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Sumoylation regulates nuclear localization and function of zinc finger transcription factor ZIC3

Li Chen [a,b], Yanlin Ma [c], Ling Qian [a], Jun Wang [a,*]

[a] The Center for Stem Cell Engineering, Texas Heart Institute, Houston, TX 77030, USA
[b] Department of Biology and Biochemistry, University of Houston, Houston, TX 77204, USA
[c] Institute of Biosciences and Technology, Texas A&M University Health Science Center, Houston, TX 77030, USA

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ABSTRACT

ZIC3, an X-linked zinc finger transcription factor, was the first identified gene involved in establishing normal left–right patterning in humans. Mutations in the Zic3 gene in patients cause heterotaxy, which includes congenital heart defects. However, very little is known about how the function of the ZIC3 protein is regulated. Sumoylation is a posttranslational modification process in which a group of small ubiquitin-like modifier (SUMO) proteins is covalently attached to targets via a series of enzymatic reactions. Here, we report for the first time that sumoylation targets human ZIC3 primarily on the consensus lysine residue K248, which is critical for the nuclear retention of ZIC3. Consequently, SUMO modification potentiates the repressive activity of ZIC3 on the promoter of its target gene *cardiac α-actin*, and the mutation of lysine 248 to arginine (K248R) abolishes its repressive function. We further revealed that ZIC3 variants with mutations found in human patients with congenital anomalies exhibit aberrant sumoylation activity, which at least partially accounts for their cytoplasmic diffusion. Improved sumoylation of human disease-associated ZIC3 variants reestablishes their nuclear occupancy in the presence of SUMO E3 ligase and SUMO-1. Thus, the altered sumoylation status of ZIC3 underpins the developmental abnormalities associated with these ZIC3 mutants. The SUMO targeting consensus sequence in ZIC3 is highly conserved in its paralogs and orthologs, pointing to sumoylation as a general mechanism underlying the functional control of ZIC proteins. This study provides a potential therapeutic strategy to regain the normal subcellular distribution and function of ZIC3 mutants by restoring SUMO conjugation.

1. Introduction

ZIC (zinc finger in the cerebellum) genes belong to the Gli superfamily of transcription factors. The ZIC gene family is composed of five members (ZIC1, ZIC2, ZIC3, ZIC4, and ZIC5) in human and mouse, all of which share highly conserved five tandem C2H2 zinc finger (ZF) motifs [1]. Mutations in ZIC genes causally underlie a variety of congenital malformations such as holoprosencephaly and neural tube defects in murine models and in humans [2–7], indicating the essentiality of ZIC proteins to proper organogenesis. Of these five family members, ZIC3 is the only X-linked gene and mutations of ZIC3 are associated with human laterality defects and sporadic congenital cardiac structural malformations [8]. The exact mechanisms underlying the various disease manifestations associated with ZIC3 mutations are not well understood; however, the role of ZIC3 in defining left–right asymmetry during vertebrate embryogenesis was at least partially attributable to its regulation of *nodal* expression in the node [9]. The incomplete phenotypic penetrance of ZIC3 mutations in humans may also be influenced by gene modifier(s) and environmental cues [10,11].

One important feature that governs ZIC3 function is nucleocytoplasmic trafficking. The mechanisms underlying its subcellular shuttling were previously studied and the existence of nuclear localization signals (NLS) and a nuclear export signal (NES) has been proposed as a mechanism whereby proper nuclear occupancy and function of ZIC3 was maintained [12,13]. Interestingly, a number of human disease-linked naturally occurring missense mutations in ZIC3 such as C253S (conversion of cysteine 253 to serine), W255G (conversion of tryptophan 255 to glycine), H286R (conversion of histidine 286 to arginine), T323M (conversion of threonine 323 to methionine) resulted in cytoplasmic retention and diminished function [14,15]. However, some of these mutations (i.e., C253S, W255G, and H286R) are not localized in any known NES or NLS. Moreover, the W255G mutation did not appear to significantly alter the structure of ZIC3 [13]. Thus, whether there are any unifying mechanisms that are associated with the extranuclear diffusion of these mutants remain to be elucidated.

* Corresponding author at: Texas Heart Institute, Center for Stem Cell Engineering, Houston, TX 77030, USA. Tel.: +1 832 355 9542; fax: +1 832 355 9333.
E-mail address: junwang@heart.thi.tmc.edu (J. Wang).

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As a dynamic regulatory mechanism, posttranslational modifications modulate functions of a variety of proteins involved in a wide spectrum of cellular events. To this end, whether and how the function of ZIC proteins is regulated by posttranslational modification(s) has not been well studied. The only clue so far is a study demonstrating that the phosphorylation of ZIC2 by DNA-PK promoted its function via enhanced stability of the protein complex containing ZIC2 and RNA helicase A, and that, conversely, dephosphorylation inhibited ZIC2's activity [16].

Sumoylation is a posttranslational modification in which SUMO (small ubiquitin-related modifier) proteins are covalently and reversibly conjugated to the specific lysine residue(s) via a series of enzymatic reactions involving the heteromeric activating enzyme E1 (SAE1/2), the conjugation enzyme E2 (Ubc9), and a number of SUMO E3 ligases represented by PIAS family; subsequently, the activity of these targets are altered via multiple mechanisms, including changes in subcellular localization and/or protein stability [17]. The SUMO-targeting consensus sequence has been identified as ΨKXE (where Ψ represents a bulky hydrophobic amino acid, and *X* represents any residue) [18]; however, SUMO conjugation can also occur on non-canonical lysine residues, particularly in the presence of SUMO E3 ligases. SUMO modification has been implicated in the pathogenesis of a number of human diseases [19–22]. Our group and others have identified a multitude of SUMO targets such as GATA4, myocardin, Nkx2.5, Prox1 [23–28] that are critical for cardiovascular development and function, and have demonstrated that decreased SUMO-1 conjugation in murine hearts, induced either by SUMO-1 knockout or by cardiac overexpression of SENP2 (a SUMO isopeptidase), caused congenital heart defects and/or cardiac dysfunction [29,30]. These studies substantiate the essential role of SUMO conjugation pathway in proper cardiac morphogenesis and function (see reviews [31,32]). Here we report for the first time that SUMO regulates nucleocytoplasmic shuttling and the function of ZIC3. A number of human disease-associated ZIC3 mutants that affect subcellular trafficking also exhibit aberrant sumoylation; restoring the sumoylation of these mutants reestablish their nuclear occupancy. Thus, sumoylation may serve as a general mechanism governing the nucleocytoplasmic shuttling of ZIC3, and the altered sumoylation status of ZIC3 mutants may be potentially associated with disease manifestations. Also, since the SUMO target sequence in the ZIC3 protein is highly conserved among its paralogs and orthologs, this posttranslational modification may represent a general mechanism controlling the function of ZIC proteins.

