 luminometer (Promega). All data are presented as the mean ± SD of triplicate independent experiments. Statistical significance was determined with the two-tailed Student t test, with a $P < 0.05$ considered statistically significant.

RESULTS

IDENTIFICATION OF HUMAN UBC9 AS A ZHX1-INTERACTING PROTEIN

To identify novel, cellular interaction partners of the ZHX1 protein, we employed the yeast two-hybrid approach to screen the cDNA library mentioned above using ZHX1 as the bait. Initial experiments were performed to test the self-activation of ZHX1 by co-transforming the *MaV203* yeast cells with BD-ZHX1 and empty pPC86 vector. The single yeast colonies containing BD-ZHX1 and empty vector pPC86 were cultured on SC-Leu-Trp-His agar plates with a series of 3AT concentrations (0, 10, 25, 50, 75, and 100 mM). The *MaV203* cells could not grow when the concentration of 3AT was higher than 25 mM (data not shown). From a total of 2×10^5 clones, the cDNA encoding the SUMO E2-conjugating enzyme (Ubc9) was obtained. To confirm the interaction between ZHX1 and Ubc9, *MaV203* yeast cells were co-transformed with BD-ZHX1 and AD-Ubc9 or other necessary controls, as shown in Figure 1A. Co-expression of ZHX1 and Ubc9, as well as a positive control, showed an evident effect on the activation of all reporter genes, but negative controls displayed negligible β-gal activity (Fig. 1A).

To determine whether ZHX1 interacts with Ubc9 in vivo in human cells, Flag-tagged ZHX1 and Myc-tagged Ubc9 were transiently co-transfected into HEK293T cells. We found that ZHX1 was present in

