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# Repression of SOX6 transcriptional activity by SUMO modification

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**Abstract SOX6 plays key functions in several developmental processes, including neurogenesis and skeleton formation. In this report, we show that SOX6 is modified in vitro and in vivo by small ubiquitin-related modifier (SUMO) on two distinct sites. Mutation of both sites abolished SOX6 sumoylation and increased SOX6 transcriptional activity. SUMO dependent repression of SOX6 transcription was promoted by UBC9 whereas siRNA to UBC9, cotransfection of inactive UBC9 or a SUMO protease increased SOX6 transcriptional activity. Furthermore, co-expression of SOX6 with SUMO2 results in the appearance of SOX6 in a punctate nuclear pattern that colocalized with promyelocytic leukemia protein, which was partially abolished by mutations in SOX6 sumoylation sites.**


*Keywords:* Small ubiquitin-related modifier; Chondroblast differentiation; SOX6; Transcriptional control; Nuclear bodies

## 1. Introduction

Recently, several ubiquitin-like proteins that act as posttranslational modificators of other proteins have been identified. Small ubiquitin-related modifier (SUMO) proteins are members of this family that have been shown to regulate diverse cellular functions of a variety of target proteins [1–4]. SUMO proteins are conjugated on the lysine residue in the consensus sequence ψKxE (where ψ represents a hydrophobic amino acid, and *x* is any amino acid). The mechanism involves a multiple-step cascade. SUMO conjugation requires a heterodimeric E1-activating enzyme and a single E2-conjugating enzyme, UBC9 [1–4]. Although in the ubiquitin pathway, at least one additional factor called E3 ubiquitin ligase is needed for substrate recognition and ubiquitin addition at the target protein, this E3 protein ligase does not appear to be required for SUMO conjugation in vitro. However, such E3 ligases play important roles in modulating the efficiency of SUMO attachment to target proteins. Three types of SUMO E3 ligases have been described: proteins of the PIAS family, RanBP2 and Pc2 [2].

Many of the functionally diverse proteins identified as SUMO targets are involved in control of gene expression and include transcription factors, co-activators and co-repressors or components of large sub-nuclear structures called promyelocytic leukemia protein (PML) nuclear bodies [1,4]. SUMO modification often represses transcriptional activity by direct sumoylation of a transcriptional factor, a co-activator, and/or increased recruitment of co-repressors. Other effects on transcription are mediated by PML nuclear bodies which, through sumoylation, control the binding/release of components of the transcriptional machinery [5].

The Sox family of transcription factors are involved in embryonic development as regulators of cellular determination and differentiation [6,7]. SOX6 has been shown to play a key role in the development of the central nervous system, chondrogenesis and cardiac and skeletal muscle cell maintenance [8–10]. Some cytokines are able to control Sox6 expression, such as BMP-2 during chondrogenesis [11]. In addition, SOX6, L-SOX5 and SOX9 physically interact and functionally cooperate in the activation of the type-II collagen alpha1 chain (*Col2a1*) [8], whereas SOX6 represses the *Fgf3* promoter through interaction with the co-repressor CtBP2 [12]. In this study we show that SOX6 is sumoylated in vivo and in vitro. Moreover, we find that sumoylation of SOX6 inhibits its transcriptional activity and its functional synergism with SOX9 on type-II collagen enhancer. Based on these observations, we suggest a potential role of SUMO in regulating SOX6-dependent transcriptional activity.

## 2. Materials and methods

### 2.1. Plasmids constructs and antibodies

pCDNA3-SOX6His was a gift from Dr. de Crombrugghe, and was used as to create the sumoylation mutants ($K_{364}R$, $K_{377}R$ and the double mutant) using PCR approaches. pCDNA3-SOX9HA was a gift from Dr. Harley. *Col2a1* enhancer reporter construct and SUMO1, 2 and 3, SENP2, hUBC9 and hUBC9 (C93S) and pIRESCFP-SUMO2 expression vectors as well as GST fusion proteins were previously described [11,13,14]. SOX6 antibodies were produced in rabbits as previously described [11]. Anti-hUBC9 was raised against the hUBC9 peptide (CEYEKRVRAQAKKFAPS). Antibodies against HA-peptide (Roche) and α-tubulin (Sigma) and SUMO2 (Zymed) were used for immunoblotting.