2. Materials and methods

2.1. Plasmid constructions

Vectors expressing HA-tagged human ZIC3 WT and its human disease-associated mutants *C253S*, *H285R*, *T323M*, *K405E*, *P217A* were generous gifts from Dr. John Belmont (Baylor College of Medicine, Houston, Texas) [33]. The cDNAs of ZIC3 WT and its mutants were then PCR-amplified and subcloned into pcDNA-4A-V5/His expression vector (Invitrogen). SUMO-1-fused human *ZIC3* expression vector was generated by PCR using corresponding oligonucleotides covering *SUMO-1* cDNA, which were thereafter subcloned into pcDNA4A-ZIC3-V5/His vector with *KpnI* and *BamHI* at the 5′ end of *ZIC3* cDNA. *ZIC3* consensus SUMO site mutant *K248R* (*ZIC3-K248R*) was generated by a two-step PCR protocol. Human ZIC3 *W255G* mutant was generated using site-directed mutagenesis kit (Stratagene). All mutants were confirmed by sequencing. The *cardiac alpha actin* promoter-driven luciferase reporter construct (*Ca-actin-Luc*) was previously described [34]. *SUMO-1*, *SUMO-2*, *SUMO-3* expression vectors, *PIAS1*, *PIAS3*, *PIASy*, *PIASxα*, *PIASxβ*, and *PIAS1-RING domain mutant* (*PIAS1-RING mut*) were detailed previously [25,35].

2.2. Antibodies

Anti-V5-HRP antibody (Invitrogen, R961-25), anti-HA-HRP antibody (Genscript, A00169), anti-GAPDH-HRP antibody (Santa Cruz, sc-20357), and anti-SUMO-1 antibody (Santa Cruz, sc-9060).

2.3. Cell culture, transient transfection

The regular Hela cells and the stable Hela cell line that expresses 6xHis-tagged SUMO-1 (su-Hela), which was generously provided by Dr. Alfred Vertegaal (Leiden University Medical Center, Leiden, Netherlands) [36], were used in the present study. Reporter transactivation assays were performed in 12-well plates, whereas transient transfections for Western blotting were carried out in 10 cm plates. At least two independent reporter assays were performed to obtain conclusive data. Hela cells were harvested 48 h post-transfection, and then luciferase activity assays were executed with Monolight ™3010 (Amersham Biosciences). For Western blotting assays, expression vectors (0.5 μg each) as indicated in each figure legend were transfected into Hela cells. Cell lysates were purified after 48 hours transfection and subjected to 4–12% Bis-Tris NuPage gel, transferred to polyvinylidene difluoride (PVDF) membrane, visualized by desired antibody using ECL plus (GE Healthcare) or Immobolin Western (Millipore).

2.4. Immunocytofluorescence

The immunofluorescence staining was detailed previously [37]. Briefly, to examine the subcellular localization of human ZIC3 WT and its mutants, V5-tagged ZIC3 WT or its mutants were transfected into Hela cells in the absence or presence of the nuclear export inhibitor Leptomycin B (LMB) (50 ng/μl) for 12 h. Transfected cells were fixed and blocked. Then sections were incubated with the primary mouse anti-V5 antibody and washed, followed by Alexa fluor® 488 donkey anti-mouse secondary antibody (Invitrogen, A21202). To examine the effects of SUMO conjugation on subcellular localization of human ZIC3 mutants, each V5-tagged ZIC3 mutant of interest was transfected into su-Hela alone, or together with HA-epitoped PIAS1 WT or PIAS1-Ring mutant (PIAS1-M), followed by double immunofluorescence staining against HA and V5 epitopes. All slides were mounted and photographed under a Leica fluorescent microscope. The number of exclusively nuclear stained cells was scored against the number of both cytoplasmic and nuclear stained cells (diffused staining) from 100 randomly selected cells per group, and was summarized from two independent assays for each staining.

2.5. In vivo sumoylation and Ni^{2+}-NTA chromatography

Ni-NTA chromatography, which was described previously [24,25], was used to evaluate the SUMO conjugation of human ZIC3 WT and its mutants in vivo. *ZIC3*-WT, or one of its human disease-associated mutants *K248R*, *C253S*, *W255G*, *H285R*, *T323M*, *K405E* or *P217A*, was transfected into Hela cells in the absence or presence of flag-tagged *SUMO-1* as indicated in each figure legend. To determine whether PIAS1 potentiates the sumoylation of ZIC3 in vivo, the similar transfection assays were also performed in the presence of the encoding vector for either *PIAS1 WT* or its RING-mut.

2.6. Statistical analysis

DATA are presented as the mean ± SEM. The unpaired Student's *t* test is applied to determine statistical significance between groups when applicable and shown in each figure legend. The p value less than 0.05 is considered as significant.

3. Results

3.1. ZIC3 is a novel SUMO substrate

Fig. 1A upper panel shows the representative structure of human ZIC3, including five zinc finger (ZF) domains, and the relative locations of NES (nuclear export signal) and NLS (nuclear localization signal) [12]. It also shows the location of each of six human disease-linked ZIC3 mutants that are selected for the assays in the present study. To identify if ZIC3 is a potential SUMO target, we first used bioinformatics program SUMOsp and revealed one putative sumoylation motif with a high score (3.389) in human ZIC3, $IK_{248}VE$, which conforms to the SUMO targeting consensus sequence previously identified [18]. This IKVE motif is well conserved in all of its paralogs as well as its orthologs examined, including human, mouse, *Xenopus*, rat and zebra fish (Fig. 1A, lower panel, boxed gray region), indicative of its potential importance for functional regulation. To determine whether SUMO targets ZIC3 and whether the K248 is a SUMO site, Ni-NTA pulldown assay was conducted on Hela cell lysates transfected with V5-6xHis-tagged ZIC3 WT or its mutant *K248R* (conversion of lysine 248 to arginine), in the presence or absence of SUMO-1 and/or PIAS1, the latter which is a potent sumoylation E3 ligase. A retarded band which is equivalent to the addition of one SUMO molecule and is responsive to anti-V5 antibody is only observed in the presence of both ZIC3 WT and SUMO-1, but not ZIC3 alone (SF1, compare lane 1 with lane 2), suggesting ZIC3 as a SUMO substrate. This slow migratory band is hardly seen by anti-V5 antibody in the lane containing the mutant *K248R* and SUMO-1 (SF1, compare lane 5 with lane 2), while free *K248R* mutant was equivalently precipitated by Ni-NTA beads as ZIC3 WT, indicating that K248 was the principal SUMO site in ZIC3. While PIAS1 enhances the SUMO conjugation to ZIC3 WT by introducing additional more slow migrating bands that are detected by anti-V5 antibody as expected (SF1, compare lane 3 with lane 2), it also induces sumoylation of the *K248R* mutant in the presence of ectopically expressed SUMO-1 (SF1, lane 6). However, the PIAS1-induced sumoylation pattern and intensity of *K248R* mutant is different from that of ZIC3 WT: the sumoylated bands of *K248R* mutant migrate faster in the western gel, and are weaker compared with their counterparts in ZIC3 WT (SF1, compare lane 6 with lane 3), suggesting an activation of non-canonical SUMO site(s) by PIAS1. To further confirm that the slow migratory band is SUMO modified ZIC3, we used the similar experimental strategy and found that the slower migratory band observed in SF1 lane 2 is reproduced and responsive to both antibodies against V5 and SUMO-1 (Fig. 1B, lane 2, asterisks), corroborating ZIC3 as a novel SUMO substrate. In line with the observation shown in SF1, SUMO conjugated K248R is much weaker, and migrates faster than that of ZIC3 WT (Fig. 1B, lane 4, asterisks). Thus, conversion of lysine 248 to arginine appears to activate a weak non-classical SUMO acceptor site, which otherwise would be latent in the presence of the intact SUMO targeting sequence. Additionally, the mutation on another SUMO consensus sequence with a low score

