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SUMOylation of DLX3 by SUMO1 Promotes its Transcriptional Activity

Olivier Duverger,[1†] Susie X. Chen,[1†] Delia Lee,[1] Tianwei Li,[2] P. Boon Chock,[2] and Maria I. Morasso[1*]

[1]*Developmental Skin Biology Section, NIAMS/NIH, Bethesda, Maryland*
[2]*Laboratory of Biochemistry, NHLBI/NIH, Bethesda, Maryland*

ABSTRACT

Small ubiquitin-like modifiers (SUMO) are post-translational modifiers that regulate target protein activity in diverse ways. The most common group of SUMO substrates is transcription factors, whose transcriptional activity can be altered positively or negatively as a result of SUMOylation. DLX3 is a homeodomain transcription factor involved in placental development, in the differentiation of structures involving epithelial-mesenchymal interactions, such as hair, teeth and nails, and in bone mineralization. We identified two potential SUMOylation sites in the N-terminal domain of DLX3 at positions K83 and K112. Among the six members of the Distal-less family, DLX3 is the only member containing these sites, which are highly conserved among vertebrates. Co-expression experiments demonstrated that DLX3 can be SUMOylated by SUMO1. Site-directed mutagenesis of lysines 83 and 112 to arginines (K83R and K112R) demonstrated that only K112 is involved in SUMOylation. Immunocytochemical analysis determined that SUMOylation does not affect DLX3 translocation to the nucleus and favors perinuclear localization. Moreover, using electrophoresis mobility shift assay (EMSA), we found that DLX3 is still able to bind DNA when SUMOylated. Using luciferase reporter assays, we showed that $DLX3^{K112R}$ exhibits a significantly lower transcriptional activity compared to $DLX3^{WT}$, suggesting that SUMOylation has a positive effect on DLX3 activity. We identified a new level of regulation in the activity of DLX3 that may play a crucial role in the regulation of hair, teeth, and bone development. J. Cell. Biochem. 112: 445–452, 2011.

KEY WORDS: DLX3; SUMOYLATION; SUMO1; NUCLEAR LOCALIZATION; DNA BINDING; TRANSCRIPTIONAL ACTIVITY

INTRODUCTION

Post-translational modifications occur to a majority of proteins to regulate their activity as a result of a particular stimulus. The small ubiquitin-like modifier, SUMO, is a post-translational modifier with distinct effects on a wide variety of targets. It can increase protein stability [Desterro et al., 1998], influence interactions between distinct proteins [Seeler and Dejean, 2001], change subcellular localization [Wilson and Rangasamy, 2001; Morita et al., 2005], and affect nuclear trafficking [Pichler and Melchior, 2002]. In mammalian cells, four SUMO isoforms (SUMO1–4) have been identified. The human SUMO1 gene encodes a 101-amino acid polypeptide related to ubiquitin. SUMO1 is known to share ~50% sequence identity with SUMO2/3, whereas SUMO2 and SUMO3 share 87% sequence identity. While SUMO4 expression appears to be tissue-specific, SUMO1–3 are widely expressed and show distinct substrate and de-sumoylating protease specificity. Albeit such differences, all SUMOs undergo a series of enzymatic reactions at the C terminus to become covalently bound to their targets.

Thus far, the most common group of SUMO substrates is transcription factors, whose transcriptional activity is altered as a result of SUMOylation. Previous studies have revealed both positive and negative regulation of transcription factor activity. Proteins whose transcriptional response is modulated as a result of SUMOylation include p53 [Gostissa et al., 1999], Dorsal [Bhaskar et al., 2002], HSF2 [Goodson et al., 2001; Tateishi et al., 2009], c-Jun [Muller et al., 2000], Androgen receptor [Poukka et al., 2000], Sox2 [Tsuruzoe et al., 2006] and several members of the DExD/H box RNA helicases family, i.e., Dhx5 and Dhx20 [Fuller-Pace et al., 2007; Jacobs et al., 2007].

Here we investigate the role of SUMOylation on DLX3, which belongs to the superfamily of homeodomain transcription factors known to be widely involved in the patterning of the developing embryo. In mouse and human, there are six *DLX* genes organized

[†]These authors contributed equally to this work
*Correspondence to: Maria I. Morasso, 50 South Drive, Building 50, Room 1525, Bethesda, MD 20892, USA
E-mail: morassom@mail.nih.gov
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into three pairs of inverted, convergently transcribed genes (*DLX1–2*, *DLX3–4*, and *DLX5–6*) [Morasso and Radoja, 2005]. *DLX3* is linked to *DLX4* on chromosome 11 in mouse and on chromosome 17 in humans. Furthermore, DLX3 is expressed in the placenta early during embryonic development [Morasso et al., 1999], while it is later found in skin as well as in structures involving epithelial–mesenchymal interactions, such as teeth and hair follicles [Robinson and Mahon, 1994]. Using a conditional knockout approach, we showed that Dlx3 plays a crucial role in hair development [Hwang et al., 2008]. There is also clinical evidence that DLX3 plays a significant role in the patterning of hair, teeth, and bone. In fact, a 4-G deletion occurring three base pairs downstream of the DLX3 homeodomain leads to a frameshifted C-terminal domain whose sequence differs completely from that of wildtype DLX3. This truncated protein is manifested in an ectodermal dysplasia called Tricho-Dento-Osseous syndrome, an autosomal dominant disorder characterized by defects in hair (kinky), teeth (enamal hypoplasia and taurodontism), and bone (increased thickness and density of craniofacial bone) [Price et al., 1998].