### 2.2. Transient transfection

HEK-293 T, HeLa, C2C12 and COS-7 cells were transfected with FuGENE 6 (Roche Diagnostics) or Lipofectamine™ (Invitrogen), $C3H10T_{1/2}$ cells were transfected with Lipofectamine 2000 (Invitrogen).

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*Abbreviations:* BMPs, bone morphogenetic proteins; *Col2a1*, type-II collagen alpha1 chain; PML, promyelocytic leukemia protein

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*2.3. RT-QPCR*

Two micrograms of total RNA, isolated using the Ultraspec™ RNA Isolation System (Biotecx), were reverse-transcribed using a Ready-to-Go First Strand Kit (Amersham). Quantitative PCRs were carried out on 96-well plates using Applied Biosystem's ABI Prism 7000 and Taqman 5′-nuclease probe method (Applied Biosystems). All transcript levels were normalized to that of GAPDH and transfection efficiency assessed by GFP expression.

*2.4. Luciferase assays*

Luciferase activities of transfected HEK-293 T cells were quantified as previously described [11].

*2.5. SUMO conjugation assays in vitro and in vivo*

SUMO conjugation assays in vitro and in vivo sumoylation assays were performed as previously described [13,14].

*2.6. Immunofluorescence assays*

HeLa and C2C12 cells were seeded onto Costar 35-mm 6-well plates containing 12-mm-coverslips. 48 h after transfection cells were fixed in 4% paraformaldehyde for 20 min, permeabilised in 0.1% Triton X-100 and blocked with 3% BSA in PBS. Cells were stained for SOX6 with rabbit anti-SOX6 at 1:200 dilution or mouse anti-His (Roche) at 1:200, followed for goat anti-rabbit or anti-mouse, respectively, IgG conjugated with Alexa 568, at 1:2000 (Molecular probes), and for PML with rabbit anti-PML at 1:200, followed by goat anti-rabbit IgG conjugated with Alexa 488, at 1:2000. The presence of SOX6, pECFP-SUMO2, and PML was detected using a Leica TCS SL inverted laser scanning confocal microscope.

*2.7. RNA interference experiments*

HEK-293-T cells were transfected with the corresponding constructs and 200 pmol of UBC9 or unrelated (mouse Id1) siRNA oligonucleotides with Lipofectamine 2000. 24 h later, luciferase assays were done and UBC9 expression was analyzed by immunoblotting.

## 3. Results

*3.1. SOX6 is sumoylated in vitro and in vivo*

Inspection of the SOX6 sequence indicated two sequences ($IK_{364}NE$ and $VK_{377}DE$) of the motif ψKxE required for modification by SUMO (Fig. 1A). We thus examined the possibility of covalent modification of SOX6 by SUMO. In vitro sumoylation assays revealed multiple forms of SOX6 conjugated to SUMO1/-2/ or -3 (Fig. 1B). To determine the requirement for UBC9 in SOX6 sumoylation, UBC9 was titrated into an in vitro sumoylation assay. Increasing amounts of recombinant UBC9 brought a dose dependent increase in SUMO modification of SOX6 (Fig. 1C). We also tested whether the known E3 ligases, RanBP2 or proteins of the PIAS family, enhanced sumoylation of SOX6 at submaximal amounts of UBC9 (12 ng). In these conditions, we could not detect a significant stimulation of SOX6 sumoylation by either RanBP2, PIAS1, PIASxα or PIASxβ (data not shown).