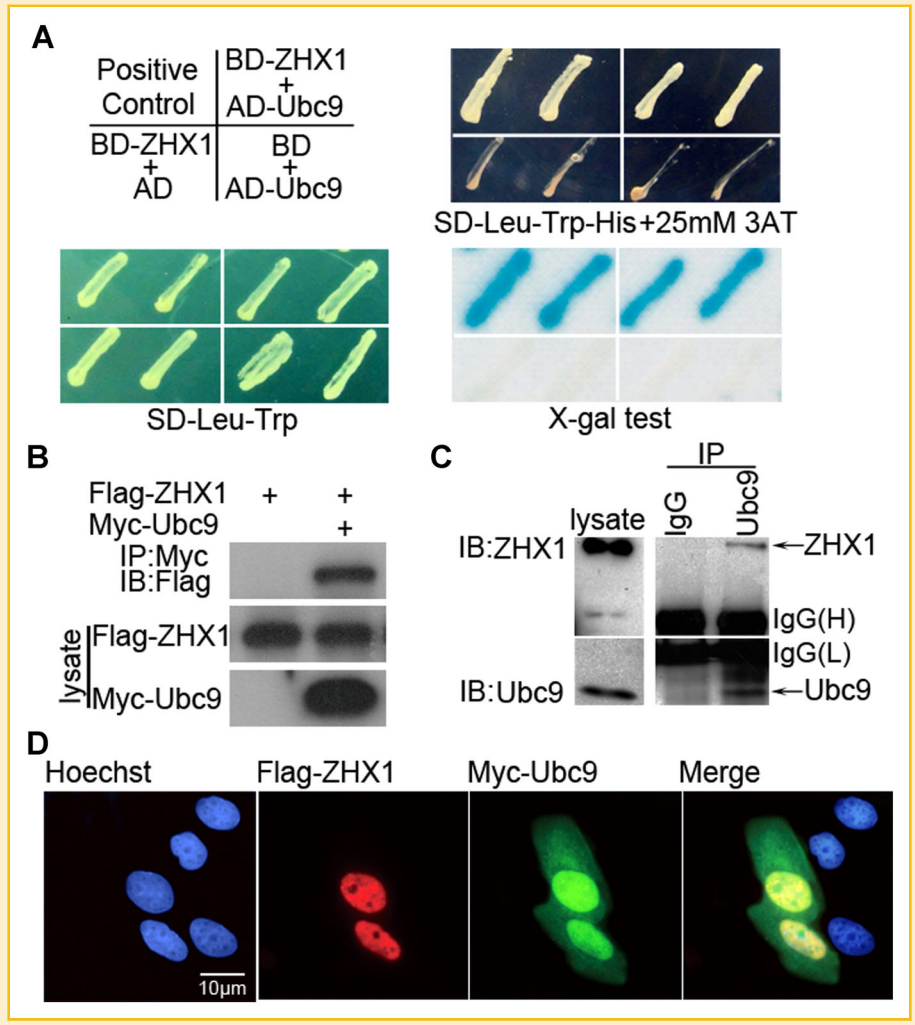

Fig. 1. Identification of Ubc9 as a ZHX1 interacting protein. A: Ubc9 interacts with ZHX1 in a yeast two-hybrid assay. Yeast *MaV203* cells were co-transformed with the indicated combinations of bait and prey plasmids (left, top). The single yeast colonies containing these plasmids were grown on SD-Leu-Trp (left, bottom) agar plates and on SC-Leu-Trp-His + 25 mM 3AT agar plates (right, top) and were tested using the X-Gal assay (right, bottom). AD and BD represent the pPC86 (containing the activation domain) and pDBLeu (containing the DNA-binding domain) vectors, respectively. Commercialized *MaV203* cells co-transformed with pPC97-Fos and pPC86-Jun are used as the positive control. B and C: Ubc9 interacts with ZHX1 in human cells. B: Flag-ZHX1 and Myc-Ubc9 were expressed together in HEK293T cells, as indicated. After 36 h, extracts containing equal amounts of protein were immunoprecipitated (IP) with an anti-Myc antibody and analyzed by immunoblotting (IB) with an anti-flag antibody (top panel). The expression of the proteins was detected in the lysates by using the indicated antibodies (lower panels). C: HEK293T cell lysates were immunoprecipitated (IP) with a control antibody (mouse IgG) or an anti-Ubc9 antibody and analyzed by immunoblotting (IB) with either an anti-ZHX1 (right, top panel) or an anti-Ubc9 antibody (right, lower panel). The left panels show the expression of ZHX1 or Ubc9 in the cell lysates. D: The co-localization of Flag-ZHX1 and Myc-Ubc9 in HeLa cells. Flag-ZHX1 and Myc-Ubc9 plasmids were co-transfected into HeLa cells. After 24 h, the cells were fixed with 4% paraformaldehyde, labeled with rabbit anti-flag antibody (1:100) and mouse anti-Myc antibody (1:100) for 2 h, then detected with FITC-tagged goat anti-mouse IgG and Cy3-tagged goat anti-rabbit IgG. The nuclei were stained with Hoechst33258, and the cells were examined by microscopy. The right panel (Merge) shows the merged images of the three panels.

the Myc-Ubc9 immunoprecipitates (Fig. 1B). To verify the interaction between endogenous ZHX1 and Ubc9, equal amounts of HEK293T cell lysates were immunoprecipitated with either a mouse anti-Ubc9 antibody or a control antibody (mouse IgG). The immunoprecipitates were then probed with an anti-ZHX1 antibody. We found that ZHX1 can bind to Ubc9 at endogenous levels (Fig. 1C). We also performed a co-localization experiment in HeLa cells, and the results showed that most of ZHX1 is expressed in the nucleus, while Ubc9 exists in both the cytoplasm and the nucleus (Fig. 1D). These data suggested that ZHX1 and Ubc9 might be associated in the nucleus.

ZHX1 IS SUMOYLATED BY SUMO1

As Ubc9 is the only E2-conjugating enzyme known to mediate the transfer of SUMO to target proteins [Meulmeester and Melchior, 2008], we examined whether the ZHX1 protein is a substrate for the SUMO modification. To address this question, we detected the subcellular localization of ZHX1 and SUMO1 by immunocytochemistry analysis using HeLa cells that were cotransfected with EGFP-ZHX1 and Red-SUMO1. As expected, a strong colocalization was observed in the nuclei (Fig. 2A). Then, we examined whether ZHX1 could be SUMOylated in cells. HEK293T