Here, we identify and characterize for the first time a SUMOylation site in DLX3. We show that SUMOylation does not dramatically affect DLX3 subcellular localization and DNA binding activity but promotes DLX3 transcriptional activity.

MATERIALS AND METHODS

PLASMIDS

The bidirectional vector pBi4 was used to simultaneously express the reporter protein EGFP with V5DLX3 (pBi-V5DLX3/GFP), under control of a unique tetracycline responsive element (TRE). Site-directed mutagenesis was then utilized to mutate the two lysines (83 and 112) that are potentially involved in DLX3 SUMOylation into arginines. These mutants were obtained by introducing A to G point mutations in DLX3 cDNA (A248G and A335G, respectively). At position 248, A was mutated into G using the following primers: Sense-GCTTACTCGCCCA**G**GTCGGAATATACC; Antisense-GGTA-TATTCCGACCTGGGCGAGTAAGC (mutated base in bold). The resulting construct was named pBi-V5DLX3^{K83R}. At position 112, A was mutated into G using the following primers: Sense-CCAGTG-TCGGTGA**G**AGAGGAGCCGGAA; Antisense-TTCCGGCTCCTCTCT-CACCGACACTGG. The resulting construct was named pBi-V5DLX3^{K112R}. The double mutant was also generated and the construct was named pBi-V5DLX3^{2K}.

SUMO1 and SUMO1-ΔGG, a mutated form of SUMO1 lacking the C-terminal double glycine which forms an isopeptide bond with the target protein, were tagged at the N-terminus with a His tag and a c-Myc tag, and cloned into pTRE2 for tetracycline inducible expression [Li et al., 2006].

CELL CULTURE AND TRANSFECTIONS

Saos2 human osteosarcoma cells expressing the tetracycline inducible transactivator rTA (Saos2-TetOFF, Clontech) were grown in DMEM (10% fetal bovine serum, 1% penicillin/streptomycin, and 1 μg/ml G418). For transfections, the cells were grown to at least 70% confluence. 2×10^6 cells were used per transfection with each construct (Amaxa Nucleofactor).

NI COLUMN PULL-DOWN

Forty-eight hours after transfection with DNA, Saos2-TetOFF cells were lysed in 1 ml Buffer A (100 mM NaH_2PO_4, 10 mM Tris-Cl, 6 M GuHCl, pH = 8.0) supplemented with 10 mM N-ethyl maleimide and 7% β-mercaptoethanol. The lysates were then sonicated, spun down for 15 min, and incubated with 40 μl of Ni-NTA agarose beads (Qiagen) for 1 h. The beads were then washed with Buffer A once, Buffer B (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M urea, pH = 8.0) twice, and Buffer C (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M urea, pH = 6.3) twice. TRIS 50 mM was used to wash beads, which were then resuspended in 2X NuPAGE LDS-sample buffer and 250 mM imidazole.

CELL LYSIS AND WESTERN BLOT ANALYSIS

Forty-eight hours after transfection, GFP was visualized and the cultured cells were rinsed with phosphate-buffered saline, scraped with lysis buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 200 mM NaCl, 0.1% Nonidet P-40,) supplemented with a protease inhibitor cocktail (Complete Mini, EDTA-free, Roche), 15 mM N-ethyl maleimide, 20 mM iodoacetamide, and sonicated. 4X NuPAGE® LDS Sample Buffer (Invitrogen) was added to these lysates after normalization of protein concentrations. These protein extracts were run on 4–12% Bis–Tris gels and MOPS SDS running buffer. Proteins were transferred onto PVDF membranes and blocked in 5% non-fat powdered milk in TBS/Tween at room temperature for 1 h. The blots were probed with primary antibody diluted in 5% non-fat powdered milk in TBS/Tween and then with secondary antibody diluted in TBS/Tween, both at room temperature for 1 h. Primary antibodies used were anti-V5 (1:2000, Serotec), anti-cMyc (1:1000, Santa Cruz). Secondary antibody used was goat anti-mouse horseradish peroxidase (1:3000, Bio-Rad). After application of each antibody, the blots were rinsed three times with TBS/Tween under similar conditions. The blots were developed by ECL (Pierce).

IMMUNOCYTOCHEMISTRY

Transfected cells were seeded on glass coverslips coated with 0.1% gelatin. Forty-eight hours after transfection, cells were washed three times in PBS and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. A 5 min incubation in 0.2% Triton in PBS was used to permeabilize the cells before blocking unspecific sites using 3% BSA in PBS for 1 h. Primary antibodies diluted in blocking solution were applied for 1 h. Primary antibodies used: anti-V5 (1:100, Serotec), anti-cMyc (1:100, Santa Cruz). Secondary antibodies diluted in blocking solution were applied for 30 min. Secondary antibodies used: Alexa Fluor® 543 goat anti-mouse IgG and Alexa Fluor® 488-conjugated anti-rabbit IgG (1:400, Invitrogen). Nuclei were stained using DAPI and coverslips were mounted on glass slides using Mowiol (Calbiochem). Images were acquired using a Zeiss 510 META confocal microscope.

ELECTROPHORESIS MOBILITY SHIFT ASSAYS

Nuclear extracts were prepared using a Nuclear Extract Kit (Active Motif). Recombinant SUMO-DLX3 fusion protein was produced using the SUMOpro kit (LifeSensors). A probe containing the DLX3 consensus binding site (GGGGGATAATTGCTGG) was radiolabeled using the High Prime DNA Labeling Kit (Roche) and [γ-^{32}P] dCTP.