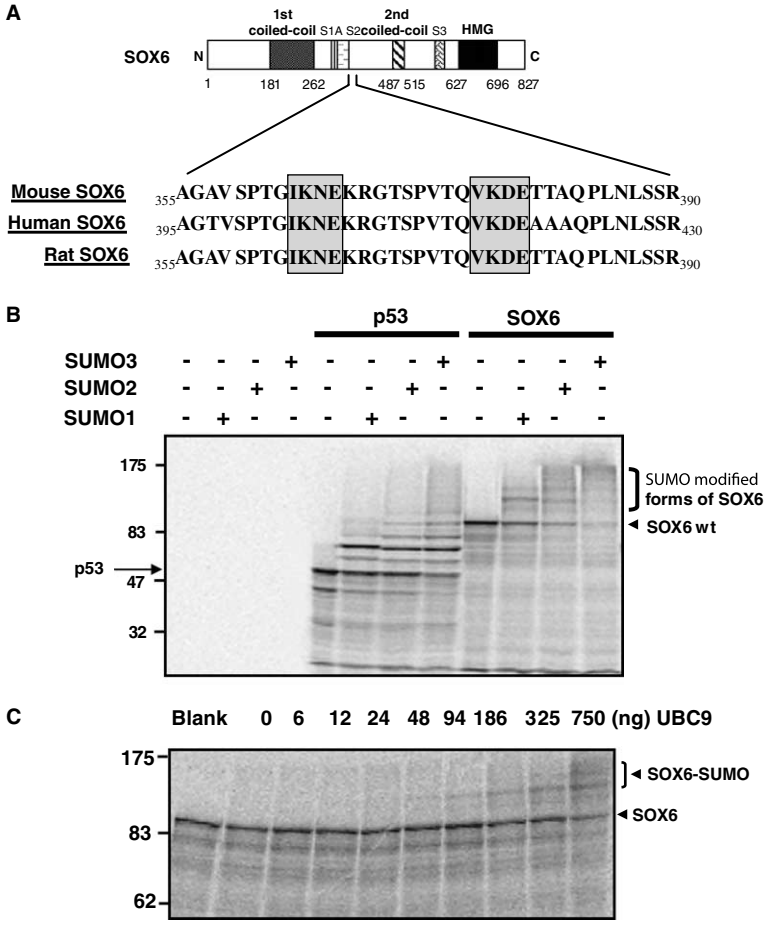


Fig. 1. SOX6 is sumoylated in vitro. (A) Schematic representation of protein domains of SOX6 and the putative SUMO conjugation residues conserved between human, mouse and rat sequences. (B) $^{35}$S-labeled SOX6 and p53 were incubated in the absence (blank) or presence of the assay mix containing recombinant E1 (SAE1/SAE2), UBC9 and SUMO1, 2 or 3 as indicated. (C) SOX6 was in vitro translated and incubated as above with the indicated amounts of UBC9.

To investigate whether the SUMO consensus sites are required for SOX6 sumoylation, these motifs ($IK_{364}NE$ and $VK_{377}DE$) were targeted for site-directed mutagenesis. Single mutants and the double mutant were tested for in vitro sumoylation. Whereas both single mutants were still sumoylated, the double mutant showed much lower formation of SOX6–SUMO complexes (Fig. 2A).

We also analyzed whether this SUMO modification occurs also in COS-7 cells. Immunoblots showed two high molecular weight bands corresponding to sumoylated forms of SOX6 suggesting that a fraction of SOX6 undergoes covalent modification when co-expressed together with SUMO1, 2 or 3. Moreover, these bands were reduced when the $K_{364}R$ and $K_{377}R$ mutants where expressed and absent when the double mutant form of SOX6 was transfected (Fig. 2B).

### 3.2. Sumoylation inhibits SOX6 transcriptional activity

In view of our findings that SOX6 is sumoylated and that sumoylation did not significantly alter SOX6 protein stability (Supplementary Fig. 1), their functional significance was studied in the context of transcriptional activation. Transcriptional assays were performed using a chondrocyte-specific *Col2a1*

