SUMO Represses Transcriptional Activity of the *Drosophila* SoxNeuro and Human Sox3 Central Nervous System–specific Transcription Factors[□]

Jean Savare, Nathalie Bonneaud, and Franck Girard

Institut de Génétique Humaine, Centre National de la Recherche Scientifique Unité Propre de Recherche 1142, 34396 Montpellier, France

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Sry high mobility group (HMG) box (Sox) transcription factors are involved in the development of central nervous system (CNS) in all metazoans. Little is known on the molecular mechanisms that regulate their transcriptional activity. Covalent posttranslational modification by small ubiquitin-like modifier (SUMO) regulates several nuclear events, including the transcriptional activity of transcription factors. Here, we demonstrate that SoxNeuro, an HMG box-containing transcription factor involved in neuroblast formation in *Drosophila*, is a substrate for SUMO modification. SUMOylation assays in HeLa cells and *Drosophila* S2 cells reveal that lysine 439 is the major SUMO acceptor site. The sequence in SoxNeuro targeted for SUMOylation, IKSE, is part of a small inhibitory domain, able to repress in *cis* the activity of two adjacent transcriptional activation domains. Our data show that SUMO modification represses SoxNeuro transcriptional activity in transfected cells. Overexpression in *Drosophila* embryos of a SoxN form that cannot be targeted for SUMOylation strongly impairs the development of the CNS, suggesting that SUMO modification of SoxN is crucial for regulating its activity in vivo. Finally, we present evidence that SUMO modification of group B1 Sox factors was conserved during evolution, because Sox3, the human counterpart of SoxN, is also negatively regulated through SUMO modification.

INTRODUCTION

Genes of sry high mobility group (HMG) box (Sox) family encode highly conserved transcription factors, involved in regulating numerous cell fate decisions during development. All Sox proteins share a conserved DNA binding domain (DBD), the HMG domain (Wegner, 1999). Phylogenetic analyses focusing on both HMG domain sequence and motifs outside led to the distinction of several subgroups, A to G (Bowles *et al.*, 2000). Studies in several organisms, including *Drosophila*, *Xenopus*, chick, and mouse, have revealed that group B Sox genes (Sox1/2/3 in vertebrates and their homologues SoxNeuro and Dichaete in *Drosophila*) are all expressed in the developing central nervous system (CNS) from the earliest stages of neurogenesis onwards (for review, see Savare and Girard, 2002). These genes were shown to play fundamental roles in neural induction, maintenance of the neural phenotype, and maintenance of the identity of the neural precursors (Mizuseki *et al.*, 1998; Bylund *et al.*, 2003; Graham *et al.*, 2003). In *Drosophila*, SoxNeuro (SoxN) expression in the neuroectoderm is regulated by the Dorsal and Dpp pathways (Crémazy *et al.*, 2000;

Stathopoulos *et al.*, 2002). Loss of function mutations of SoxN are responsible for severe hypoplasia in the embryonic CNS, resulting from a drastic loss of neuroblasts in the lateral and intermediate regions of the neuroectoderm (Buescher *et al.*, 2002; Overton *et al.*, 2002). The target genes regulated by these Sox, and the molecular mechanisms that regulate their activity, are largely unknown.

The activity of transcription factors is regulated by several posttranslational modifications, including phosphorylation, acetylation, or ubiquitylation. Covalent modification by small ubiquitin like modifier (SUMO) has recently emerged as an important posttranslational modification involved in regulating several nuclear events, including transcription, nucleocytoplasmic shuttling, DNA replication and repair, chromosome dynamics, localization into discrete nuclear structures (for review, see Gill, 2003, 2004; Seeler and Dejean, 2003; Verger *et al.*, 2003; Girdwood *et al.*, 2004; Johnson, 2004). SUMO modification of transcription factors often has been associated with transcriptional repression, although the precise molecular mechanisms underlying this regulation are still a matter of debate (Gill, 2004; Girdwood *et al.*, 2004). SUMOs are highly conserved in all eukaryotes. Whereas invertebrates contain only one SUMO, known as Smt3, four types of SUMO exist in vertebrates, SUMO1/2/3 and a recently identified SUMO4. SUMO modification (SUMOylation) consists in the covalent attachment of SUMO to a lysine residue in the protein substrate, at a consensus site ΨKXE (where Ψ is a hydrophobic residue, either isoleucine, valine, or leucine) (Rodriguez *et al.*, 2001; Sampson *et al.*, 2001). Two enzymatic activities are involved in SUMO attachment: an E1 activating enzyme and an E2 conjugating enzyme known as Ubc9. A third class of enzymes was further identified, E3, that is not absolutely required for SUMO attachment in vitro but that might enhance specific-

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Address correspondence to: Franck Girard (fgirard@igh.cnrs.fr).