Nuclear extracts or recombinant proteins were pre-incubated in 1X gel shift binding buffer (Promega) for 15 min at 4°C, with an excess of unlabeled probe for competition assays or with appropriate antibody (anti-DLX3 or anti-cMyc) for supershift assays. After this pre-incubation, each sample was supplemented with 5×10^4 DPM of radiolabeled probe and incubated for 30 min at 4°C. The binding reactions were resolved on 6% DNA retardation gels (Invitrogen). The gels were dried and DNA–protein complexes were visualized by autoradiography.

LUCIFERASE REPORTER ASSAY

To determine the transcriptional activity, a synthetic oligonucleotide containing three tandem copies of the DLX3 responsive element (DRE; GCGATAATTGCGGCGATAATTGCGGCGATAATTGCG) followed by the HSV thymidine kinase proximal promoter region was cloned into the pGL3-promoter vector (pGL3–3XDRE) driving a Firefly luciferase reporter cassette [Duverger et al., 2008]. Saos2-TetOff cells were transiently transfected with pBi-V5DLX3^{WT} or pBi-V5DLX3^{K112R} constructs, together with pTRE2-SUMO1, pGL3–3xDRE, and the pRL-TK vectors (*Renilla* luciferase used for normalization). Twenty-four hours after transfection, relative luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (Promega). Statistical analysis was performed using Prism 5.02.

RESULTS

DLX3 IS THE ONLY MEMBER OF THE DLX FAMILY CONTAINING PUTATIVE SUMOYLATION SITES, ONE OF WHICH IS CONSERVED AMONG SEVERAL VERTEBRATES

The DLX3 amino acid sequence was analyzed and two potential SUMOylation sites ψKXE, where ψ represents a hydrophobic amino acid, X represents any amino acid, were discovered at lysine 83 (pkse) and lysine 112 (vkee) (Fig. 1A). Because proline is less hydrophobic than valine, we hypothesized that lysine 83 is less susceptible to SUMOylation than lysine 112. The DLX3 amino acid sequence in several vertebrates was aligned, and the SUMOylation site at position K112 was found to be conserved among human, mouse, chicken, zebrafish, and Xenopus (Fig. 1B). An amino acid sequence comparison was made between all human DLX proteins. DLX3 is the only member of the DLX family containing these two potential SUMOylation sites (Fig. 1C). This specificity and the conservation among vertebrates led us to further investigate the effect of SUMOylation on DLX3.

DLX3 IS SUMOYLATED BY SUMO1

To determine the functionality of the two potential SUMOylation sites in DLX3, we co-expressed V5DLX3 (DLX3 tagged with a V5 epitope) with wildtype SUMO1 in Saos2-TetOFF cells. As a control, we also co-expressed V5DLX3 with a mutated form of SUMO1

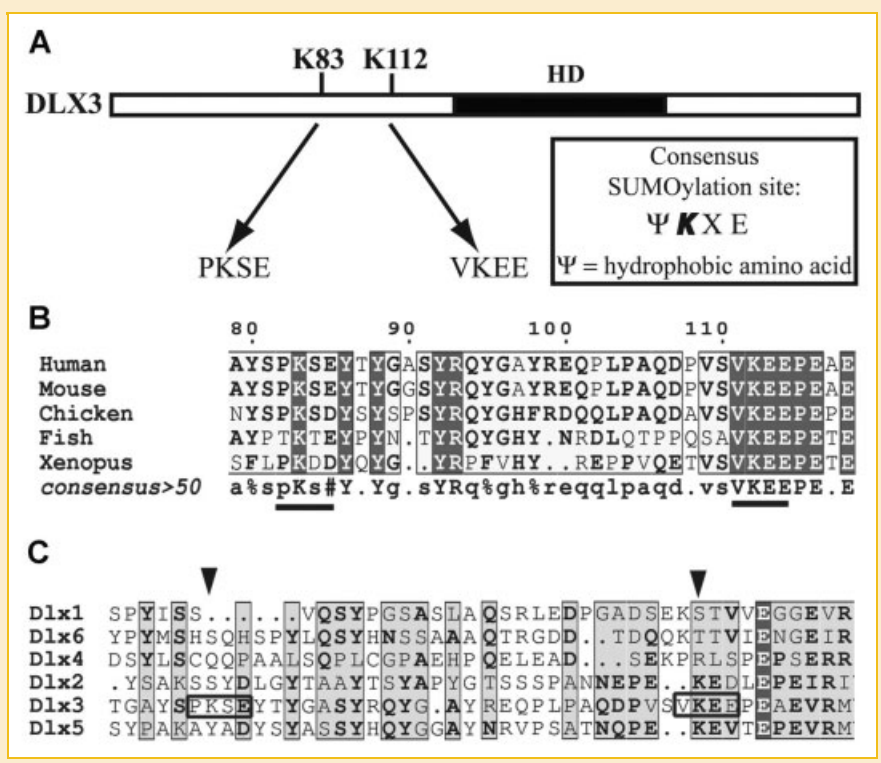

Fig. 1. Potential SUMOylation sites in the DLX3 protein sequence. (A) Diagram showing the position of the two potential SUMOylation sites in DLX3. (B) Alignment of the DLX3 sequence from several Vertebrates showing a conservation of the potential SUMOylation site at position 112. (C) Alignment of the sequence of all six human DLX proteins showing that potential SUMOylation sites are present only in DLX3.