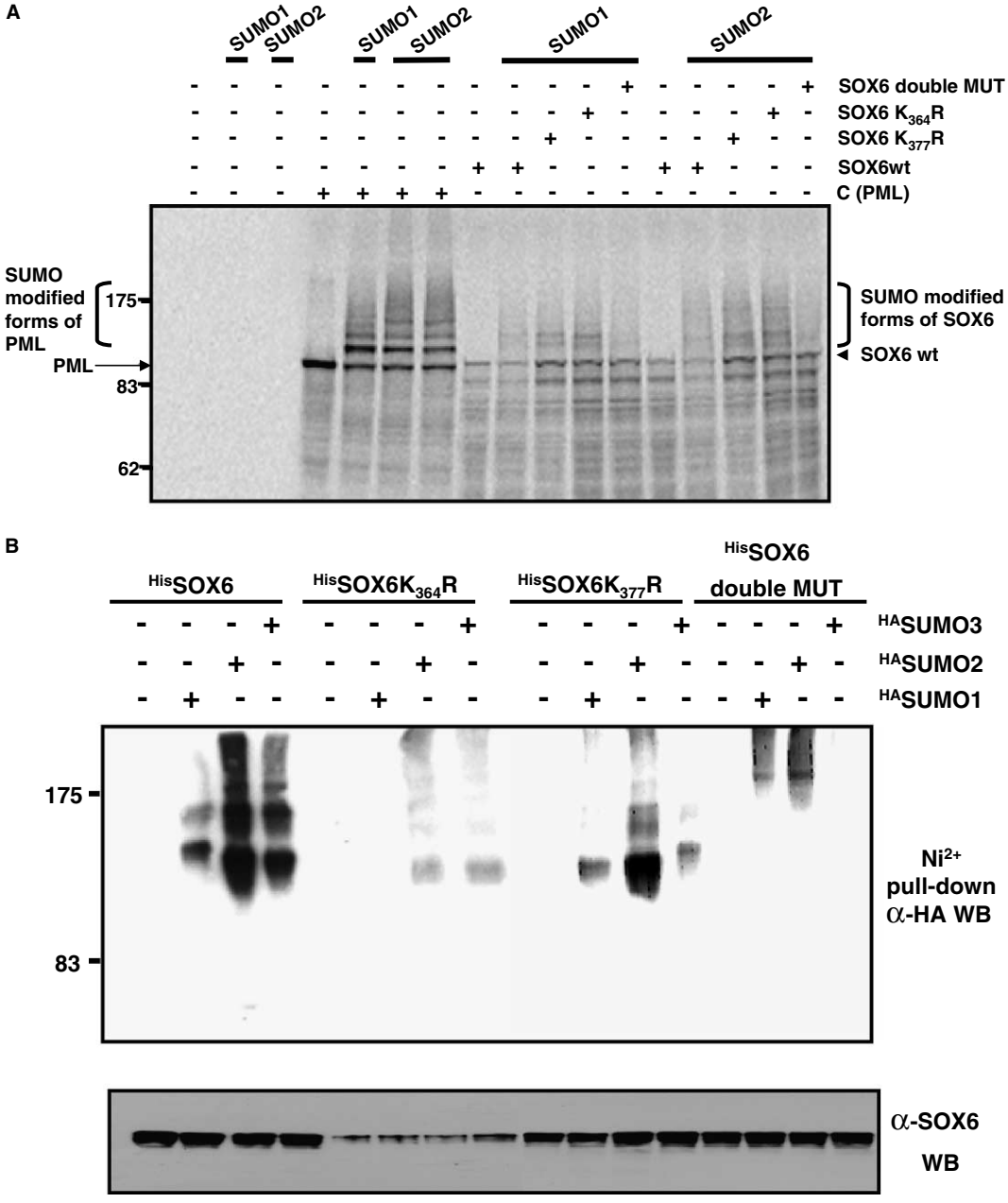


Fig. 2. Sites of SOX6 sumoylation in vitro and in vivo. (A) $^{35}S$-labeled PML (as a control), SOX6 wild type and mutants were incubated as above and sumoylated products were visualized by SDS–PAGE. (B) COS-7 cells were transfected with either wild type SOX6, $K_{364}R$, $K_{377}R$ single or double mutants together with HA-tagged SUMO1, 2 or 3. SOX6 proteins were purified by nickel-pull-down of the cells extracts and sumoylated SOX6 was detected by immunoblotting with the HA-epitope. Expression levels of the different SOX6 constructs were determined by immunoblotting with antibody against SOX6.

enhancer reporter construct, previously described to be SOX6-responsive [11]. We observed that co-expression of increasing amounts of UBC9 increased sumoylation of SOX6 (Fig. 3A) and gradually reduced SOX6-mediated transcription in HEK 293 T cells (Fig. 3B). In contrast, co-expression of either increasing amounts of a UBC9 dominant negative or a SUMO specific protease (SENP2), which decreased sumoylation of SOX6 (Fig. 3A), increased the reporter gene expression (Fig. 3B).

Next, we determined the transcriptional activity of wild type and sumoylation mutants of SOX6 in HEK 293 T cells. Whereas SOX6 single mutants displayed only slightly higher reporter responses, SOX6 double mutant enhanced about 50% the transcriptional activity of SOX6 wild type (Fig. 3C). Moreover, SOX6 double mutant became partially refractory to the inhibitory transcriptional effects of overexpression of UBC9 or activation by UBC9 DN (Fig. 3D). These results indicate that SUMO modification exerts a repressive effect on SOX6-dependent transactivation.