Abbreviations used: DBD, DNA binding domain; HMG, high mobility group; Sox, sry HMG box; SoxN, SoxNeuro; SUMO, small ubiquitin-like modifier; TAD, transactivation domain.

ity to the substrate *in vivo*. Several classes of SUMO E3 were discovered, including members of the PIAS family, RanBP2, and the polycomb group protein Pc2 (Sachdev *et al.*, 2001; Kagey *et al.*, 2003; Pichler *et al.*, 2004). These enzymes were shown to enhance SUMO attachment *in vitro* and to target the substrate protein to particular nuclear compartments *in vivo*, such as promyelocytic leukemia (PML) bodies, nuclear pore, or polycomb bodies. SUMO modification is a reversible process, catalyzed by SUMO cleaving enzymes of the Ulp family (for recent review, see Johnson, 2004).

How Sox factor transcriptional activity is regulated still remains poorly understood. Sox often pair off with specific partners, leading to synergistic and context-dependent transcriptional regulation (for review, see Kamachi *et al.*, 2000). In Sox9, several posttranslational events were shown to be essential for its activity, including protein kinase A-dependent phosphorylation (Huang *et al.*, 2000), SUMO modification (Komatsu *et al.*, 2004), and regulation of nucleocytoplasmic shuffling (Gasca *et al.*, 2002). In this report, we present evidence that SoxNeuro is SUMO modified, both in *Drosophila* S2 cells and in HeLa cells. Lysine acceptor site maps within an inhibitory domain surrounded by two adjacent transactivation domains. SUMO modification of SoxN is associated with transcriptional repression. Our data also show that Sox3, the human counterpart of SoxN, is similarly regulated by SUMO modification. Finally, overexpression in *Drosophila* embryos of a SoxN form in which the SUMO acceptor lysine was mutated to arginine perturbs CNS development, strongly suggesting that the regulation of SoxN activity through SUMO modification is essential for CNS development.

MATERIALS AND METHODS

Plasmid Constructs

pGlomyc-SoxN was described previously (Bonneaud *et al.*, 2003). Lysine 439 and 375 were mutated to arginine in pGlomyc-SoxN and pSG424-Sox3, respectively, by using PCR-based site-directed mutagenesis (Stratagene, La Jolla, CA), and confirmed by sequencing. SoxN and human Sox3, and deletion mutants, were cloned in frame with GAL4 DNA binding domain in pSG424 (kindly provided by P. de Santa Barbara, Institut de Génétique Humaine, Montpellier, France), by using *KpnI-XbaI* PCR fragments. Details of the primers used are available upon request. pMK26-FlagHA-SoxN wild-type and K439R, and pUAS-SoxNK439R, were constructed as follows. An *EcoRI* fragment from pGlomyc-SoxN (wild-type and K439R) was first cloned in pcDNA-FlagHA (kindly provided by F. Poulat, Institut de Génétique Humaine, Montpellier, France) *EcoRI*-digested. The FlagHA-SoxN cassette was then removed by *HindIII/NotI* digestion, filled in with Klenow, and cloned in pMK26 (Act5C promoter) (kindly provided by A. Pélissou, Institut de Génétique Humaine, Montpellier, France) *EcoRV*-digested, and in pUAS. pcDNA-6His-SUMO2 and pcDNA-Ubc9 were provided by R. Hay (University of St. Andrews, St. Andrews, Scotland, United Kingdom). pSG5-6His-SUMO1 was provided by A. Dejean (Institut Pasteur, Paris, France). pPac-Smt3 and pPac-Ubc9 were provided by A. Courey (University of California, Los Angeles, CA). pcDNA-DN Ubc9 (C93S substitution) was provided by A. Sharrocks (University of Manchester, Manchester, United Kingdom).