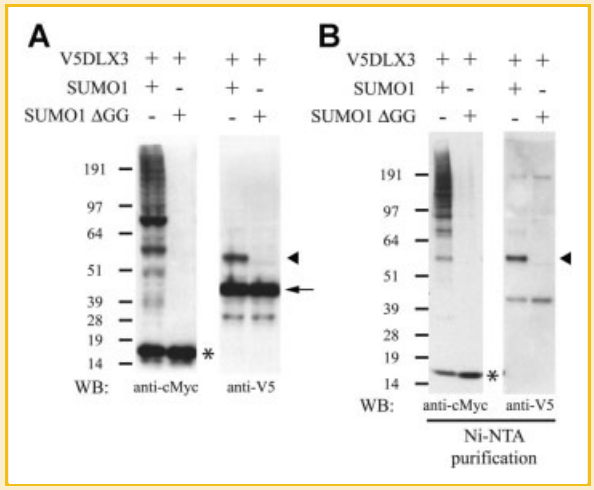

Fig. 2. Detection of DLX3 SUMOylation by SUMO1, (A) Western blot performed on cells expressing V5DLX3 and SUMO1, or V5DLX3 and SUMO1-ΔGG. SUMO1 and SUMO1-ΔGG were tagged with both a cMyc tag and a His tag. Anti-cMyc was used to detect all SUMO1-conjugated proteins and SUMO1-ΔGG. Anti-V5 was used to detect V5DLX3 and SUMOylated V5DLX3. (B) Same Western blot as in A performed after purification of the protein extracts on Ni-NTA beads (pulls down all SUMO1-conjugated proteins and SUMO1-ΔGG that are His-tagged). Arrow: V5DLX3; arrowhead: SUMOylated V5DLX3; asterisk: monomeric SUMO1.

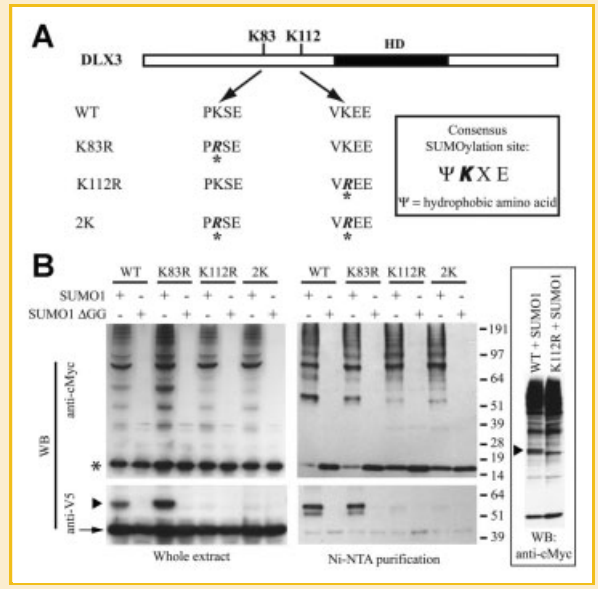

Fig. 3. Identification of the lysine involved in DLX3 SUMOylation, (A) Diagram displaying the different mutants that were produced to determine which is the lysine involved in DLX3 SUMOylation: mutation of lysine 83 into an arginine (K83R), mutation of lysine 112 into an arginine (K112R), and mutation of both lysines into arginines (2K). (B) Same approach as in Figure 2 was used to analyze the SUMOylation capacity of DLX3 and the three mutants described in A. Arrow: V5DLX3; arrowhead: SUMOylated V5DLX3; asterisk: monomeric SUMO1. The inset on the right shows a long exposure of a western blot using anti-cMyc antibody, showing the detection of SUMOylated DLX3 among all SUMOylated proteins.

lacking the C-terminal double glycine which forms an isopeptide bond with the target protein (SUMO1-ΔGG). SUMO1 and SUMO1-ΔGG are tagged with both a His tag and a c-Myc tag. Western blot was performed to analyze the size pattern of c-Myc tagged and V5-tagged proteins in these cells (Fig. 2A). Results monitored with anti-cMyc showed a low molecular weight band, around 15 kDa, corresponding to the monomer of SUMO1 or SUMO1-ΔGG, as well as a smear corresponding to all endogenous proteins SUMOylated by SUMO1, but not SUMO1-ΔGG (Fig. 2A, left panel). The anti-V5 blot revealed two major bands for V5DLX3 when co-expressed with SUMO1: the lower band corresponding to the normal size of V5DLX3, and the upper corresponding to a V5DLX3 derivative with additional 15–20 kDa (Fig. 2A, right panel). This upper band was not detected with SUMO1-ΔGG. These observations suggest that V5DLX3 is SUMOylated by SUMO1. To confirm that the upper complex detected is indeed SUMOylated V5DLX3, we performed the same western blot analysis after purifying the protein extracts with a Ni-column (Ni-NTA, binding His-tagged SUMO1) to pull-down all SUMOylated proteins. Using this approach, we detected SUMOylated V5DLX3 among the proteins purified on the Ni-column (Fig. 2B). These results demonstrate that V5DLX3 is SUMOylated by SUMO1. Moreover, the fact that we detected only one band for SUMOylated V5DLX3 suggests that only one lysine is involved in DLX3 SUMOylation.