### 3.3. SUMO represses SOX6/SOX9 synergism

SOX6 and SOX9 have been shown to functionally cooperate in the activation of the *Col2a1* [8]. As previously described, cotransfection of SOX6 induced a 2-fold increase

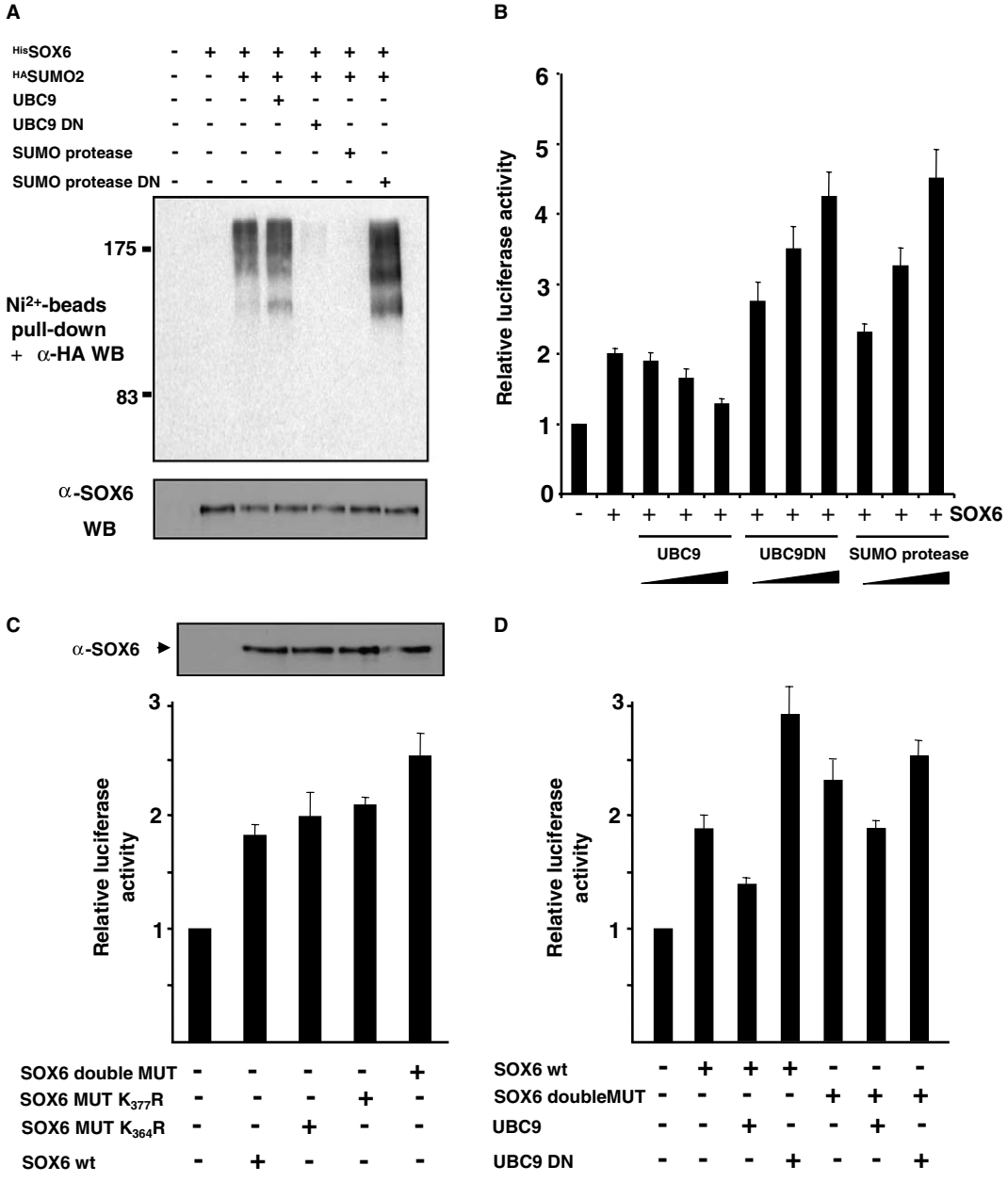


Fig. 3. Sumoylation represses SOX6 transcriptional activity. (A) SOX6 proteins from HEK-293-T transfected cells were analyzed for sumoylation as above. (B) HEK-293 T cells were transfected with the *Col2a1*-enhancer reporter and expression vector for SOX6 with increasing amounts of the indicated expression vectors (0.05, 0.2 and 1 μg). Luciferase activities represent the average of three independent experiments ± S.E.M. after normalization by β-galactosidase. (C, D) HEK 293-T cells were transfected with the *Col2a1*-enhancer reporter and the indicated constructs and luciferase activity measured as above indicated.