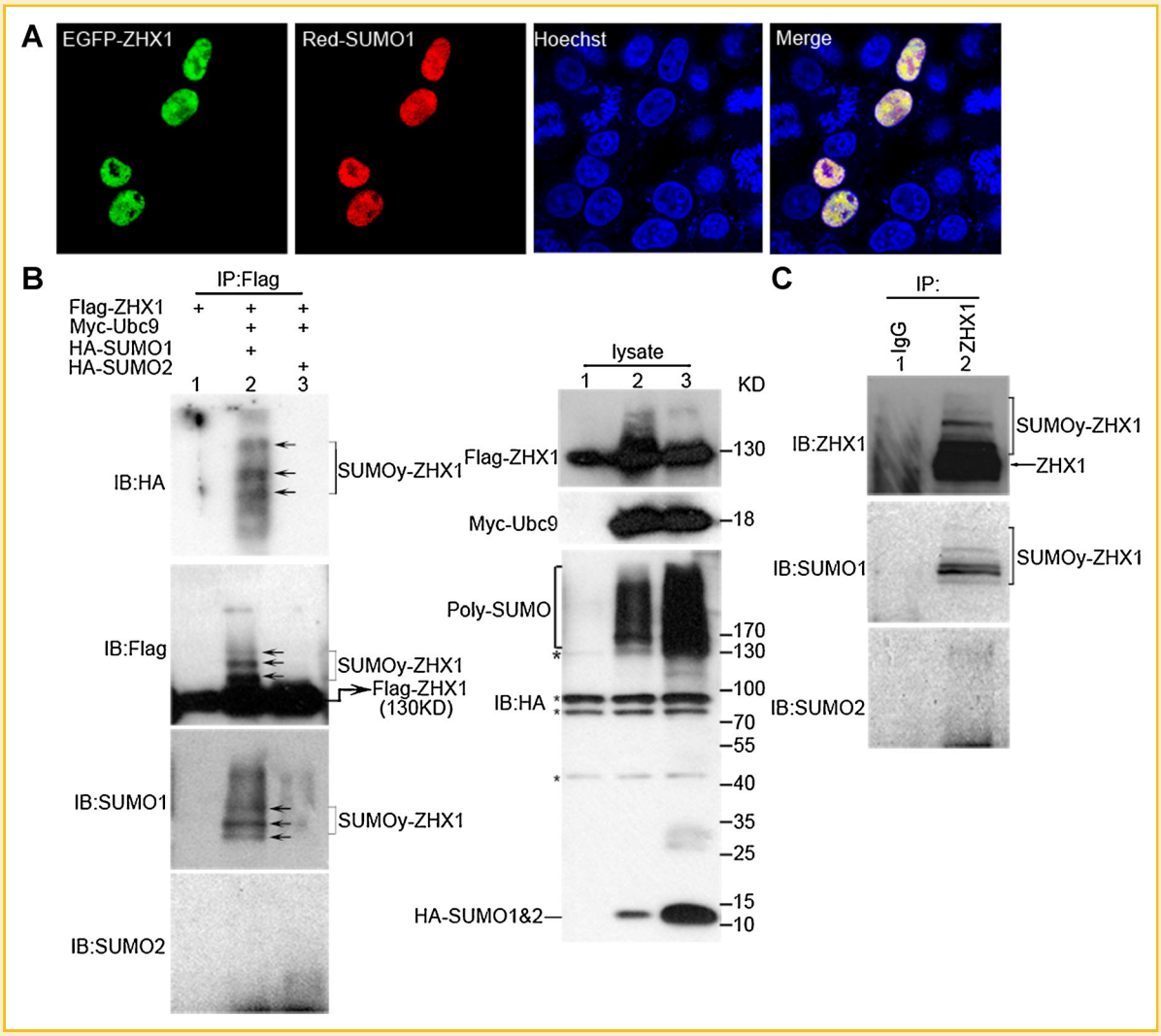

Fig. 2. ZHX1 is SUMOylated by SUMO1. A: The co-localization of EGFP-ZHX1 and Red-SUMO1 in HeLa cells. Hoechst33258 was used to stain the nuclei. The merged image is shown on the right. B: HEK293T cells were transfected with the indicated plasmids. After 36 h, the cell lysates were immunoprecipitated with an anti-flag antibody and analyzed by immunoblotting with anti-HA, anti-flag, anti-SUMO1 or anti-SUMO2 antibodies (left panels). The expression of Flag-ZHX1, HA-SUMO1, HA-SUMO2 and Myc-Ubc9 in the lysates was detected by immunoblot analysis with the indicated antibodies (right panels). C: HEK293T cell lysates were immunoprecipitated with control IgG or a ZHX1 antibody. The immunoprecipitates were analyzed by immunoblotting with anti-ZHX1, anti-SUMO1 or anti-SUMO2 antibodies.

cells were transfected with Flag-ZHX1, Myc-Ubc9 and HA-SUMO1 or HA-SUMO2. Co-immunoprecipitation was performed with an anti-flag antibody. Western blot analysis showed that the co-transfection of SUMO-1 caused a shift in ZHX1 to multiple, slower migrating bands that were immunoreactive with the anti-HA, anti-flag, and anti-SUMO-1 antibodies. In the same experiments, the co-transfection of SUMO-2 did not cause a shift in ZHX1 to slower-migrating bands (Fig. 2B). These data suggested that ZHX1 was specifically SUMOylated by SUMO-1 in HEK293T cells. To confirm that endogenous ZHX1 was modified by SUMO-1, equal amounts of cell extracts from HEK293T cells were immunoprecipitated with a rabbit anti-ZHX1 antibody or a control antibody (rabbit IgG), and then the immunoprecipitates were probed with anti-ZHX1, anti-SUMO-1 or anti-SUMO2 antibodies. Several high molecular weight bands were observed that were immunoreactive with the anti-ZHX1 and anti-SUMO-1 antibodies. These bands could not be detected with an anti-SUMO2 antibody (Fig. 2C). These data demonstrated that ZHX1 is subjected to SUMOylation by endogenous SUMO-1.

THE K159, K454, AND K626 ARE SUMOYLATION SITES OF ZHX1

SUMOylation often occurs at the lysine residue of substrates containing a ψKxE/D motif, where ψ represents hydrophobic residues and x is any amino acid [Gareau and Lima, 2010]. Sequence analysis of the ZHX1 protein revealed three putative SUMOylation sites at positions K159, K454, and K626. To examine whether these potential SUMO modification sites are in fact SUMOylated in vivo, we replaced these three conserved lysines with arginines, as indicated (Fig. 3A). Plasmids expressing HA-SUMO-1, Myc-Ubc9 and Flag-tagged plasmids containing the mutated ZHX1 cDNAs were cotransfected into HEK293T cells. Co-immunoprecipitation assays showed that