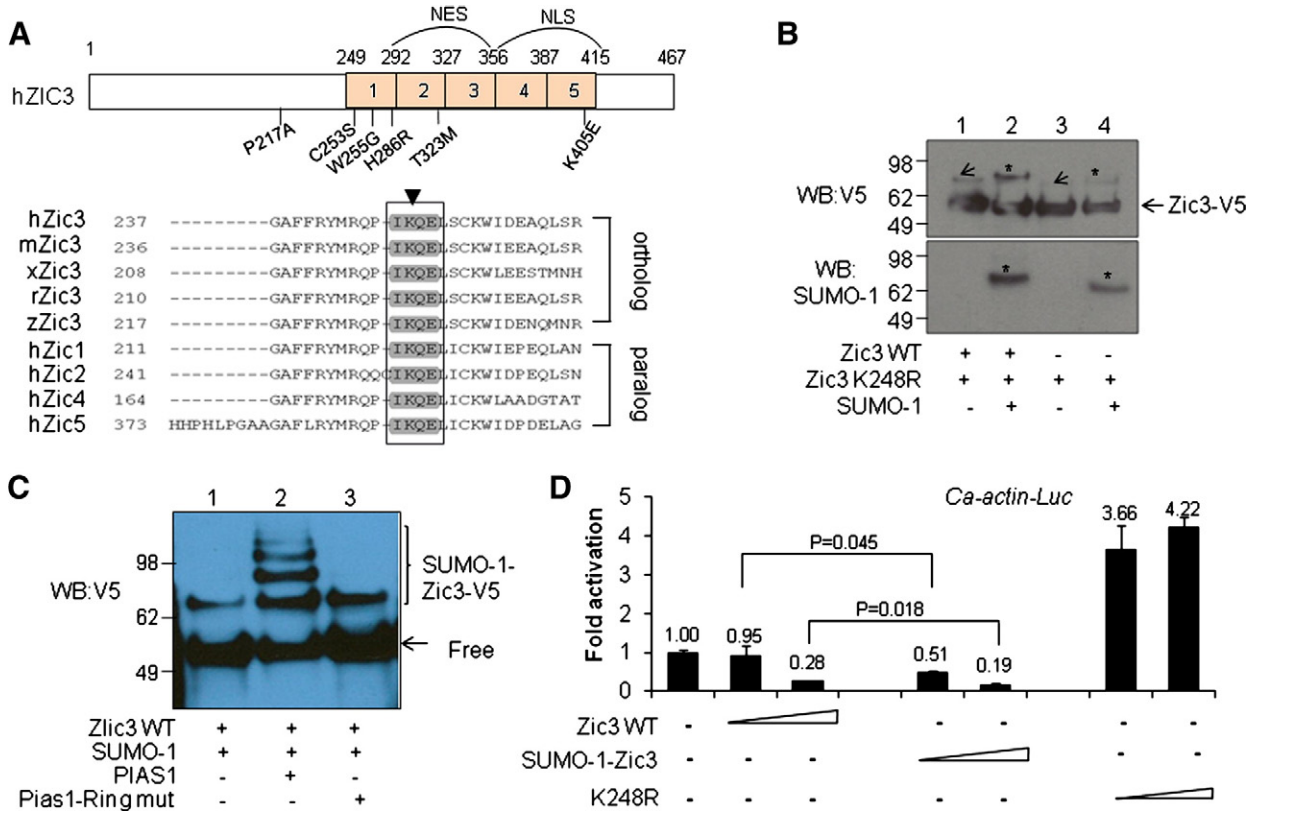

Fig. 1. ZIC3 is a target of post-translational sumoylation. (A) A schematic representation of the major structures in human ZIC3. The upper panel shows the five zinc finger domains (#1–#5, 249–415 aa), relative locations of NES (nuclear export signal) and NLS (nuclear localization signal), and the relative position of each of six human-linked ZIC3 variants selected for the assays in the present study. The lower panel shows lysine 248 (K, arrow), the primary SUMO site, which is highly conserved among ZIC3 orthologs and paralogs. Constraint-based Multiple Alignment Tool (COBALT) is used for sequence alignment. h: human; m: mouse; x: *Xenopus*; r: rat; z: zebrafish. (B–C) Identification of lysine 248 as the primary SUMO acceptor site in ZIC3. (B) Western blotting using either anti-V5-HRP antibody or anti-SUMO-1 antibody confirms that the SUMO modification of ZIC3-K248R mutant (lane 4) is greatly reduced compared to that in ZIC3 WT (lane 2). Ni-NTA pulldown assays were performed on Hela cell lysates expressing V5-6xHis-tagged ZIC3 WT or K248R mutant in the presence or absence of exogenous SUMO-1. The blot was first probed with anti-V5 antibody (upper panel), and then was stripped and reprobed with anti-SUMO-1 antibody (lower panel). Asterisks indicate SUMO-conjugated ZIC3. Arrows indicate non-specific bands which are not reactive with anti-SUMO-1 antibody. (C) Ni-NTA was performed on cell lysates purified from Hela cells transfected with V5-6xHis-tagged ZIC3 WT/SUMO-1, or in the presence of either PIAS1 WT or PIAS1 RING-mut. PIAS1 WT, but not PIAS1 RING-mut, enhances the SUMO modification of ZIC3-WT (compare lane 2 with lane 3). Blot: anti-V5. (D) The repressive activity of ZIC3 on cardiac α actin promoter is enhanced by SUMO-1 fusion (SUMO-1-ZIC3), but is abolished by the mutation of lysine 248 to arginine (K248R). Promoter activity is expressed as the ratio of luciferase activity induced by the presence of specific factor(s) to the control group with the presence of empty vector only. The luciferase activity in the control group is taken as 1.