Antibodies

Mouse anti-myc (Tebu, Le Perray en Yvelines, France), mouse anti-FLAG (Sigma-Aldrich, St. Louis, MO), mouse anti-hemagglutinin (HA) (Roche Diagnostics, Indianapolis, IN), horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse (Amersham Biosciences, Piscataway, NJ), mouse anti-GAL4 DBD (Ozyme, St. Quentin en Yvelines, France), goat anti-Sox3 (Tebu), anti-mouse and anti-goat Alexa Fluor 568 (Molecular Probes, Eugene, OR), Cy3-conjugated mouse anti-HRP (Jackson ImmunoResearch Laboratories, West Grove, PA), monoclonal anti-Fasciclin II (FasII) (Developmental Studies Hybridoma Bank, Iowa City, IA), and biotinylated goat anti-mouse and anti-rabbit (Amersham Biosciences) were all purchased commercially and used according to the manufacturer's instructions. Affinity-purified rabbit anti-SoxN was described previously (Crémazy *et al.*, 2001). In Figure 6, anti-FasII and SoxN primary antibodies were detected with biotinylated secondary antibody and the ABC Elite kit (Vectastain; Vector Laboratories, Burlingame, CA). Whole mount embryo immunostainings were performed

on formaldehyde-fixed *Drosophila* embryos by using standard protocols (Ashburner, 1989).

Cell Culture, Transfection, Immunostaining, and Luciferase Assay

HeLa cells were cultured at 37°C in DMEM supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics (penicillin and streptomycin) (Invitrogen, Carlsbad, CA). Cells were transiently transfected with jetPEI (Qbiogene, Illkirch, France) according to the manufacturer's instructions. *Drosophila* S2 cells were cultured at room temperature in SF900-II medium with glutamine supplemented with antibiotics (penicillin and streptomycin) (Invitrogen). S2 cells were transiently transfected with Effectene (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

For immunostainings, HeLa cells were fixed 18–24 h after transfection in 3.7% formaldehyde for 5 min followed by permeabilization in phosphate-buffered saline (PBS), 0.2% Triton for 10 min. S2 cells were plated on glass coverslips 48 h after transfection. Twenty-four hours later, cells were fixed in the same conditions. Cells were preincubated with PBS containing 1% bovine serum albumin and processed for immunostaining with either anti-myc or anti-FLAG, in HeLa and S2 cells, respectively, and Alexa Fluor 568 goat anti-mouse. The procedure was identical for HeLa cells transfected with pSG424-Sox3 derivatives, which were detected with goat anti Sox3/Alexa Fluor 568 anti-goat.

For luciferase assays, HeLa cells were transfected with pG5-Luc (reporter plasmid containing five multimerized Gal4 binding sites upstream of luciferase) (Promega, Madison, WI), pRL-SV40 (Promega) as internal control, and pSG424 effector plasmids. Luciferase activities were monitored 18–24 h after transfection using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. The firefly luciferase activities were normalized with *Renilla* luciferase activities from pRL-SV40.

SUMOylation Assays

HeLa cells were transfected with various combinations of plasmids (1.5 µg each, unless specified in the text, for a total amount of 7.5 µg of DNA), as indicated in figure legends. 6His-tagged SUMO-conjugated products were isolated and analyzed as follows. Eighteen to 24 h after transfection, cells were washed twice in PBS and lysed in 500 µl of lysis buffer (6 M guanidium chloride, 100 mM Na₂HPO₄, pH 8, 10 mM imidazole, and protease inhibitor cocktail). Extracts were then sonicated (8 pulses, 75 W), centrifuged, and the supernatant was subjected to nickel-agarose precipitation (QIAGEN) for 4 h at 4°C. After extensive washes (2 washes with 1 ml of lysis buffer, 2 washes with 1 ml of lysis buffer diluted 5 times in 50 mM Tris, pH 7.5 and containing 20 mM imidazole, and 2 washes with 1 ml 50 mM Tris, pH 7.5, containing 20 mM imidazole), samples were recovered in Laemmli buffer supplemented with 200 mM imidazole. Protein samples were resolved by SDS-PAGE and immunoblotted with anti-myc. For human Sox3, SUMOylation assay was conducted as described for SoxN, except that samples were immunoblotted with anti-GAL4 DBD to detect GAL4 DBD-Sox3 fusion proteins.