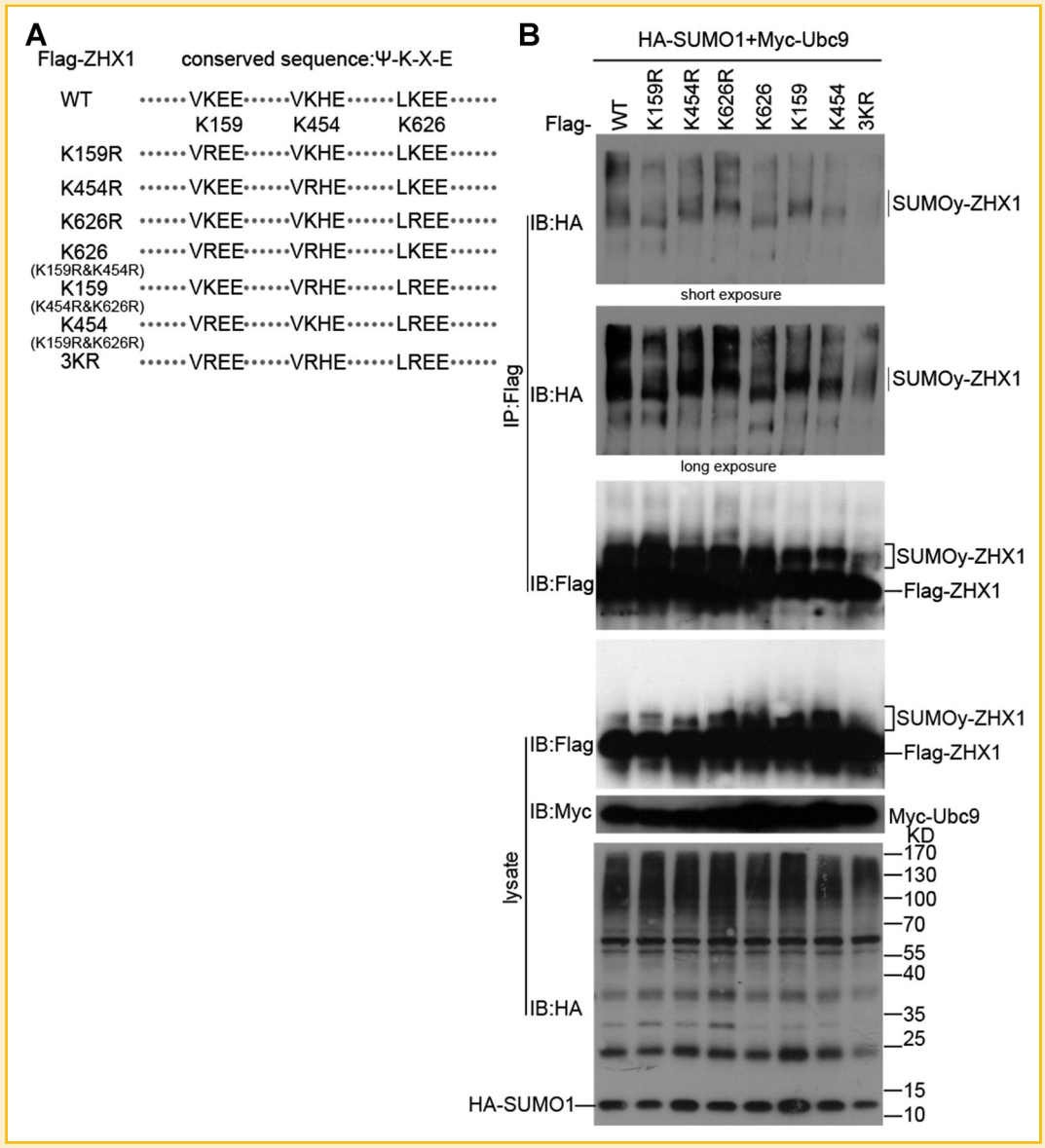

Fig. 3. Identification of three conserved SUMOylation sites (K159, K454, and K626) on ZHX1. A: An alignment of wild type and related mutants containing potential SUMOylation sites in ZHX1. The potential locations of modified lysines are marked by numbers. B: SUMOylation of wild type and mutant ZHX1. HEK293T cells were co-transfected with the indicated expression plasmids. The cell lysates were immunoprecipitated with an anti-flag antibody 36 h after transfection. The immunoprecipitates were analyzed by immunoblotting with anti-HA or anti-flag antibodies (upper panels). The expression levels of the transfected plasmids in the lysates were analyzed by Western blot analysis with the indicated antibodies (lower panels).

SUMOylated bands with slower mobility appeared when ZHX1 contains at least one of the wild type consensus lysines [K626 (K159R/K454R), K159 (K454R/K626R), K454 (K159R/K626R), K159R, K454R and K626R], while the mutation of all three lysines (3KR) leads to a less SUMOylated protein (Fig. 3B). We also observed that these migrating bands had a different mobility when the immunoprecipitates were loaded onto a 6% SDS–PAGE and analyzed by Western blot analysis with an anti-HA antibody (Fig. 3B, top two panels). These results indicated that ZHX1 is actually SUMOylated at the K159, K454, and K626 sites.

SUMOYLATION DOES NOT AFFECT THE ZHX1 SUBCELLULAR LOCALIZATION

To assess the effect of SUMOylation on the ZHX1 subcellular localization and to determine whether the ZHX1 subcellular localization was affected by its inability to be SUMOylated, we used confocal microscopy to detect the expression of EGFP-ZHX1 (WT) or EGFP-ZHX1 (3KR) in COS7 cells (Fig. 4A,C). We also performed immunohistochemical analysis on COS7 cells transfected with Flag-ZHX1 (WT) or Flag-ZHX1 (3KR) (Fig. 4B,D). Fluorescence imaging (Fig. 4A,C) and immunostaining using anti-flag antibody