of SOX9 activation of *Col2a1* reporter [11]. More interestingly, in HEK 293 T cells, cotransfection of SOX6 double mutant induced a further 2-fold activation compared to wild type SOX6 (Fig. 4A). Similar to the data from transfection of SOX6 alone, UBC9 inhibited and UBC9 DN activated transcriptional activity of wild type SOX6 whereas SOX9 alone and SOX6 double mutant became refractory to these effects (Fig. 4A). We also analyzed the effects of mutation of SOX6 on endogenous *Col2a1* mRNA expression. Although $C3H10T_{1/2}$ cells express *Col2a1* at low levels, following transient transfection of SOX9 and SOX6 plasmids, *Col2a1* mRNA accumulated in the cells within 24 h. Whereas wild type SOX6 induced a modest 30% increase on *Col2a1* mRNA, transfection with either SOX6 double mutant or cotransfection of UBC9 DN induced 2-fold increase in *Col2a1* mRNA levels (Fig. 4B). To further confirm that sumoylation of SOX6 at these sites mediates their effects on transcription we ablated UBC9 expression with siRNA and measured SOX6/SOX9 effects to *Col2a1* enhancer in HEK 293 T cells. siRNA directed against UBC9 mRNA, which significantly reduced endogenous UBC9 expression and blocked SOX6 sumoylation (Fig. 4C), enhanced the SOX6 effects on *Col2a1* enhancer whereas had much lower effects on the effects of the SOX6 double mutant (Fig. 4D).