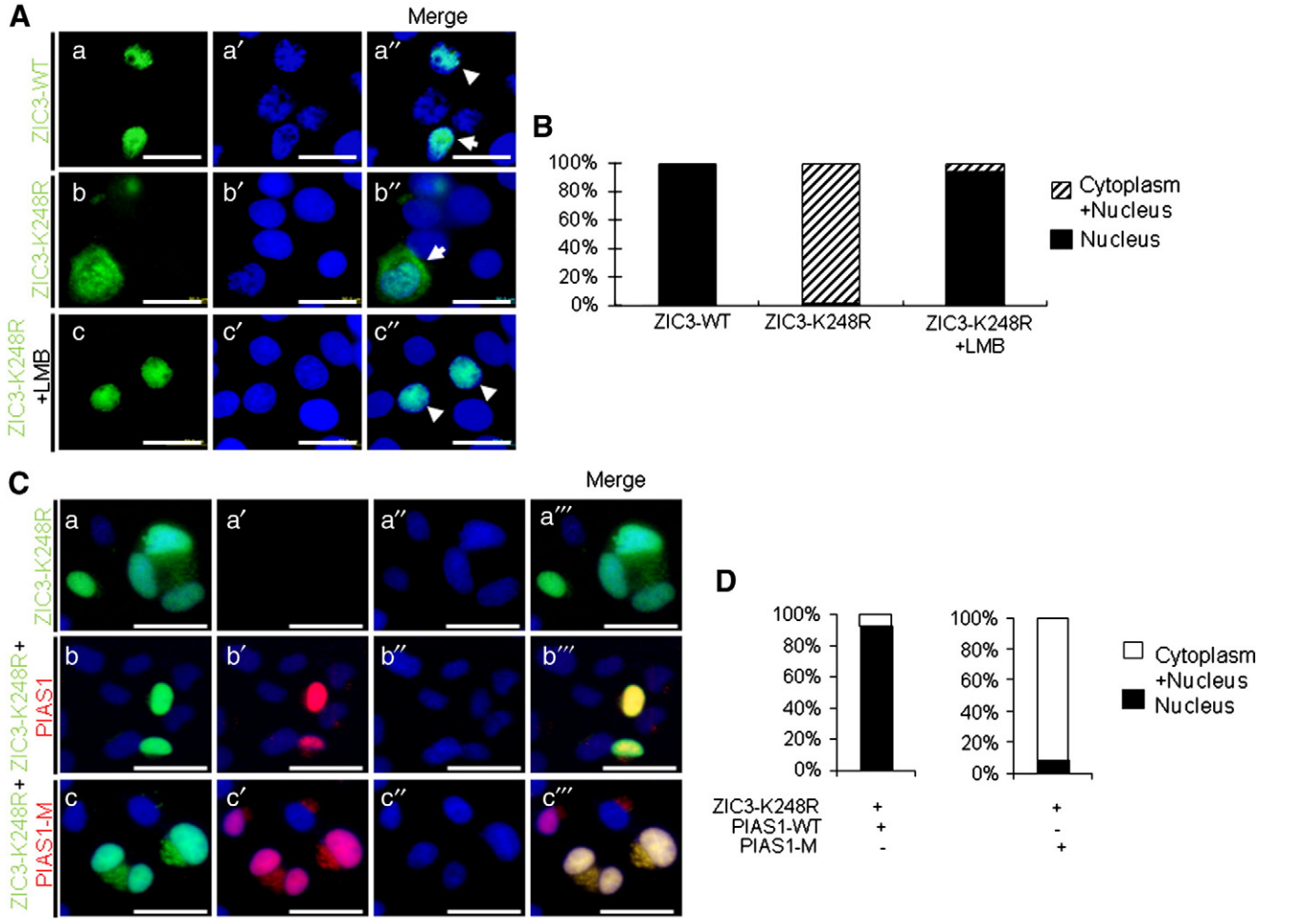

Fig. 2. SUMO site K248 is required for nuclear localization of ZIC3. (A–B) SUMO site mutation leads to diffused subcellular localization of ZIC3. (A) Immunocytofluorescence staining was performed on Hela cells transfected with HA-tagged ZIC3 WT (a–a″), or K248R mutant (b–c″) using antibody against HA. While HA-ZIC3 WT is exclusively located in the nucleus (a–a″), while the HA-K248R mutant exhibits diffused subcellular localization (in both nucleus and cytoplasm) (b–b″). The presence of nuclear export inhibitor Leptomycin B (LMB) reestablishes the nuclear occupancy of K248R (c–c″). Green: ZIC3 WT in a–a″, ZIC3 K248R in b–c″; blue: DAPI. Amplification, 40×. The score of the number of cells showing exclusively nuclear staining vs. that showing diffused staining in each group is shown in B. The scores were obtained from randomly selected 100 cells/group, and were summarized from two independent assays. Black column, exclusively nuclear; shaded column, diffused. (C–D) PIAS1 WT/SUMO-1 restores the nuclear localization of K248R mutant. Double immunocytofluorescence staining was performed on SUMO-1-stably expressed Hela cells (su-Hela) transfected with V5-tagged ZIC3 alone (a–a‴), or in the presence of HA-epitoped PIAS1 WT (b–b‴) or PIAS1 RING-mut (PIASA1-M, c–c‴). Note that PIAS1 restores the nuclear localization of K248R (b–b‴), while PIAS1 Ring domain mutant (PIAS1-M) does not (c–c‴). Green: V5-ZIC3-K248R; red: HA-PIAS1 WT-HA in b–b‴, HA-PIAS1 Ring domain mutant in c–c‴, blue: DAPI. The scores were obtained from randomly selected 100 cells/group, and were summarized from two independent assays. Black column, exclusively nuclear; white column, diffused. Amplification, 40×. Scale bar: 100 μm.

(0.422), $FK_{359}CE$, does not show any significant effect on ZIC3 sumoylation (data not shown). While PIAS1 WT stimulates sumoylation of ZIC3, its RING-mut does not (Fig. 1C). Collectively, these findings indicate that lysine 248 in ZIC3 is the primary SUMO site, and that PIAS1 substantiates SUMO attachment to ZIC3 via its RING domain.

Next, to assess the functional consequence of SUMO conjugation to ZIC3, luciferase activity assay was performed on Hela cells transfected with Ca-actin-Luc in the presence of ZIC3 WT, SUMO-1-fused ZIC3 WT (SUMO-1-ZIC3), or the mutant *K248R*, respectively. Consistent with the previous report [33], ZIC3 WT suppresses the activity of Ca-actin-Luc. While SUMO-1-ZIC3 shows more repressive effects on this promoter compared with ZIC3 WT, the mutation of lysine 248 to arginine abolishes it (Fig. 1D). Taken together, these data suggest that the SUMO conjugation potentiates the repressive function of ZIC3.