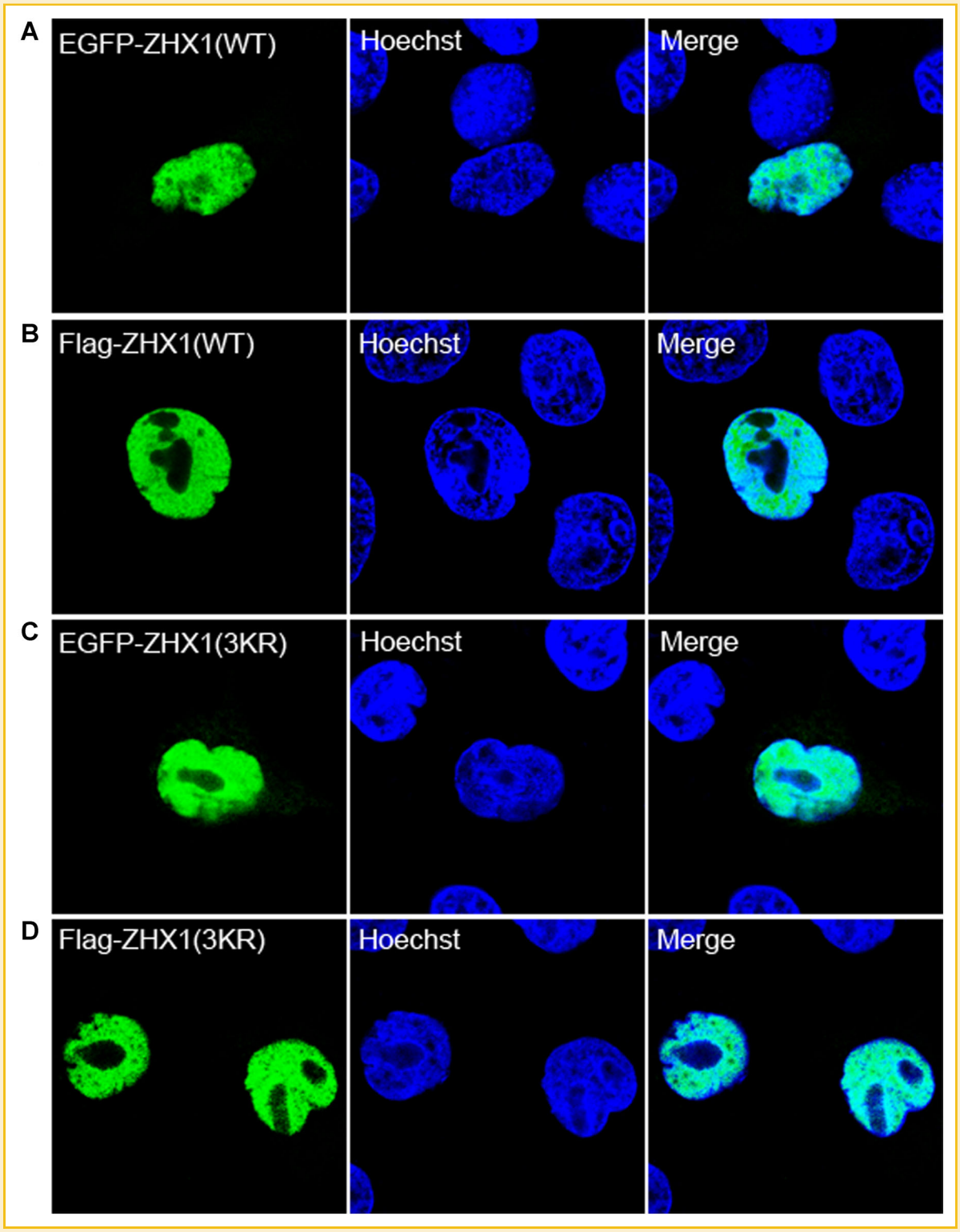

Fig. 4. Effect of SUMOylation on ZHX1 subcellular localization. A and C: Fluorescence imaging. B and D: Immunocytochemical analysis was performed on COS7 cells expressing EGFP-ZHX1 (WT), EGFP-ZHX1 (3KR) Flag-ZHX1 (WT), or Flag-ZHX1 (3KR). For B and D: An anti-flag antibody was used to detect the flag-tagged proteins. Hoechst33258 was used to stain the nuclei. The merged images are shown in the third panel.

(Fig. 4B,D) showed that both Flag-ZHX1 (WT) and Flag-ZHX1 (3KR) were present in the nucleus. Thus, preventing ZHX1 from being SUMOylated does not affect its subcellular localization. These data suggested that SUMOylation does not play a pivotal role in determining the distribution pattern of ZHX1 in cells.

SUMOYLATION REGULATES THE PROTEIN STABILITY AND UBIQUITINATION OF ZHX1

Because ZHX1 is SUMOylated in vivo, we wanted to determine whether SUMOylation regulates the protein stability of ZHX1. To answer this question, a CHX chase assay was performed. HEK293T cells were transfected with flag-tagged wild-type or 3KR-mutant ZHX1, followed by treatment with the protein translation inhibitor cycloheximide (CHX, Beyotime), either alone or together with the 26S proteasome inhibitor MG132 (10 μM, Beyotime), for six different time periods (0, 3, 6, 9, 12, and 24 h). We found that the half-life of wild-type ZHX1 was shorter than that of the 3KR mutant when treated with CHX (Fig. 5A). This showed that the stability of ZHX1 could be affected by the SUMO modification.

In this experiment, we also observed that the 26S proteasome inhibitor MG132 could partially inhibit wild-type ZHX1 degradation (Fig. 5A). This result suggested that the ZHX1 protein might be degraded by the ubiquitin proteasome pathway. By measuring the ubiquitination levels of ZHX1 deletion mutants, we have found that more ubiquitin was detected in the regions containing amino acids 272–564 and 565–732 of ZHX1 (Fig. 5C). Further research showed