SUMOylation in *Drosophila* S2 cells was analyzed by immunoprecipitation. Cells were transfected with wild-type or K439R FLAG-HA-tagged SoxN and components of the SUMO machinery (Smt3 and Ubc9). Cells were lysed in lysis buffer (120 mM NaCl, 50 mM Tris, pH 8, 5 mM EDTA, 0.5% NP-40, 1 mM dithiothreitol, and protease inhibitors) containing 50 mM N-ethyl maleimide and incubated overnight at 4°C with anti-FLAG M2 affinity gel (Sigma-Aldrich). After two washes in lysis buffer and one in washing buffer (300 mM NaCl, 50 mM Tris, pH 7.5, 0.1% SDS, and 0.1% sodium deoxycholate), samples were resuspended in Laemmli buffer, resolved by SDS-PAGE, and immunoblotted with anti-HA to detect SoxN.

Fly Stocks

All *Drosophila* stocks were maintained on standard yeast-cornmeal medium at 25°C. Oregon R was used as wild-type stock. The following fly lines were used: pUAS SoxN (Blanco *et al.*, 2005), da-GAL4 (Bloomington Stock Center, Indianapolis, IN), and ey-GAL4 (a gift of W. Gehring, Biozentrum, Basel, Switzerland). The pUAS-SoxN K439R stock was obtained by P element-mediated germline transformation by using standard protocols (Karess, 1985).

RESULTS

SoxNeuro Is SUMO Modified in Cultured Cells

The ΨKXE motif in substrate proteins, in which lysine is targeted for SUMOylation, was shown to be the binding site for the Ubc9 E2 ligase (Rodriguez *et al.*, 2001; Sampson *et al.*, 2001). The SUMO pathway is functionally conserved in vertebrates and invertebrates. Indeed, it was shown that the *Drosophila* dSmt3 conjugation system could efficiently recognize and modify human SUMO1 substrates (Sapetschnig *et al.*, 2002). Thus, because SoxN sequence contains a consen-

Figure 1. SoxN is SUMO modified on lysine 439 in transfected HeLa and S2 cells. HeLa cells were transfected with the indicated plasmids (1.5 μg each, except in C where 0.75 μg of both myc-SoxN and 6His-SUMO2 were used, and in A and D where the quantities of 6His-SUMO1 and DN-Ubc9 are indicated). For SUMOylation assay, cell extracts were incubated with Ni-NTA resin to bind 6His-tagged SUMO-modified proteins. Samples were then resolved by SDS-PAGE, and immunoblotted with anti-myc to detect SoxN. (A) SoxN is SUMO modified both with SUMO1 and SUMO2. Note that SUMO1-modified SoxN is visible only when increasing the quantity of transfected 6His-SUMO1. Controls included myc-SoxN, 6His-SUMO1 or 6His-SUMO2 transfected alone. Arrowhead points to SUMO modified SoxN. Note that unmodified SoxN is bound by the resin, because it contains a stretch of five histidines (marked with an asterisk). (B and C) Cotransfection of the E2 SUMO ligase Ubc9 does not significantly increase the quantity of SUMO2-modified SoxN bound to the Ni-NTA resin (B), whereas cotransfecting a dominant negative form of Ubc9 significantly inhibits SoxN SUMOylation (C). (D) SUMOylation assay in HeLa cells was performed as described above, with 6His-SUMO2 cotransfected with wild-type myc-SoxN or a mutated version in which lysine 439 in the IKSE motif was mutated to Arginine. K439R SoxN is not SUMO modified, showing that lysine 439 is the major SUMO acceptor site. In all cases, total cell extracts were immunoblotted with anti-myc to control the levels of SoxN expression in each experimental condition. Molecular weights are given in kilodaltons. (E) *Drosophila* S2 cells were transfected with wild-type or K439R FLAG-HA-tagged SoxN, in the presence or absence of Smt3 and *Drosophila* Ubc9. Cell extracts were immunoprecipitated with anti-FLAG and immunoblotted with anti-HA. A SUMO-modified SoxN (marked by arrowhead) is detected and is absent in the K439R SoxN mutant. Asterisk marks the position of SoxN. Molecular weights are given in kilodaltons. Note that a SUMO-modified SoxN form is detected even in conditions where Smt3/Ubc9 are not overexpressed.