DLX3 IS SUMOYLATED BY SUMO1 ON K112, BUT NOT K83

To test which of the two potential SUMOylation sites in DLX3 is actually involved in SUMOylation, we mutated the lysine residues at positions 83 and 112 into arginines (Fig. 3A). Thus, we generated two single mutants ($DLX3^{K83R}$ and $DLX3^{K112R}$) as well as a double mutant in which both lysine residues were mutated ($DLX3^{2K}$). In Saos2-TetOff cells, we co-expressed SUMO1 or SUMO1-ΔGG with $DLX3^{WT}$, $DLX3^{K83R}$, $DLX3^{K112R}$, or $DLX3^{2K}$, respectively. Western blot analysis was performed using anti-cMyc and anti-V5 antibodies, both on whole extracts and on Ni-column purified protein fraction (Fig. 3B). This assay revealed that mutation in position 83 does not preclude SUMOylation of V5DLX3, since a band corresponding to SUMOylated DLX3 appears for both $DLX3^{WT}$ and $DLX3^{K83R}$. This band, however, was absent for $DLX3^{K112R}$. As predicted, the double mutant $DLX3^{2K}$ is not SUMOylated. SUMOylated DLX3 could be identified among all SUMOylated proteins using anti-cMyc antibody and with a longer exposure (Fig. 3B, inset). These observations demonstrate that lysine K112 is the only SUMOylation site in DLX3.

SUMOYLATION LEADS TO A PREFERENTIAL PERINUCLEAR LOCALIZATION OF DLX3

In order to assess the effect of SUMOylation on DLX3 subcellular localization, we performed immunohistochemical analysis on Saos2-TetOFF cells co-transfected with V5DLX3 and SUMO1 or SUMO1-ΔGG (Fig. 4A). Immunostaining using anti-cMyc antibody showed that SUMO1 was present in both the cytoplasm and the nucleus: the nuclear expression was rather diffuse, however, it tended to be stronger at the nuclear periphery, while in the cytoplasm it formed dense aggregates (Fig. 4A, c). SUMO1-ΔGG exhibited a completely different distribution pattern: it was

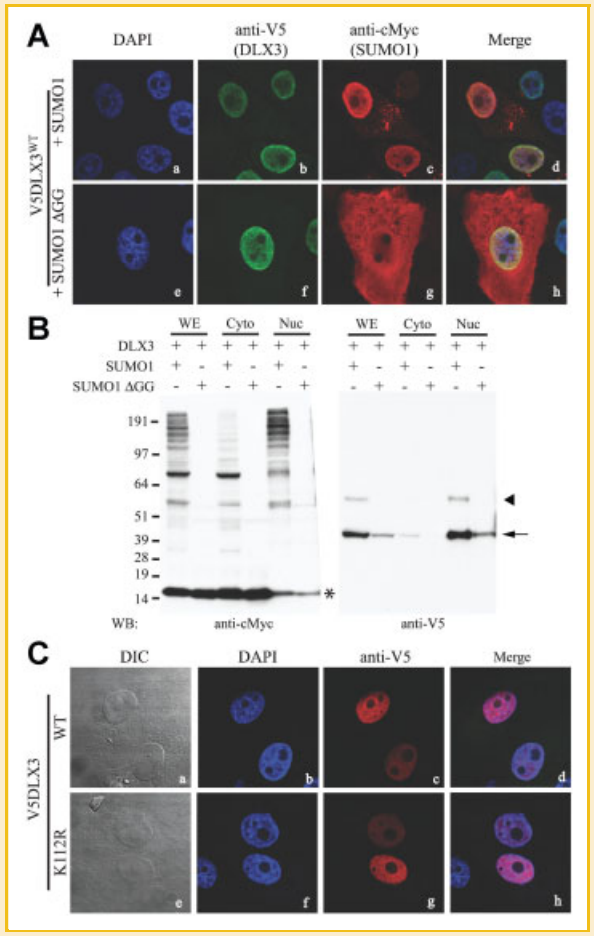

Fig. 4. Effect of SUMOylation on DLX3 subcellular localization, (A) Immunohistochemical analysis performed on Saos2-TetOff cells expressing V5DLX3 and SUMO1, or V5DLX3 and SUMO1-ΔGG. Anti-V5 was used to detect V5DLX3 and SUMOylated V5DLX3 (b and f). Anti-cMyc was used to detect SUMO1, all SUMO1-conjugated proteins and SUMO1-ΔGG (c and g). DAPI was used to stain nuclei (a and e). Merge images are shown in d and h. (B) Western blot on fractionated protein extracts (cytoplasm/nucleus) from Saos2-TetOff cells expressing V5DLX3 and SUMO1, or with V5DLX3 and SUMO1-ΔGG. Anti-cMyc was used to detect all SUMO1-conjugated proteins and SUMO1-ΔGG. Anti-V5 was used to detect V5DLX3 and SUMOylated V5DLX3. WE: whole extract; Cyto: cytoplasmic fraction; Nuc: nuclear fraction; Arrow: V5DLX3; arrowhead: SUMOylated V5DLX3; asterisk: monomeric SUMO1. (C) Immunohistochemical analysis performed on Saos2-TetOff cells expressing $V5DLX3^{WT}$ or $V5DLX3^{K112R}$. Anti-V5 was used to detect $V5DLX3^{WT}$ and $V5DLX3^{K112R}$ distribution (c and g). DIC: differential interference contrast (a and e). DAPI was used to stain nuclei (b and f). Merge images are shown in d and h.