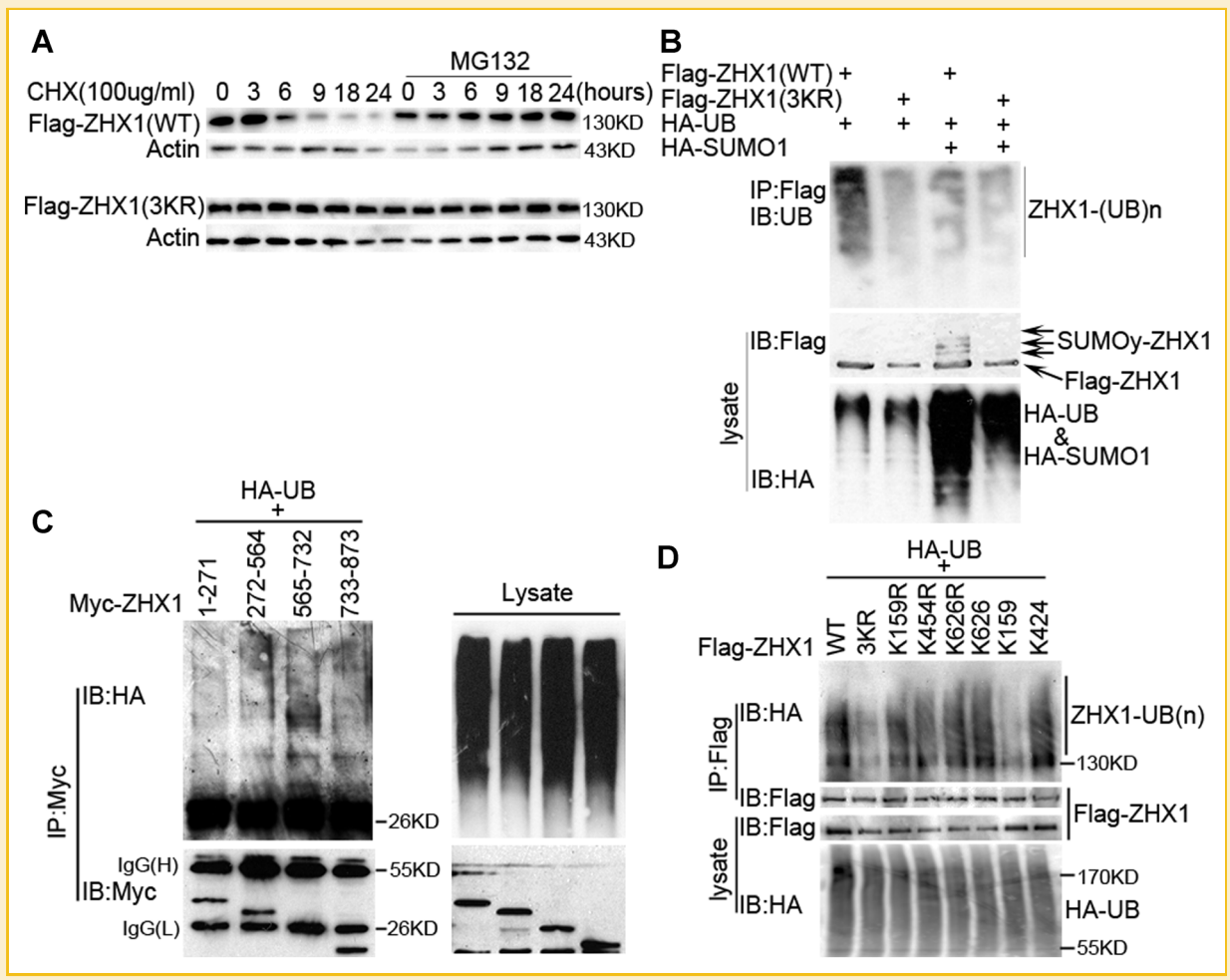

Fig. 5. Effect of SUMOylation on protein stability and ubiquitination of ZHX1. A: A CHX chase assay of the wild type and 3KR-mutant ZHX1. HEK293T cells were transfected with flag-tagged wild type or 3KR-mutant ZHX1. The cells were treated with CHX1 (100 μg/ml) and MG132 (10 μM) for the indicated times 24 h after transfection. The cell lysates were collected and analyzed by Western blot analysis with anti-flag or anti-β-actin antibody. B: The effect of SUMOylation on the ubiquitination of ZHX1. HEK293T cells were transfected with the expression plasmids, as indicated. The cell lysates were immunoprecipitated with an anti-flag antibody 36 h after transfection. The immunoprecipitates were analyzed by immunoblotting with an anti-ubiquitin antibody (upper panel). The expression levels of the transfected plasmids in the lysates were analyzed by Western blot analysis with anti-flag or anti-HA antibodies (lower panels). C and D: K454 and K626 were the ubiquitination sites on ZHX1. HEK293T cells were transfected with the expression plasmids, as indicated. The cell lysates were immunoprecipitated with anti-Myc or anti-flag antibodies 36 h after transfection. The immunoprecipitates were analyzed by immunoblotting with the indicated antibodies (upper panel). The expression levels of the transfected plasmids in the lysates were analyzed by Western blot analysis with anti-Myc, anti-flag, or anti-HA antibodies (lower panels).

that the ubiquitination levels of 3KR and K159 mutants were less than that of the wild-type ZHX1 (Fig. 5B,D), indicating that the K454 and K626 sites may function as ubiquitination sites as well. The SUMOylation of wild-type ZHX1 reduced its ubiquitination, while the SUMOylation-deficient ZHX1 mutant did not show any apparent change in the level of ubiquitination (Fig. 5B). These results demonstrated that the SUMOylation of ZHX1 could suppress its ubiquitination and that SUMO and ubiquitin may compete for the K454 and K626 sites of ZHX1.