3.2. Specificity of the SUMO conjugation machinery in ZIC3 sumoylation

SUMO family consists of three conjugatable members, SUMO-1, SUMO-2 and SUMO-3, which possess substrate specificity. To explore if differential modification of ZIC3 by SUMO-1, -2 and -3 exists, Ni-NTA pulldown assays were performed on Hela cell lysates containing V5-6xHis-tagged ZIC3 WT in the presence of encoding vectors for SUMO-1, SUMO-2 or SUMO-3, respectively. As shown in SF2.A, ZIC3 is modified by all these three SUMO proteins at equivalent levels. There are mainly five members in the SUMO E3 ligase PIAS family (PIAS1, PIAS3, PIASy, PIASxα and xβ). To investigate if PIAS proteins differentially regulate SUMO-1 modification of ZIC3, the combination of V5-6xHis-tagged ZIC3 WT and SUMO-1 was co-transfected into Hela cells together with one of those PIAS proteins, followed by above-mentioned Ni-NTA pulldown assays. In the absence of PIAS protein, the combination of ZIC3/SUMO-1 displays one clear slow migrating band as expected (SF2.B, lane 2), and all PIAS proteins are able to promote the appearance of at least one additional retarded migratory band, with PIAS1 as the strongest enhancer of SUMO-1 conjugation of ZIC3 (SF2.B, compare lane 3 with lane 4–7). However, only PIAS1, 3 and y, but not PIASxα and xβ, enhance the sumoylation of ZIC3 by SUMO-3 by introducing at least one additional retarded migratory band (SF2.C). Therefore, while SUMO isoforms exhibits equivalent capacity to modify ZIC3, PIAS E3 ligases display differential discrimination for SUMO proteins to be covalently attached to ZIC3.

3.3. SUMO conjugation regulates nucleocytoplasmic trafficking of ZIC3

Nuclear localization of ZIC3 plays an important role in regulating its function [12,13]. Since sumoylation may regulate the activity of its substrates via altering its subcellular distribution, we asked if K248 contributed to maintain ZIC3's nuclear localization. Immunostaining against HA tag was performed on Hela cells transfected with HA-epitoped ZIC3 WT or K248R mutant, respectively. Consistent with previous reports, ZIC3 WT is predominantly localized within the nucleus (nearly 100%) (Fig. 2A, a–a″, and B), however, ~90% of K248R exhibits both nuclear and cytoplasmic localizations (Fig. 2A, b–b″, and B), indicating that K248 is critically involved in ZIC3's nucleocytoplasmic shuttling. Next, to probe if nuclear export is involved in K248R cytoplasmic diffusion, leptomycin B (LMB), a repressor of nuclear export, was applied to the Hela cells expressing HA-tagged K248R. The presence of LMB results in the nuclear retention of K248R up to ~90% (Fig. 2A, c–c″, and B). Collectively, these findings prove that the lysine 248 is implicated in mediating the subcellular localization of ZIC3.

Since lysine 248 is the primary SUMO acceptor site, we next asked if SUMO conjugation is directly involved in regulating the nuclear occupancy of ZIC3. Since PIAS1 WT but not PIAS1 RING-mut promotes the sumoylation of K248R in the presence of overexpressed SUMO-1 (Fig. 1C), we used this system to test whether the diffused subcellular localization of K248R is affected by PIAS1 WT/SUMO-1. V5-6xHis-tagged *K248R* mutant was transfected into su-Hela cells stably expressing SUMO-1 alone, or together with HA-tagged PIAS1 WT or RING-mut, respectively. As expected, *K248R* mutant shows extranuclear distribution (Fig. 2C, a–a″). The addition of PIAS1 WT results in ~90% nuclear accumulation of K248R (Fig. 2C, b–b″ and D), whereas PIAS1 RING-mut does not significantly alter the diffused subcellular localization of K248R (~10%) (Fig. 2C, c–c″ and D). In accordance with a previous report [38], both PIAS1 WT and RING-mut exhibit no significant difference in their subcellular localization patterns (data not shown), and RING-mut is physically associated with ZIC3 as well as PIAS1 WT (data not shown), demonstrating that PIAS1 does not sequester ZIC3 within the nucleus via physical association. We therefore conclude that sumoylation contributes to retain the nuclear localization of ZIC3 K248R mutant.

3.4. Defective SUMO conjugation involved in aberrant subcellular distribution of human ZIC3 mutants

A number of disease-associated human ZIC3 mutants exhibited impaired nuclear localization [15]. To interrogate if those naturally occurring mutations affect ZIC3 sumoylation, six ZIC3 variants with various localizations of mutated sites (Fig. 1A), three in ZF1 (C253S, W255G, H286R), one in ZF2 (T323M), one in ZF5 (K408E), and one in the N-terminal domain (P217A), were tested for SUMO modification. Ni-NTA pulldown was performed on the Hela cells transfected with V5-6xHis-tagged ZIC3 WT or one of those mutants in the absence or presence of SUMO-1. While free ZIC3 WT and mutants are precipitated at comparable levels, the mutants C253S, W255G, H286R, and T323M display substantially decreased sumoylation (Fig. 3, asterisks). The other two mutants, P217A and K405E, show no significant alteration of SUMO modification (Fig. 3, asterisks). The above observations coincide respectively with the diffused subcellular localization of C253S, W255G, H286R, and T323M, and with relatively normal subcellular distribution of P217A and K408E [15]. We then hypothesized that an improvement of sumoylation of these mutants would improve their nuclear occupancy. Three mutants C253S, W255G and H286R are selected to test this hypothesis. First, we investigated if PIAS1 WT was able to potentiate the sumoylation of these ZIC3 mutants. Ni-NTA pulldown assays were performed on 6xHis-SUMO-1 stably expressed Hela cells containing one of the V5-6xHis-tagged three mutants alone, or together with PIAS1 WT or RING-mut, respectively. As expected, PIAS WT, but not RING-mut, substantially improves sumoylation of these three mutants (Fig. 4A). Next, each of these V5-tagged mutants was transfected with either HA-tagged PIAS1 WT or RING-mut into the su-Hela cells stably expressing SUMO-1, followed by double immunostaining against V5 and HA. All these three mutants show significantly improved nuclear accumulation in the presence of HA-PIAS1 WT, but not with HA-PIAS1-RING-mut (Fig. 4B, b–g″, see double HA+/V5+ cells, and 6D–F). As a control, P217A, which exhibits no significantly altered subcellular distribution and sumoylation, mainly stays in the nucleus in the presence of PIAS1-M (Fig. 4B, a–a″ and C), in agreement with the previous report [15]. Taken together, these findings demonstrate that sumoylation is negatively affected in a number of naturally occurring disease-associated ZIC3 variants that also exhibit aberrant subcellular distribution, and that improved sumoylation of these mutants restores their nuclear accumulation.