distributed throughout the cytoplasm in a filament-like pattern and was also present in the nucleus (Fig. 4A, g). V5DLX3 was found in the nucleus in the presence of both wild-type and mutant SUMO1 (Fig. 4A, b and f). This observation suggests that SUMO1 does not affect DLX3 translocation to the nucleus. However, in the presence of SUMO1, it is frequent to see DLX3 accumulation at the periphery of the nucleus where it co-localizes with SUMO1 (Fig. 4A, d). We used cell fractionation and western blot analysis to corroborate our immunohistochemical observations (Fig. 4B). The anti-cMyc blot revealed that the cytoplasm contained both monomeric SUMO1 and SUMO1 conjugated with target proteins, while in the nucleus SUMO1 was primarily present in its conjugated form. SUMO1-ΔGG, that can only be monomeric, exhibited a much stronger expression in the cytoplasm than in the nucleus. The anti-V5 blot showed that both V5DLX3 and SUMOylated V5DLX3 were accumulated almost exclusively in the nuclear fraction. These data confirm that SUMO1 does not affect DLX3 nuclear localization. We then investigated whether DLX3 subcellular localization was affected by its inability to be SUMOylated. To address this question, immunohistochemical analysis was carried out with Saos2-TetOff cells transfected with $V5DLX3^{WT}$ or $V5DLX3^{K112R}$. As shown in Figure 4C, both wild type and K112R mutant were exclusively located in the nucleus. Thus, preventing DLX3 from being SUMOylated does not affect its subcellular localization. Taken together, these data suggest that SUMOylation does not play a significant role in determining the distribution pattern of DLX3 in cells, but leads to a preferential perinuclear localization.

SUMOYLATED DLX3 IS ABLE TO BIND DNA

To determine the role of SUMOylation on DLX3 function, we next explored the effect of SUMOylation on DLX3 binding activity. We first asked if SUMOylated DLX3 was able to bind DNA. To address this question, we generated a recombinant SUMO-DLX3 fusion protein and tested its ability to bind to the DLX3 consensus binding site in an electrophoresis mobility shift assay (EMSA), using a probe containing a DLX3 binding site. A protein–DNA complex was formed, that could be competed using an excess of non-radioactive probe (self competitor), but not using an excess of a mutated non-radioactive probe (mutant competitor) (Fig. 5A, lanes 2–4). To confirm that this protein–DNA complex contained SUMO-DLX3, we performed a supershift assay using anti-DLX3 antibody and were able to detect a protein–DNA–antibody complex (Fig. 5A, compare lanes 5 and 6). Even though the SUMO-DLX3 fusion protein does not perfectly mimic the tertiary structure of SUMOylated DLX3, this strategy is commonly used in the field [Ouyang et al., 2009] and gives a preliminary suggestion that having SUMO bound to DLX3 does not affect its ability to bind DNA. To test this hypothesis in a more physiologically meaningful context, we performed EMSA using nuclear extracts from cells expressing V5DLX3 with SUMO1 or SUMO1-ΔGG (Fig. 5B, inset). In both cases (wild-type and mutant SUMO1), we could detect a protein–DNA complex formed between DLX3 and the probe (Fig. 5B, arrow). In order to detect if SUMOylated DLX3 is involved in a complex with the probe, we performed a supershift assays using anti-cMyc antibody (recognizing SUMO1 and SUMO1-ΔGG). As shown in Figure 5B, a partial supershift could be detected in the presence of anti-cMyc when DLX3 was expressed with SUMO1, but not with SUMO1-ΔGG (Fig. 5B, arrowhead, compare lanes 3 and 5). We performed a similar experiment in which we prepared nuclear extracts from cells expressing SUMO1 with $DLX3^{WT}$ or $DLX3^{K112R}$ (Fig. 5C, inset), and performed an EMSA as described above. As expected, using anti-cMyc antibody, we could detect a partial supershift for $DLX3^{WT}$ but not for $DLX3^{K112R}$ (Fig. 5C, compare lanes 3 and 5). These observations demonstrate that when SUMO1 is bound to DLX3, DLX3 is still able to bind to its consensus binding site. We also showed that $DLX3^{K112R}$ is able to bind DNA (Fig. 5C, arrowhead,