SUMOYLATION MODULATES THE TRANSCRIPTIONAL REPRESSION ACTIVITY OF ZHX1

ZHX1 was identified as a transcriptional regulatory factor. The transcriptional regulatory activity of ZHX1 is often measured by the mammalian one-hybrid system [Yamada et al., 2002]. Using this reporter system, our results (Fig. 6A) were consistent with the previous report that GAL4 DBD fused with ZHX1 (GAL4-ZHX1) inhibits the activity of the 5xGAL4-GL3 reporter, which contains five copies of the GAL4-binding site that were inserted into the pGL3-Control plasmid upstream of the SV40 promoter. To determine the effect of SUMOylation on the transcriptional repression activities of ZHX1, HEK293T cells were transfected with plasmids expressing GAL4 DBD fused with wild-type ZHX1 (GAL4-ZHX1 WT), either alone or together with plasmids expressing Myc-Ubc9 and HA-SUMO1. By measuring the activity of the 5xGAL4-GL3 reporter, we found that Myc-Ubc9 combined with HA-SUMO1 could increase the activity of the reporter (Fig. 6B), indicating that the SUMOylation of ZHX1 may decrease the transcriptional repressive activity of ZHX1. To determine the contribution of all three lysines to the repression activity of ZHX1, the GAL4 reporter assays were performed by transfecting HEK293T cells with the GAL4 tagged WT or mutant ZHX1 plasmids. Notably, the repressive activity of GAL4-ZHX1 (3KR) was higher than that of GAL4-ZHX1 (WT) but lower than the activity of the SUMO1 fused ZHX1 (Gal4-ZHX1-SUMO1) (Fig. 6C). These results indicated that the SUMOylation of ZHX1 inhibited the repressive activity of ZHX1 and may positively regulate the target genes of ZHX1.

DISCUSSION

In this study, we demonstrated the interaction of ZHX1 and Ubc9, an E2-conjugating enzyme involved in the SUMOylation of target proteins, by a yeast two-hybrid system, co-immunoprecipitation and co-localization assays. We further showed that ZHX1 could be SUMOylated via Ubc9. Using bioinformatics and the yeast two-hybrid method, a previous report showed that several zinc finger-containing proteins such as ZCCHC7, ZCCHC12, ZNF237, ZNF198, and ZHX1 can interact with both SUMO1 and SUMO2 [Hecker

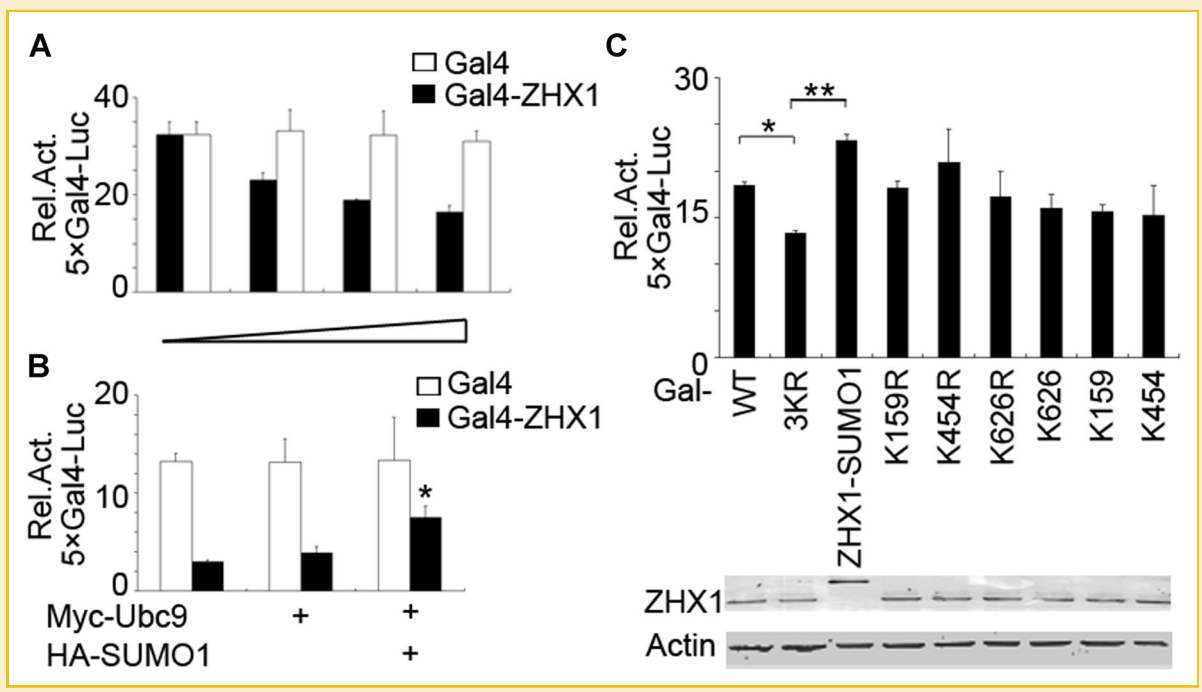

Fig. 6. Effect of SUMOylation on ZHX1 transcriptional repressive activity. A: ZHX1 is a transcriptional repressor. HEK293T cells were transfected with a 5xGal4-Luc reporter together with an increasing dose of the Gal vector or Gal4-ZHX1 (0, 0.1, 0.2, or 0.3 μg). The empty vector pcDNA3.0 was added to each transfection to maintain an equal amount of total DNA. pRL-TK was transfected into the cells to normalize the transfection efficiency. The reporter assays were processed 36 h after transfection. B: The transcriptional repressive activity of ZHX1 was inhibited by SUMO1. HEK293T cells were transfected with the Gal vector or Gal4-ZHX1 (0.3 μg), Myc-Ubc9 (0.2 μg) and HA-SUMO1 (0.2 μg) plasmids, as indicated. Empty plasmid was added to maintain an equal amount of total DNA, and pRL-TK was transfected into the cells to normalize the transfection efficiency. The reporter assays were processed 36 h after transfection. C: The relative transcriptional activity of wild type and mutant ZHX1. The reporter assays were performed as described in B: Except that cells were transfected with Gal4-fused wild type or mutant ZHX1 (0.3 μg). $^*P < 0.05$; bar graphs show the mean $\pm$ SD, n = 3. The lysates were collected and analyzed by Western blot analysis with anti-flag or anti-β-actin antibodies for detecting the expression of the ZHX1 mutants (lower panels).

et al., 2006]. However, in the current work, we demonstrated that ZHX1 was only SUMOylated by SUMO1. We also identified three SUMOylation sites (K159, K454, and K626) in ZHX1.