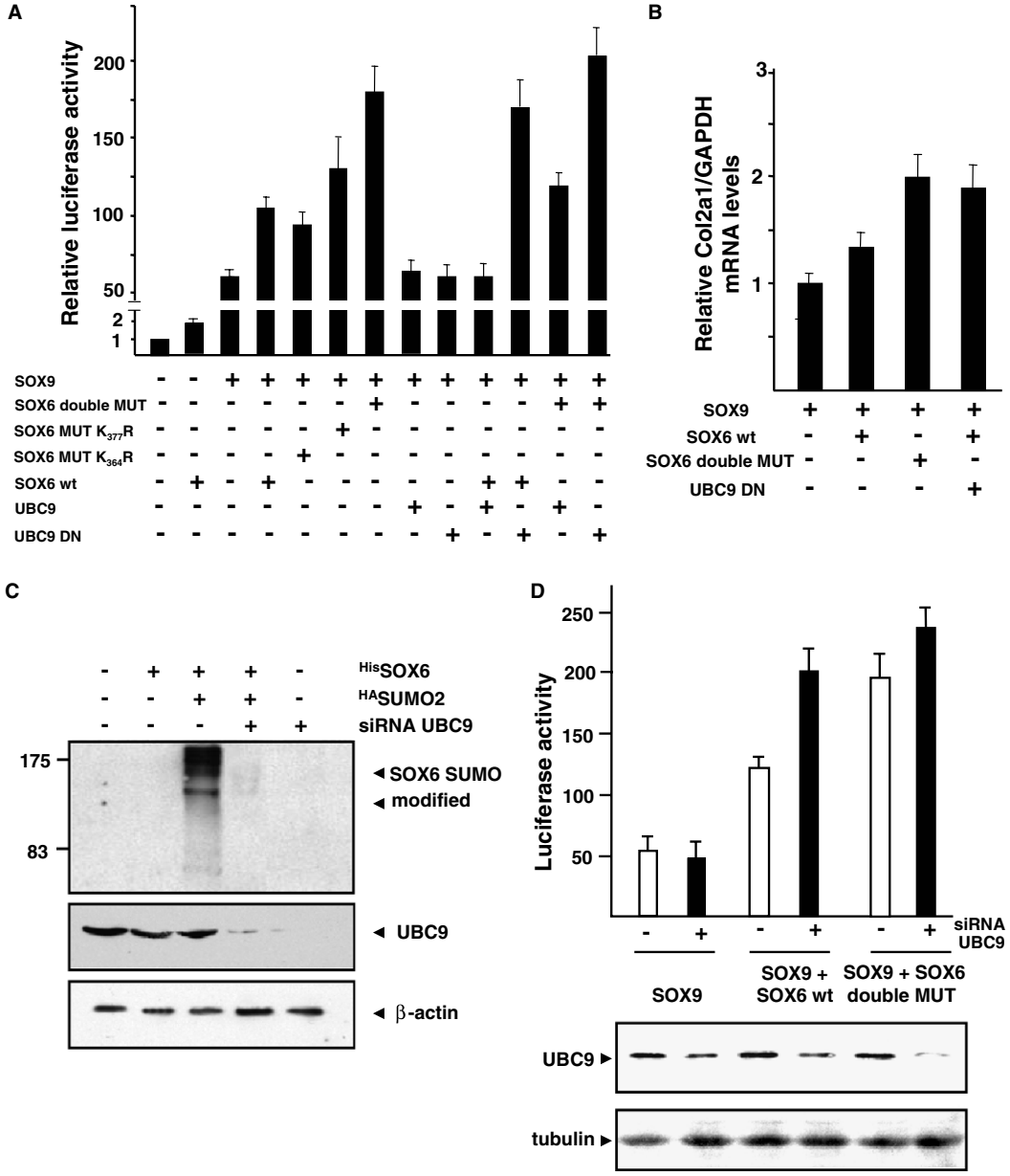


Fig. 4. SUMO represses SOX6/SOX9 additive effects. (A) HEK 293-T cells were transfected with the *Col2a1*-enhancer reporter and the indicated constructs. Luciferase activity was determined 24 h later, and represents the average of four independent experiments ± S.E.M. after normalization by β-galactosidase. (B) $C3H10T_{1/2}$ cells were transfected with the indicated plasmids and real-time PCR analysis was performed 36–48 h later. Relative values of *Col2a1* expression represent the average of four independent experiments ± S.E.M. after normalization by GAPDH expression. (C, D) HEK-293 T cells were transfected with the indicated constructs together with 200 pmol of a siRNA targeted against UBC9 or an unrelated siRNA (mouse Id1) as indicated. Sumoylated SOX6, UBC9 or β-actin levels were detected by immunoblotting and luciferase activity was determined as above.

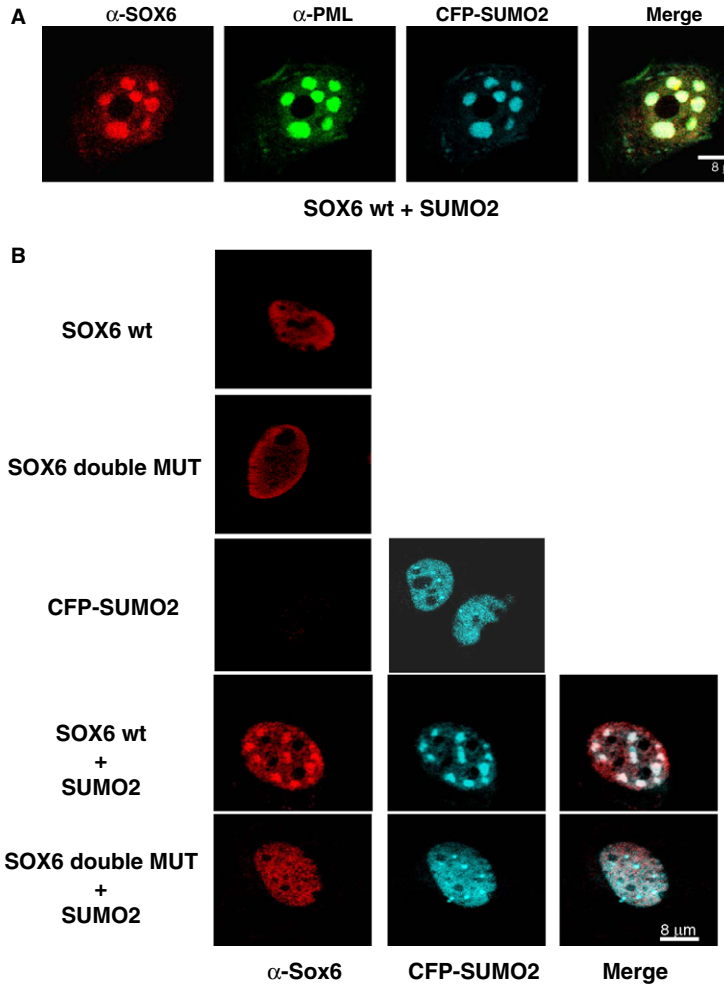


Fig. 5. Sub-cellular localization of SOX6 in a SUMO-dependent manner. (A) HeLa cells were transfected with SOX6 and CFP-SUMO. Cells were stained as indicated with anti-His antibody, anti-PML antibody and CFP-SUMO2 was directly visualized. (B) C2C12 cells were transfected as indicated. Cells were stained with anti-SOX6 antibody and CFP-SUMO2 was directly visualized.