sus site for SUMO modification, IKSE, in which lysine 439 could be potentially targeted, we used HeLa cells as a paradigm to analyze SoxN SUMOylation. Our assay consisted in transfecting cells with myc-tagged SoxN, together with 6His-tagged SUMO1 or SUMO2. Cell extracts were prepared under denaturing conditions to inhibit SUMO protease activities, and incubated with nickel-nitrilotriacetic acid (Ni-NTA) resin, to specifically bind the 6His-tagged, SUMO-modified proteins. Samples were then probed by Western blot for the presence of myc-SoxN. Because SoxN contains in its sequence a stretch of five histidines (amino acid position 58–62), it was able to bind efficiently to the resin when myc-SoxN plasmid was transfected alone (Figure 1A). Cotransfecting 6His-SUMO1 or 6His-SUMO2 resulted in the purification of a slower migrating, SUMO-modified, SoxN form. The stoichiometry of this SUMO-modified SoxN form was directly proportional to the quantity of SUMO (1 and 2) plasmid transfected. In SUMO1, because it was apparently less expressed, it was necessary to increase the quantity of transfected 6His-SUMO1 plasmid to detect SUMO-modified SoxN (Figure 1A, right). Cotransfecting Ubc9, the SUMO E2 ligase, did not significantly increase the ratio of SUMO-modified SoxN, likely because HeLa cells contains sufficient amounts of endogenous Ubc9 (Figure 1B). Finally, we use a dominant negative form of Ubc9 (DN-Ubc9), able to interfere with the endogenous SUMO path-

way (Yang *et al.*, 2003) and analyzed SoxN SUMOylation. We observed that cotransfecting increasing quantities of DN-Ubc9 together with SoxN and 6His-SUMO2 resulted in a significant decrease in SUMO modification of SoxN (Figure 1C). Collectively, these data demonstrate that SoxN is efficiently SUMO modified in transfected HeLa cells, both with SUMO1 and SUMO2.

Lysine 439 in the IKSE SUMO consensus site was mutated to arginine, and tested for SUMO modification using the same assay. As shown in Figure 1D, no SUMO modified SoxN form was detected when K439R mutant was transfected in HeLa cells in the presence of 6His-SUMO2. The same result was obtained when SUMO1 was used instead of SUMO2, and when Ubc9 was cotransfected together with SUMO1 or SUMO2 (our unpublished data). This result showed that Lysine 439 within the IKSE motif is the major SUMO acceptor site in SoxN.

We next analyzed whether SoxN was also SUMO modified in cultured *Drosophila* S2 cells. Reverse transcription-PCR and immunostaining experiments revealed that these cells contained no detectable levels of SoxN RNA and protein. The SUMOylation assay consisted in transfecting FLAG-HA tagged SoxN, together with components of the SUMO machinery (Smt3 and *Drosophila* Ubc9). Cell lysates were immunoprecipitated with anti-FLAG, and immunoblotted with anti-HA to detect SoxN. A SUMO-modified

Figure 2. Mapping the domains responsible for SoxN transcriptional activity. Shown is a schematic representation of SoxN sequence. The following domains are highlighted: DNA binding domain (HMG box), alanine repeats (Ala1 and Ala2), glutamine-rich C-terminal regions, and the SUMO acceptor lysine K439. Various domains of SoxN were fused in frame with GAL4 DBD, and the resulting constructs were tested in HeLa cells for their ability to activate a reporter construct containing multiple GAL4 binding sites monitoring luciferase activity. Cells were transfected with 2 μ g of pEG5-Luc reporter, 20 ng of pRLSV40, and 200 ng of pSG24-SoxN derivatives. Results are given as a ratio to the control (GAL4 DBD, taken as 1). In each case, the expression of the transfected construct was monitored by Western blot with anti-GAL4. Left, schematic representation of the different constructs tested. Shown are representative experiments, with each point done at least in triplicate (SEs are shown).