SUMOylation plays an important role in the regulation of protein subcellular localization, stability, ubiquitination, transcriptional activity and the protein-protein interactions of transcription factors [Percherancier et al., 2009; Cai et al., 2010]. ZHX1 is synthesized in the cytoplasm, then translocated to the nucleus via its nuclear localization signal sequence. In our experiments, fluorescence imaging and immunostaining assays showed that most of the wild type and 3KR-mutant ZHX1 were expressed in the nucleus (Fig. 4A, B). These data showed that SUMOylation did not affect the distribution pattern of ZHX1 in cells. Regarding the physiological function of SUMOylation on ZHX1, we found that the 3KR mutant is more stable than wild type ZHX1 upon CHX treatment. These results suggested that the mutation of these sites protects ZHX1 from degradation. Thus, the K159, K454, and K626 sites could serve as elements for regulating ZHX1 degradation. At the same time, the 26S proteasome inhibitor MG132 partially inhibits the degradation of wild type ZHX1. This revealed that ZHX1 may be degraded though the 26S-proteasome-mediated pathway. As SUMOylation and ubiquitination are both lysine-targeted modifications, the antagonistic relationship between SUMOylation and ubiquitination may play an important role in regulating ZHX1 activity. We have found that SUMOylation inhibits the ubiquitination of ZHX1 and that the mutation of all three SUMOylation sites decreases ZHX1 ubiquitination. Through ubiquitination assays, we also found that the ubiquitination of ZHX1 was decreased when two or three of the SUMOylation sites were mutated (K454/626R or 3KR). Therefore, SUMO and ubiquitin may competitively associate with lysines 454 and 626 of ZHX1, in vivo. This concept may be similar to that of IκBα, for which SUMOylation antagonizes ubiquitination through the same attachment residue, Lys21 [Desterro et al., 1998]. In addition, ZHX1 is also similar to Smad4, in which the SUMO modification sites are also targeted by ubiquitin. Mutation of these sites in Smad4 blocks SUMOylation and reduces its ubiquitination, resulting in the enhancement of its stability [Lin et al., 2003]. However, the cooperation between SUMOylation and ubiquitination has also been documented [Ulrich, 2005]. For example, NEMO can be modified sequentially by SUMO and ubiquitin, which mediates NF-κB activation in genotoxic stress [Huang et al., 2003]. However, these modifications take place at different amino acid residues.

Although, the effects of SUMOylation on transcription factors are diverse, there is much evidence that modifications by SUMO play pivotal roles in regulating transcription factor activity. In most cases, SUMOylation has been found to inhibit transcription. For example, SUMOylation negatively regulates the transcriptional activity of Elk1 [Yang and Sharrocks, 2004], C-Jun [Muller et al., 2000], and Sp3 [Sapetschnig et al., 2002] by inhibiting their trans-activation function. In another transcriptional factor, IRF2, mutations in the SUMOylation sites decreases the ability of IRF2 to inhibit IRF1-mediated transcription [Han et al., 2008]. However, the SUMO-1 modification of p53 increases its DNA binding activity and activates GATA4-dependent cardiogenic gene activity [Rodriguez et al., 1999; Wang et al., 2004]. Thus far, the DNA binding sites of ZHX1 have not been identified, and the specific genes regulated by the transcription factor ZHX1 are not known. Therefore, we are unable to examine the influence of SUMOylation on ZHX1 transcriptional activity in a gene-specific manner. However, ZHX1 was found to interact with NFYA [Yamada et al., 1999a], a general transcription factor that binds to CCAAT motifs in the promoter region of a variety of genes [Xu et al., 2012]. Using a Miwi promoter reporter containing a CCAAT motif as a model [Hou et al., 2012], we determined whether the SUMOylation of ZHX1 has any effect on the transcriptional activity of NFYA. The results showed that neither the wild type nor the 3KR-mutant ZHX1 has any effect on the activity of the Miwi promoter reporter (data not shown), suggesting that ZHX1 may not be involved in the regulation of NFYA-mediated transcription. The transcriptional activity of ZHX1 is often tested by a mammalian one-hybrid system [Yamada et al., 2002]. In the present study, we demonstrated that the 3KR-mutant ZHX1 is a stronger repressor than the wild type ZHX1, by measuring the 5xGal4-luc reporter. Meanwhile, expression of Ubc9 together with HA-SUMO1 inhibits the transcriptional repressive activity of wild-type ZHX1. These data suggested that SUMO modification plays a positive role in the transcription of ZHX1-regulated genes. The revelation of the SUMOylation of ZHX1 and the effects of SUMOylation on ZHX1 functions may help further the understanding of the molecular mechanisms and biological functions of ZHX1 in the future.

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SUPPORTING INFORMATION

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Table S1. The primers used in the study