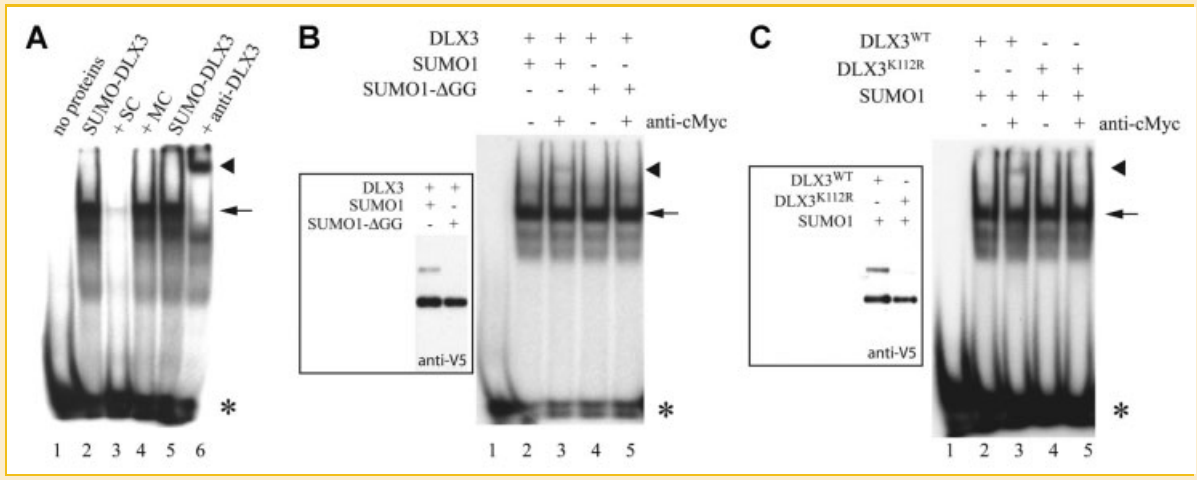

Fig. 5. Effect of SUMOylation on DLX3 DNA binding activity, (A) Electrophoresis mobility shift assay (EMSA) using recombinant SUMO-DLX3 fusion protein and a radiolabeled probe containing a consensus DLX3 binding site (lanes 2 and 5). An excess of non-radioactive probe was used to compete with the radioactive DLX3 consensus probe (SC: self competitor; lane 3). An excess of a mutated non-radioactive probe was used as a control (MC: mutant competitor; lane 4). Anti-DLX3 antibody was used to supershift protein–DNA complexes involving SUMO-DLX3 (lane 6). (B) EMSA using nuclear extracts from cells expressing V5DLX3 and SUMO1 (lanes 2 and 3), or V5DLX3 and SUMO1-ΔGG (lanes 4 and 5). Anti-cMyc antibody was used to supershift protein–DNA complexes involving SUMOylated DLX3 (lanes 3 and 5). The inset on the left shows a western blot performed on the nuclear extracts using anti-V5 antibody. (C) EMSA using nuclear extracts from cells expressing V5DLX3^{WT} and SUMO1 (lanes 2 and 3), or V5DLX3^{K112R} and SUMO1 (lanes 4 and 5). Anti-cMyc antibody was used to supershift protein–DNA complexes involving SUMOylated DLX3 (lanes 3 and 5). The inset on the left shows a western blot performed on the nuclear extracts using anti-V5 antibody. Asterisk: free probe; arrow: protein–DNA complex; arrowhead: antibody–protein–DNA complex.

lanes 4 and 5), demonstrating that preventing DLX3 from being SUMOylated does not affect its ability to bind DNA.

SUMOYLATION HAS A POSITIVE EFFECT ON DLX3 TRANSCRIPTIONAL ACTIVITY

In order to test the effect of SUMOylation on DLX3 transcriptional activity, we performed luciferase reporter assays to address the effect of overexpressing SUMO1 on DLX3 transcriptional activity. We transfected Saos2-TetOFF cells with pCMV-V5DLX3 (constitutive expression of V5DLX3), pTRE2-SUMO1 (inducible expression of SUMO1), pGL3–3xDRE (3 copies of the DLX3 responsive element upstream of the Firefly luciferase reporter gene), and pRL-TK (constitutive expression of Renilla luciferase gene used for normalization). After transfection, the cells were grown for 24 h with or without doxycycline, and assayed for Firefly and Renilla luciferase activity. Using this strategy, we found that DLX3 transcriptional activity was slightly higher in the presence of SUMO1 (-Dox) than in its absence (+Dox), but this difference was not statistically significant (data not shown). Considering that this moderate effect of SUMO1 overexpression on DLX3 transcriptional activity could be due to the effect of SUMO1 on other endogenous targets that may interact with DLX3, we decided to focus on the comparison between DLX3^{WT} and DLX3^{K112R}. In this next assay, Saos2-TetOFF cells were transfected with pBi-GFP, pBi-V5DLX3^{WT}/GFP, or pBi-V5DLX3^{K112R}/GFP, together with pTRE2-SUMO1, pGL3–3xDRE, and pRL-TK. After transfection, the cells were grown for 24 h with or without doxycycline, and relative luciferase activity (Firefly/Renilla) was measured (Fig. 6). As expected, in the absence of doxycycline, we detected a significant increase in relative luciferase activity in the presence of DLX3^{WT} compared to the GFP control. The transcriptional activity measured for DLX3^{K112R} was significantly lower than that of DLX3^{WT}, demonstrating that preventing DLX3 SUMOylation in a context where SUMO1 is active significantly reduces its transcriptional activity. In the presence of doxycycline that shuts down the expression of the transgenes in Saos2-TetOff cells, the relative luciferase activity was reduced to a

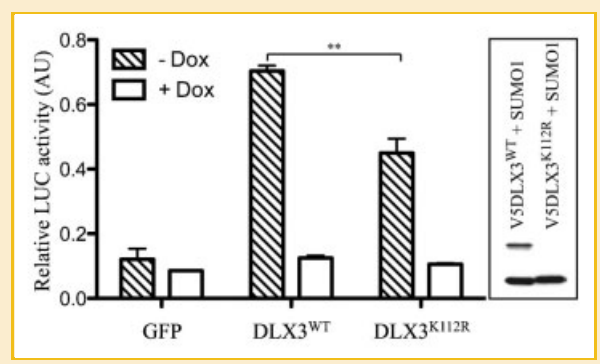

Fig. 6. Effect of SUMOylation on DLX3 transcriptional activity, Luciferase reporter assay comparing the transcriptional activity of DLX3^{WT} and DLX3^{K112R}. Saos2-TetOff cells were transfected with pBi-GFP, pBi-V5DLX3^{WT}/GFP, or pBi-V5DLX3^{K112R}/GFP, together with pTRE2-SUMO1, the reporter construct pGL3–3xDRE (Firefly luciferase under control of three copies of a DLX3 responsive element) and the normalization vector pRL-TK (Renilla luciferase under control of a TK promoter). Transfected cells were grown for 24 h in the absence (transgenes ON) or presence (transgenes OFF) of doxycycline and relative luciferase activity (Firefly/Renilla) was measured. In the presence of SUMO1, DLX3^{K112R} transcriptional activity is significantly lower than DLX3^{WT} transcriptional activity (*t*-test, $P = 0.008$). The inset on the right shows the levels of DLX3^{WT} and DLX3^{K112R} expressed in this assay.

basal level in all conditions, confirming that the transcriptional activities measured are related to the transgenes expression. Taken together, these data suggest that SUMOylation promotes DLX3 transcriptional activity.