SoxN form was detected at the expected molecular size when wild-type SoxN was overexpressed in S2 cells, and this form was absent when K439R mutant was transfected (Figure 1E). This experiment shows that SoxN is SUMO modified in *Drosophila* and confirmed that K439 is the major SUMO acceptor site. The SUMO-modified SoxN form was detected in extracts prepared from S2 cells transfected with FLAG-HA-SoxN alone (Figure 1E). This suggested that even when the components of the SUMO machinery were not overexpressed, SoxN was endogenously SUMO modified and that SoxN SUMO modification does not simply result from Smt3/Ubc9 overexpression.

Mapping Activation and Repression Domains in SoxNeuro

Because SUMO modification has been associated with transcriptional regulation, we first examined which domains in SoxN protein were responsible for intrinsic transcriptional activity. For this purpose, full-length SoxN and various deletion constructs were fused to the GAL4 DBD, and the resulting derivatives were tested for their ability to activate in HeLa cells a reporter construct containing multimerized GAL4 binding sites upstream of a luciferase gene. Several domains are present in SoxN: the HMG box (DBD), two alanine stretches (amino acid position 306–317 and 471–484) surrounding the SUMO acceptor lysine at position 439, and a C-terminal glutamine rich region (Figure 2). Constructs containing the HMG box showed repressive activity toward the reporter gene compared with the GAL4 DBD construct

(Figure 2A). These include full-length construct 182–573 (deletion of the region N-terminal to the HMG box), construct 1–258 (deleted of the region C-terminal to the HMG box), and construct 180–258 (containing the HMG box alone). This suggests that apart from its function in DNA binding and DNA bending, HMG box also might have a repressive effect on transcription. Alternatively, one cannot exclude that SoxN HMG box is titrated by endogenous Sox consensus DNA binding sites, resulting in lower levels of GAL4 DBD-SoxN fusion proteins available to activate the reporter construct. In marked contrast, construct 256–573, containing the region C-terminal to the HMG box, exhibited a luciferase activity severalfold higher than the control (5- to 10-fold higher, depending on the experiment), suggesting that this region contains potential domain(s) responsible for transcriptional activation (Figure 2A). Immunoblot with anti-GAL4 revealed that all constructs were expressed at similar levels (Figure 2A), showing that the differences in luciferase activity reflect intrinsic properties of the SoxN derivatives and are not due to changes in expression levels.

The C-terminal region was further deleted, and the corresponding constructs were monitored for their transcriptional activity (Figure 2B). Deleting the region between the HMG box and the SUMOylation site (constructs 321–573 and 425–573) significantly reduced the transcriptional activity. Deleting the C-terminal domain up to the SUMOylation site (constructs 256–499 and 256–469) resulted in a similar loss of transcriptional activation. This suggests that SoxN

Figure 3. SUMO modification has a repressive role on SoxN-mediated transcriptional activity. (A) Cells were transfected with 2 μ g of pEG5-Luc reporter, 20 ng of pRLSV40, and 200 ng of pSG24-SoxN derivatives. Several GAL4 DBD-SoxN fusions were tested: wild-type and K439R full-length SoxN, wild-type, and K439R C-ter SoxN (amino acids 256–573). Luciferase assay in HeLa cells was done as described in Figure 2. Relative luciferase activity is given as a ratio to control (GAL4 DBD, taken as 1). Each point was done at least in triplicate (SEs are shown). In each case, the expression of the transfected construct was monitored by Western blot with anti-GAL4 DBD. (B) Cells were transfected with 1 μ g of pEG5-Luc reporter, 10 ng of pRLSV40, 100 ng of pSG24-SoxN256–573, and increasing quantities of DNUbc9 as indicated. Relative luciferase activity is given as a ratio to control (GAL4 DBD-SoxN256–573 in the absence of DNUbc9, taken as 1). Shown is a representative experiment, with each point done in triplicate (SEs are shown).

contains two regions important for transcriptional activation, both containing alanine repeats. Constructs 256–469, 256–434, 425–573 and 442–573 were used to study the importance of the SUMOylation site in SoxN transcriptional activity. We observed that the removal of the IKSE motif in these constructs led to a dramatic increase in transcriptional activity. Indeed, whereas constructs 256–469 and 425–573 exhibited, respectively, luciferase activity 1.1- and 2.3-fold higher than the control, the same constructs deleted of the SUMO motif displayed luciferase activity 42- and 22.5-fold higher than the control, respectively. These results show that the region 434–442 containing the IKSE motif exerts an inhibitory effect *in cis* on the two adjacent transcriptional activation domains (TADs), localized at positions 256–434 and 442–573.