### *3.4. SUMO conjugation induces sub-nuclear re-localization of SOX6*

Various studies have reported that SUMO conjugation can alter the subcellular localization of the modified protein [5]. Immunofluorescence analysis in HeLa cells showed that after co-expression with SUMO2, SOX6 was distributed in punctate nuclear structures reminiscent of nuclear bodies (Fig. 5A). We confirmed that the nuclear punctate structures where SOX6 is localized completely overlapped with anti-PML staining. Furthermore, whereas SOX6 co-expressed with SUMO2 in C2C12 cells was localized in punctate nuclear structures, co-expression of SUMO2 with the SOX6 double sumoylation mutant resulted in a significant loss of the punctate staining pattern of SOX6 (Fig. 5B). Altogether, these results indicate that, although sumoylation is not required for SOX6 nuclear localization, SUMO proteins influence SOX6 sub-nuclear distribution.

## 4. Discussion

Sumoylation of transcription factors has been reported to have a range of different effects on their activity. For instance, SUMO modification of Tcf-4 [15] or the heat shock factors HSF1 [16] has been shown to increase their transactivation capacity. However, it is becoming clear that in the vast majority of the cases described to date, conjugation of SUMO represses the activity of transcriptional activators [1–4]. In this report, we show that sumoylation of SOX6 has a repressive effect on transcriptional activation. Data from the SOX6 sumoylation mutants further support that the transcriptional effects seen by modulating UBC9 activity, at least partially, rely on direct effects of sumoylated SOX6 in transcriptional complexes. For instance, the double mutant is partially refractory to the effects of either overexpression of UBC9 or "knocking down" endogenous UBC9 expression. In addition, the SOX6 double mutant has stronger ability to activate the chondrocyte-specific *Col2a1* enhancer as well as to increase transcription of endogenous *Col2a1* mRNA.

Interestingly, it was found that not only SOX6, but also SOX9 is sumoylated, and these sumoylation sites occur within synergy control motif (SC) of SOX9 [17]. The SC protein motif was identified in transcription factors that activate transcription synergistically when their binding sites are present in multiple copies in the promoter region, which is the case of the *Col2a1* enhancer [18]. This could be also the case of SOX6

since both sumoylation sites are included in two tandem repeated SC motifs and synergism of SOX6/SOX9 on the *Col2a1* enhancer is profoundly affected by sumoylation of SOX6 at these sites.

In most sumoylated transcription factors the sumoylation site lies within a negative regulatory region [18,19]. Furthermore, recruitment of SUMO to a promoter, in the context of Gal4-SUMO fusion, is sufficient for repression, suggesting that SUMO itself can directly bind transcriptional co-repressors [20]. Interestingly, SOX6, which mainly function as transcriptional activator, has been shown to have repressive effects on the *Fgf-3* promoter through interaction with the co-repressor CtBP2. Interaction between SOX6 and CtBP2 takes place in a short sequence located next to the SOX6 sumoylation sites [12]. Although we could also confirm interaction between CtBP2 and SOX6, this interaction was independent of the SOX6 sumoylation state (data not shown).

Many SUMO-modified proteins have been found in PML nuclear bodies [1–4]. Mutation of the SUMO acceptor sites in PML induces nuclear body components such as Sp100 and CBP to relocalize in the nucleus [21]. Similarly, SUMO modification of certain proteins like p53 or Sp100 correlates with their partition to sub-nuclear domains such as PML bodies [21,22]. This is also the case of SOX6 since the mutant lacking both sumoylation sites shows a partially impaired recruitment to PML nuclear bodies. It has been proposed that PML-NBs may play a specific, though indirect, role in transcription by sequestration and/or modification of transcription factors [1–4].

Thus, three possible separate, but non-mutually exclusive, mechanisms of SUMO mediated repression of SOX6 can be envisioned. Directly, through disruption of the transcriptional activation complex with SOX9 or by recruitment of co-repressor to the sumoylated complex and/or indirectly, by sequestration of SOX6 to PML bodies, thus removing it from transcriptionally active sites. As the role of SUMO in the chondrocytic differentiation program has not been previously addressed, the identification of SOX6 as a substrate for SUMO modification, suggest the possibility that sumoylation may be relevant for regulation of chondrocyte differentiation.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.01.031.

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