3.5. LMB promotes nuclear accumulation of human ZIC3 variants with impaired SUMO conjugation

Since SUMO conjugation is involved in nucleocytoplasmic trafficking of ZIC3, and LMB promotes nuclear accumulation of sumoylation-deficient ZIC3 mutant K248R, we asked if LMB could also improve nuclear localization of those human mutants that exhibit abnormal subcellular trafficking. Immunostaining was performed on Hela cells transfected with one of the expression vectors encoding V5-tagged P217A (SUMOylation-normal mutant as a control), C253S or W255G alone or together with LMB. In line with the previous reports, P217A primarily stays in the nucleus, and the sumoylation-deficient mutants C253S and W255G exhibit diffused distribution (Fig. 5A, a–c). While LMB has little effects on the subcellular localization of P217A (Fig. 5A, a′, B), it substantially improves nuclear localization of C253S and W255G (Fig. 5A, b′–c′, and C-D, from ~10% to ~95% for C253S and from ~18% to ~95% for W255G). LMB also efficiently diminishes the extranuclear diffusion of H286R (data not shown). Thus, these observations argue that the similar molecular phenotype(s) underpin the abnormal subcellular localization of sumoylation-deficient naturally occurring ZIC3 variants and the SUMO site mutant K248R.

4. Discussion

In the present study, we report for the first time that ZIC3 is a SUMO substrate. We first reveal that lysine residue 248, which is localized immediately upstream of ZF1 of ZIC3, is the principal SUMO acceptor

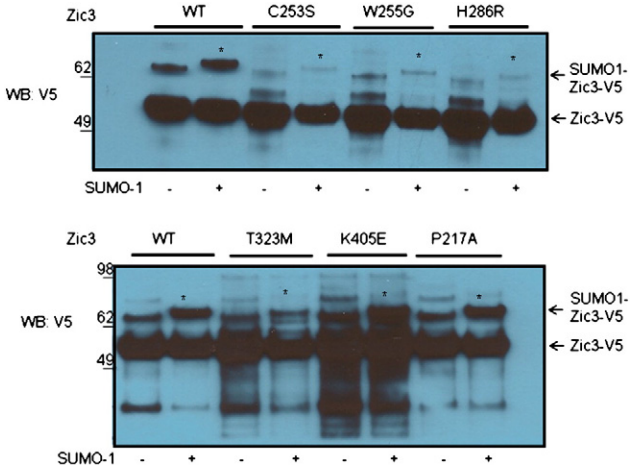

Fig. 3. Human ZIC3 variants displayed deficient SUMO conjugation. Ni-NTA pulldown assays were performed on Hela cell lysates containing V5-6xHis-tagged ZIC3 WT or one of its mutants in the absence or presence of ectopically expressed SUMO-1 as indicated. Upper panel, three mutants (C253S, W255G, H286R) show decreased SUMO conjugation compared with ZIC3 WT; lower panel, one mutant (T323M) shows decreased SUMO conjugation compared with ZIC3 WT. Asterisks indicate SUMO-conjugated ZIC3. Blot, anti-V5.