SUMO Modification Represses SoxN Transcriptional Activity

The presence of the IKSE motif in the SoxN inhibitory domain prompted us to examine more directly the effect of SUMO modification on SoxN transcriptional activity. For this purpose, HeLa cells were transfected with wild-type and K439R SoxN proteins fused to GAL4 DBD, and luciferase activity was monitored as described above. Full-length SUMO-deficient SoxN (K439R) showed a threefold higher transcriptional activity compared with wild type. Mutating lysine 439 in the C-ter construct (amino acids 256–573) had dramatic effect on SoxN transcriptional capacity. Indeed, although wild-type C-ter construct induced a fourfold transcriptional activation, its K439R mutated counterpart exhibited a 150-fold transcriptional activation (Figure 3A). Immunoblot with anti-GAL4 confirmed that all four constructs were expressed at similar levels, showing that the differences observed in luciferase activity were not due to differ-

ences in SoxN protein levels. These results demonstrate that SUMO modification of lysine 439 within the inhibitory domain plays a repressive role in SoxN mediated transcriptional activity.

We also examined SoxN transcriptional activity when DNUbc9 was cotransfected to diminish endogenous SUMO machinery activity. We observed a dose-dependent increase in SoxN transcriptional activity, with relative luciferase activity ranging from 1 when SoxN was transfected alone to 5.6 when DNUbc9 was coexpressed (Figure 3B). Hence, DNUbc9 was effective in inhibiting SUMO modification of SoxN (Figure 1C), which resulted in increased SoxN activity (Figure 3B). By contrast, we found that cotransfecting DNUbc9 up to 2 μ g had little or no effect on the luciferase activity of the GAL4 DBD alone (Figure 4D). Together, these two experiments show that SoxN SUMO modification represses its transcriptional activity.

Conservation of SUMO Modification in Mammalian Group B Sox Proteins

In mammals, three group B Sox proteins, Sox1, Sox2, and Sox3, are structurally and functionally homologous to SoxN (for review, see Wegner, 1999; Bowles *et al.*, 2000). Closer examination of their sequence revealed that all three proteins contain a Ψ KXE motif located in their C-terminal region (Figure 4A). In these three proteins, C-terminal region was shown to contain sequences necessary for transcriptional activity (Bowles *et al.*, 2000, and references therein). We used SUMOylation assay in HeLa cells to determine whether human Sox3 was SUMO modified. Cotransfecting 6His-SUMO1 (Figure 4C, left) or 6His-SUMO2 (right) together with GAL4 DBD-Sox3 C-ter resulted in the purification of a slowly migrating SUMO-modified band, which was not detected when lysine 375 in the putative SUMO acceptor

Figure 4. SUMO modification of human Sox3. (A) Alignment of C-terminal region of group B Sox factors, *Drosophila* SoxN, and human Sox1, Sox2, and Sox3, highlighting the conserved ΨKXE motif. (B) Schematic representation of human Sox3 and GAL4 DBD derivative, highlighting several domains: HMG box DBD, Ala repeats, and VKSE motif. (C) SUMOylation assay in HeLa cells. Cells were transfected with GAL4DBD-Sox3 derivatives 216–447 (wild type and K375R), in the presence or absence of plasmids expressing Ubc9, 6His-SUMO1/2, and DN Ubc9 as indicated. Top, cell extracts were incubated with Ni-NTA to bind 6His-tagged, SUMO-modified proteins. Samples were then resolved by SDS-PAGE and immunoblotted with anti-GAL4 DBD to detect Sox3. Bottom, total cell extracts were immunoblotted with anti-GAL4 DBD to monitor expression levels of the various Sox3 derivatives. (D) SUMO modification impairs Sox3 transcriptional activity, as assessed by cotransfecting DN Ubc9 (D) or mutating K375 to R (E). Luciferase assay was performed as described in Figure 2. Relative luciferase activity is given as a ratio to control (D, GAL4 DBD in the absence of DN Ubc9, taken as 1; E, GAL4 DBD taken as 1). Shown are representative experiments, with each point done in triplicate (SEs are shown). Western blot with anti-GAL4 DBD shows that all proteins are expressed at similar levels.

site was mutated to arginine. As already observed for SoxN, DN Ubc9 when cotransfected in the cells is able to significantly impair Sox3 SUMO modification (Figure 4C). This demonstrated that lysine 375 is the major SUMO acceptor site in human Sox3.