DISCUSSION

In the last decade, the number of proteins identified as being post-transcriptionally modified by SUMOylation has shown an exponential rise. Although these modifiers are related to the ubiquitin family, they have not been associated with protein degradation and exhibit a large variety of effects on their target proteins. The knockout of SUMO1 is lethal, and SUMO1 haploinsufficiency has been associated with cleft lip and palate in human [Alkuraya et al., 2006]. Even though these observations do not determine specific targets of SUMOylation and how their function is altered in vivo, they demonstrate that SUMOylation plays an essential role in embryonic development. Altered SUMOylation of p63α contributes to the Split-Hand/Split-Foot malformation phenotype [Huang et al., 2004], which further supports the importance of SUMOylation of specific proteins in the regulation of developmental processes. The reports published so far on SUMOylated proteins show that it is difficult to predict the effect of SUMOylation on the activity of a protein. A large majority of the proteins that have been identified as targets of SUMO are transcription factors. In this study, we identified for the first time a SUMOylation site in the homeodomain transcription factor DLX3.

We identified lysine K112 as the unique SUMOylation site in DLX3. This lysine branches SUMO in the N-terminal domain of DLX3, less that 20 amino acids upstream of its nuclear localization signal (NLS) located right before the homeodomain [Bryan and Morasso, 2000]. In spite of the proximity between lysine K112 and the NLS, SUMOylation does not affect DLX3 nuclear localization. Recent reports showed that it is quite frequent to find a SUMOylation site near a nuclear export signal (NES). For example, KLF-5 SUMOylation favors its retention within the nucleus by inhibiting its NES and thus preventing its translocation to the cytoplasm [Du et al., 2008]. SUMO is also involved in the subnuclear localization of protein complexes, particularly in controlling the assembly of PML-nuclear bodies [Heun, 2007]. We did not observe any obvious change in the distribution of DLX3 within the cell, neither by inducing nor inhibiting SUMOylation. However, we noticed a tendency for DLX3 to exhibit a perinucleal localization in the presence of SUMO1, suggesting that SUMO1 may have an effect on DLX3 subnuclear localization.

DNA binding can be affected by SUMOylation. Although the first report of the SUMOylation of the Heat Shock Factor HSF2 suggested a positive effect of SUMO1 on HSF2 DNA binding activity [Goodson et al., 2001], a more recent study showed that HSF2 is unable to bind DNA when bound by SUMO [Tateishi et al., 2009]. The same effect was observed for Sox2 [Tsuruzoe et al., 2006]. Here we show that DLX3 is still able to bind DNA when it is bound to SUMO, and that mutating lysine K112 does not prevent DNA binding, suggesting that SUMOylation does not dramatically affect the ability of DLX3 to bind DNA.

SUMOylation is a very dynamic, reversible, and unstable process, which makes it difficult to analyze in a physiological context. Although SUMOylation has been associated with both activation and repression of transcriptional activities, a large majority of the reports published so far have shown a repressor effect of SUMO on transcriptional activity [Yang et al., 2003]. Among the transcription factors whose activity is promoted by SUMOylation are two factors involved in muscle differentiation: myocardin and nkx2.5 [Wang et al., 2007; Wang et al., 2008]. Here, we show that, in a context where SUMO1 activity is high, the transcriptional activity of a mutant of DLX3 that cannot be SUMOylated is significantly lower relative to that of wild-type DLX3. This suggests that SUMOylation has a positive effect on DLX3 transcriptional activity. The mechanism responsible for this effect may involve interactions with transactivation partners that remain to be identified. Among the potential candidates are other members of the DLX family, as well as members of the MSX family, since members of these two families have been shown to form homodimers and heterodimers [Zhang et al., 1997]. The interaction and interplay between DLX3, DLX5 and MSX2 has been shown to play an essential role in the regulation of Runx2 and osteocalcin expression during osteoblast differentiation [Hassan et al., 2004; Hassan et al., 2006]. Future studies should investigate the possible involvement of SUMOylation in this process. DLX3 is also involved in ectodermal appendages development such as hair and teeth, where interacting partners potentially have essential regulatory roles as well. The identification of such factors will be an essential pre-requisite to the analysis of the impact of DLX3 SUMOylation in these tissues.

Interestingly, the SUMOylation site in DLX3 is conserved among vertebrates and none of the five other members of the Distal-less family contains a SUMOylation site. This high specificity suggests that SUMOylation may play a major role in the regulation of DLX3 activity during embryonic development. DLX3 also distinguishes itself from other members of the Distal-less family by its distribution and function during embryogenesis. First, it is the only Distal-less member that has not been detected in the mammalian central nervous system. Second, mice that are null for Dlx3 die at E9.5 from placental defects [Morasso et al., 1999], while Dlx1, Dlx2, and Dlx5 were shown to be essential for mouse craniofacial development but not for placental development [Depew et al., 2005]. Furthermore, DLX3 is one of the few transcription factors in which mutations have been linked to a human ectodermal dysplasia, namely Tricho-Dento-Osseous syndrome [Price et al., 1998; Morasso and Radoja, 2005]. These differences suggest that DLX3, while keeping common features with its family members, acquired specific characteristics during evolution, including its potential to be regulated by SUMOylation.

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