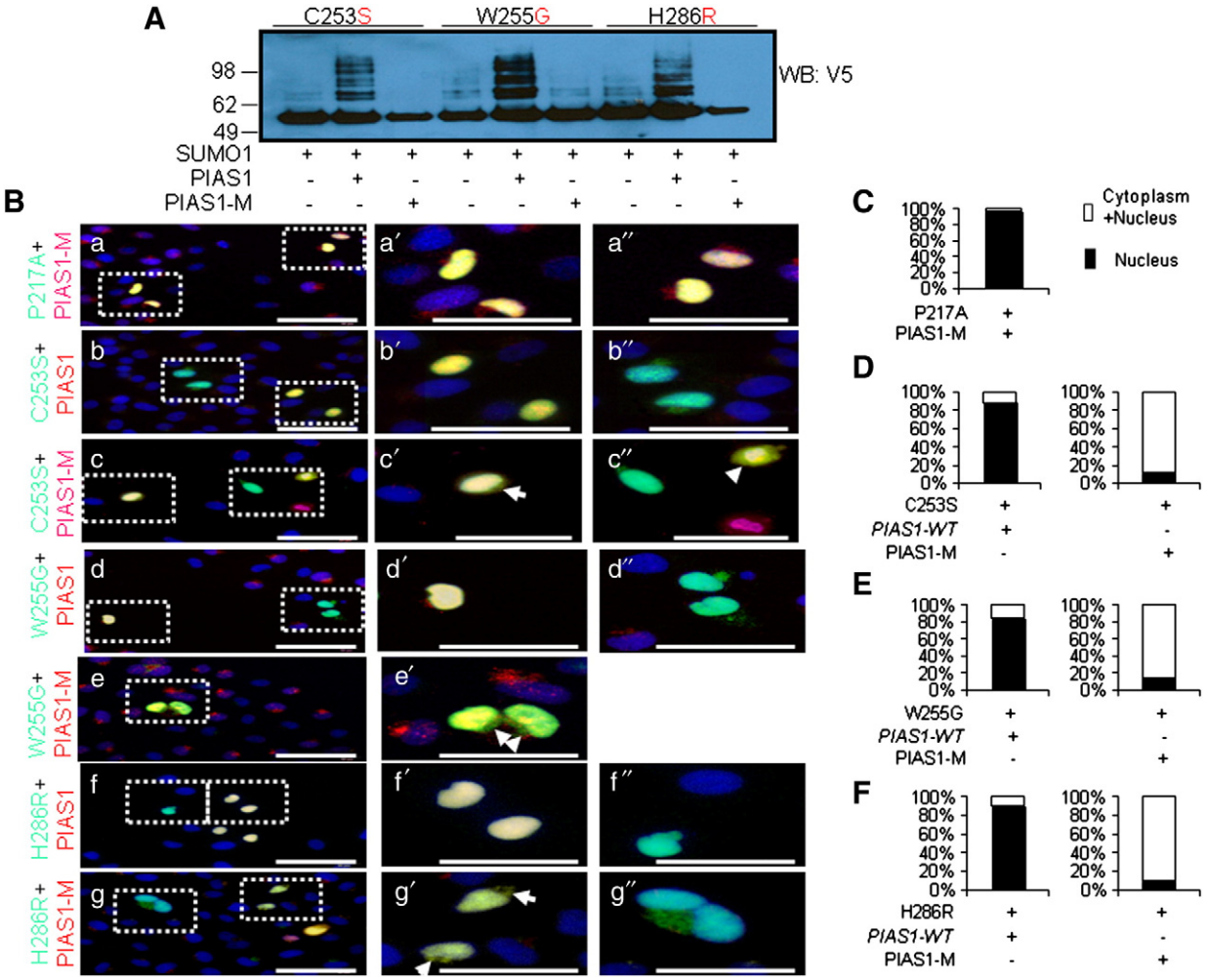

Fig. 4. Improved sumoylation of human ZIC3 variants restores their nuclear occupancy. (A) SUMO E3-ligase PIAS1 WT, but not the RING-mut, enhances the sumoylation of human mutants C253S, W255G and H286R. Ni-NTA pulldown assays were performed in su-Hela cell lysates containing V5-6xHis-tagged ZIC3 mutant alone, or in the presence of either HA-epitoped PIAS1 WT or RING-mut (RING-M). Blot, anti-V5. (B) The presence of PIAS1 WT, but not PIAS1-RING-mut, restores the nuclear occupancy of sumoylation-deficient ZIC3 variants in su-Hela cells. Double immunocytofluorescence staining using antibodies against V5 (green) and HA (red) was performed on su-Hela cells transfected with one of V5-tagged ZIC3 variants together with either HA-epitoped PIAS1 WT or RING-mut (RING-M). Note that the sumoylation-deficient human ZIC3 mutants C253S, W255G and H286R display substantially improves nuclear accumulation in the presence of PIAS1 WT compared with that in the presence of PIAS1-M. C253S, compare b–b″ with c–c″; W255G, compare d–d″ with e–e′; H286R, compare f–f″ with g–g″. The sumoylation-normal human ZIC3 mutant P217A, which exhibits primarily nuclear localization, serves as a control in the presence of HA-epitoped PIAS1-M (a–a″). Arrows indicate diffused staining. Green: V5-tagged human ZIC3 mutants-V5; red: HA-tagged PIAS1 WT or Ring-M. Blue: DAPI staining. The middle and right panels of B (40× magnification) are the amplification of the boxed areas in the right panels of B (20× magnification). Scale bar: 100 μm. (C–F) The scores were obtained from randomly selected 100 cells/group, and were summarized from two independent assays. Black column, exclusively nuclear; white column, diffused localization.

site, although the mutation of this site activates a latent non-classical SUMO domain in ZIC3. We further examine the effects of SUMO conjugation on ZIC3 activity using two variants, SUMO-1 fused ZIC3 (SUMO-1-ZIC3) and a SUMO site mutant of ZIC3 (K248R), in the well-established model system in which ZIC3 represses the activity of Ca-actin-Luc reporter [33]. SUMO-1-ZIC3 displays a more inhibitory effect than ZIC3 WT, while the mutation of lysine 248 to arginine totally abolishes its repressive effect. Although SUMO-1-ligated ZIC3 is not identical to the natively SUMO-conjugated form, this type of variant has often been employed to test the activity of SUMO-conjugated targets in vivo in the SUMO field [39–42]. The precise molecular basis underlying the inhibitory effects of ZIC3 on cardiac α-actin promoter remains unclear, as are the mechanisms by which Ca-actin-Luc exhibits higher activity in the presence of K248R than WT ZIC3. It is possible that ZIC3 recruits some corepressor(s) to execute this repressive function. Alternatively, ZIC3 may inhibit this cardiac promoter by competing with some (as yet unknown) activator(s) for binding to this promoter. Thus, the removal of the suppressive impact of ZIC3 on Ca-actin-Luc by the mutation of K248R could be at least partially attributed to its cytoplasmic relocalization (see below). The effect of this relocalization may be to either "piggyback" the corepressor(s) to cytoplasm and/or to increase the binding of activator(s) to this promoter, leading to the activation of this cardiac promoter. Whether these hypotheses hold true requires further investigation. Given that SUMO-1-Zic3 represses Ca-actin-Luc more than ZIC3 WT, we conclude that SUMO attachment is associated with ZIC3's repressive activity.

SUMO conjugation was involved in regulating the subnuclear or subcellular trafficking of a number of target proteins [41,43,44]. Previous studies defined NLS and NES between ZF2 and ZF5 in ZIC3 [12,13], and these signals were suggested to cooperatively modulate ZIC3's nucleocytoplasmic trafficking. The major SUMO site lysine 248, despite being distant from the NLS and NES, is critically involved in regulating the nucleocytoplasmic shuttling of ZIC3, and the extranuclear relocation caused by this site mutation is blocked by LMB. For any given mutated nuclear protein, the abnormal cytoplasmic distribution may be explained basically by the following: 1) the protein is less efficiently imported into the nucleus but still exported at the same rate, or 2) the nuclear import rate is unchanged but export rate is enhanced. In either of these two

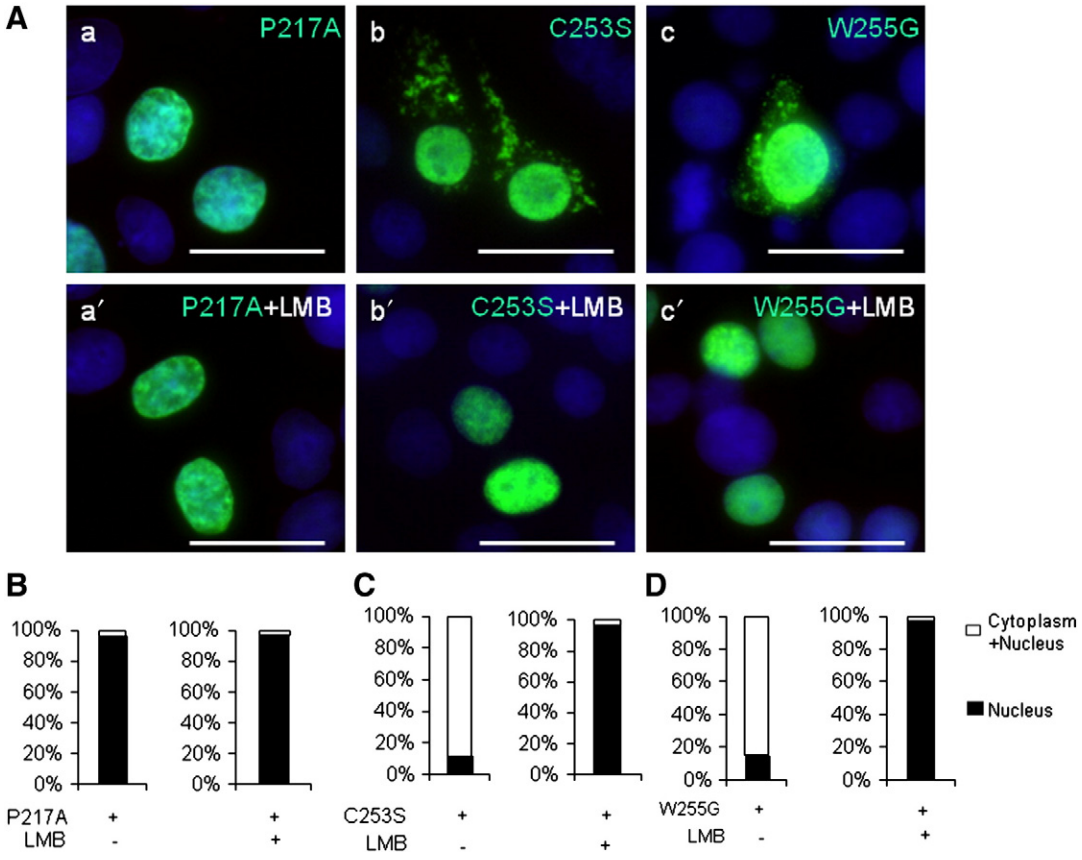

Fig. 5. LMB improves nuclear localization of sumoylation-deficient human ZIC3 Mutants. (A) Immunocytofluorescence staining using antibody against V5 was performed on Hela cells transfected with V5-tagged ZIC3 mutants in the absence or presence of LMB as indicated. The sumoylation-normal human ZIC3 mutant P217A displays primarily nuclear localization (a), while C253S and W255G mutants are diffused in both nucleus and cytoplasm (b & c). LMB has no effects on the nuclear localization of P217A (a′), but greatly restores the nuclear accumulation of C253S and W255G (b′ & c′). Green: V5-tagged ZIC3 mutant; blue: DAPI. (B–D) The scores were obtained from randomly selected 100 cells/group, and were summarized from two independent assays. Black column, exclusively nuclear; white column, diffused localization. Amplification, 40×. Scale bar: 100 μm.

cases, the application of LMB, an export inhibitor, in this test system would lead to the nuclear accumulation of the mutant protein. To this end, we currently do not have evidence for either scenario for the K248R mutant. However, given that sumoylation was shown to be associated with nuclear import [45], it is likely that the mutation of K248 impacts the function of NLS. Another interesting but unaddressed issue is whether SUMO modification leads to ZIC3's nuclear localization or whether nuclear localization of ZIC3 leads to its sumoylation.

Unexpectedly, K248R mutation introduces a secondary weak sumoylation site, which is probably covered in the presence of the intact SUMO consensus motif. We noticed that under overexpression conditions, only ~20% of ZIC3 WT is SUMO modified, and the majority of the unmodified free ZIC3 WT still stays exclusively in the nucleus. Also, we did not detect sumoylation of exogenous ZIC3 by the endogenous sumoylation machinery in the regular HeLa cells (data not shown). Thus, we cannot conclude that SUMO conjugation is required for the exclusive nuclear localization of ZIC3 WT unless the majority of WT ZIC3 is sumoylated, but is only partially detectable due to the transient and dynamic nature of this modification in vivo. However, given the findings that PIAS1 WT—but not its RING-mut—substantially restores the nuclear occupancy of K248R mutant in the presence of SUMO-1, we argue that sumoylation is involved in mediating the nuclear retention of ZIC3. The fact that significant augmentation of the nuclear accumulation of K248R after SUMO conjugation is enhanced also suggests that even SUMO attachment to non-canonical site(s) can contribute to ZIC3's nucleocytoplasmic shuttling. Thus, SUMO conjugation plays a critical role in determining the subcellular localization of ZIC3. However, we were unable to identify the non-canonical SUMO site that is activated by K248R mutation, which otherwise could be used to further test whether the existence of PIAS1/SUMO-1 would be able to sequester a non-sumoylatable ZIC3 mutant within the nucleus.

A multitude of genes encoding SUMO targets are disease related. Presumably, the mutations that occurred within the SUMO targeting sequence negatively affected their sumoylation, thereby contributing to disease development, as evidenced by the mutations in lamin A and in the microphthalmia-associated transcription factor (MITF) [46,47]. However, the mutation of sites that are not in the SUMO attachment sequence could also indirectly affect sumoylation and was potentially involved in pathogenesis, such as those mutations of *Tbx22* and *Nkx2.5* [48,49]. In the present study, we provide another example of these indirect effects. In the six human ZIC3 mutants examined, all three ZF1 mutants (*C253S*, *W255G*, *H286R*) and one ZF2 mutant (*T323M)* suppress sumoylation, while two other mutations (*K405E*, a mutation located in the ZF5, and *P217A*, a mutation of the N-terminal domain of ZIC3) do not. Interestingly, while sumoylation-normal mutants *P217A* and *K405E* do not display significant changes in the subcellular distribution pattern compared with WT ZIC3, all sumoylation-deficient mutants tested (*C253S*, *W255G*, *H286R* and *T323M*) exhibit impaired nuclear occupancy. More importantly, the abnormal cytoplasmic diffusion of these mutants is reversed by restored sumoylation that is achieved by co-expression of WT PIAS1/SUMO-1. It is noteworthy that the mutations on ZF1 such as *C253S*, *W255G* and *H286R* are not part of any NLS defined previously [12,13].

Sumoylation-deficient mutant *K248R* shows altered nuclear export, which in turn, is blocked by LMB. To assess whether there is any similar

molecular phenotype underlying K248R and these sumoylation-defective human ZIC3 variants, we selected three mutants *C253S*, *W255G* and *H286R* for further examination. Indeed, LMB impedes the cytoplasmic relocalization of all three of these mutants. Thus, these observations together argue that sumoylation might serve as a unifying mechanism that dictates the nuclear occupancy of ZIC3. Given the diverse phenotypic manifestations associated with alternative ZIC3 mutants, it is likely that the degree of altered sumoylation activity of these ZIC3 mutants contributes to the variable expressivity of phenotypes caused by these mutants.

In *Xenopus* embryo, the *C253S*, *W255G* and *H286R* mutants were reported to be primarily located in the nucleus [14]. Given the observation that increased SUMO conjugation promotes the nuclear retention of sumoylation-deficient ZIC3 mutants, it would be interesting to see if high SUMO conjugation activity exists in *Xenopus* embryo compared with that of cell lines such as HeLa and NIH 3T3. We also noted that LMB showed no significant effects on the cytoplasmic localization of some mutants (i.e., *W255G* and *H286R)* [12,13]. It is currently not clear what accounts for the difference between our observation and theirs. However, we realize that under our experimental conditions, the cytoplasmic diffusion of those mutants is not as "extreme" as observed in those reports. Numerous experimental conditions could contribute to the differences in experimental outcomes mentioned above.

5. Conclusion

We have demonstrated that zinc finger transcription factor ZIC3 is modified by SUMO conjugation on the primary SUMO site lysine 248 and that lysine 248 is involved in regulating ZIC3's subcellular localization. Mutations in human ZIC3 in ZF1 and 2 cause a decrease in SUMO conjugation and abnormal subcellular distribution; and enhanced sumoylation of these mutants in the presence of SUMO E3 ligase and SUMO-1 reestablishes their nuclear accumulation. These findings collectively support the notion that deficient sumoylation of human ZIC3 variants underpins the aberrant extranuclear localization and provides a potential strategy to salvage their normal subcellular distribution by restoring SUMO conjugation. Whether other ZIC family proteins are SUMO targets need further exploration. However, given the evolutionally conserved SUMO consensus motif among paralogs and orthologs of ZIC3, the sumoylation pathway must represent a common pathway that governs the function of ZIC proteins.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2013.07.009.

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