As shown previously, SoxN contains two TADs, in which long stretches of alanine repeats are present ($n = 12$ in TAD1 and $n = 14$ in TAD2). Similarly, human Sox3 contain four alanine repeats ($n = 15, 7, 6$, and 10), all being localized N-terminally to the VKSE SUMOylation motif (Figure 4B). We monitored luciferase activity of GAL4 DBD-Sox3 216–447 derivative, in the absence or presence of DN Ubc9 (Figure 4D). Sox3 displayed a moderate transcriptional activity (2-fold over the GAL4 DBD alone), which was increased when the endogenous SUMO machinery was challenged with DN Ubc9 cotransfection (4-fold over the GAL4 DBD alone). By contrast, the activity of the GAL4 DBD was similar in the absence or presence of DN Ubc9. Finally, the activity of the K375R Sox3 mutant was compared with its

wild-type counterpart (Figure 4E). As already observed for SoxN, the single K-to-R mutation in the SUMO acceptor site resulted in a significant increase in transcriptional activity (23-fold higher than the GAL4 DBD alone, and 9-fold higher than its wild-type counterpart). Collectively, these data show that human Sox3 is SUMO modified on lysine 375 in HeLa cells and that Sox3 SUMO modification is associated with transcriptional repression.

SUMO Modification Is Not Associated with Changes in SoxN and Sox3 Cellular Localization

SUMO modification has been shown to regulate subcellular localization of several targets, including transcription factors, although it does not seem to be a general mechanism by which SUMO affects transcription. For example, SUMO was shown to be involved in nuclear import/export, targeting to the nuclear pore and to discrete nuclear compartments such as PML bodies (for review, see Seeler and Dejean, 2003; Gill, 2004). In transfected HeLa cells, myc-tagged SoxN is found

Figure 5. SUMO modification is not associated with changes in Sox nuclear localization in transfected HeLa and S2 cells. HeLa and S2 cells were transfected with the indicated plasmids and immunostained with either anti-myc or anti-FLAG to detect SoxN. Similar nuclear localization was observed in all the conditions tested: wild-type SoxN, K439R mutated SoxN, in the presence of SUMO1/2 and Smt3, or when the endogenous SUMO machinery was perturbed by DNUbc9 cotransfection. Similarly, GAL4 DBD-Sox3 C-ter wild-type and K375R derivatives showed comparable nuclear localization.

exclusively in the nucleus, localized at many punctuate sites, and with higher staining found at the nuclear membrane (Figure 5). We found that this nuclear pattern was not modified in the following conditions: when SUMO acceptor lysine was mutated to arginine, when SUMO1/2 were cotransfected with SoxN, or when the endogenous SUMO pathways was challenged with DNUbc9 transfection (Figure 5). Similar conclusions were reached when comparing SoxN nuclear distribution in *Drosophila* S2 cells with its K439R counterpart or when cotransfecting Smt3 (Figure 5). Finally, we monitored the distribution of GAL4 DBD-Sox3 C-ter wild-type and K375R de-

rivatives with anti-Sox3 antibody and observed similar nuclear distribution for the wild-type and the SUMO-deficient K375R form (Figure 5). Collectively, these data implies that SUMO modification of SoxN and Sox3 is not associated with major redistribution of the proteins to specific subnuclear compartments in transfected cells.

Overexpression of SoxN K439R Perturbs *Drosophila* CNS Development

To analyze the impact of SUMOylation on SoxN function in vivo, we compared the phenotypic consequences of overex-

Figure 6. Overexpression of SUMO-deficient K439R SoxN strongly impairs embryonic CNS development. (A–C) Adult phenotypes resulting from the ectopic expression of SoxN K439R (A) or SoxN wild type (B and C) in the developing eye. (D and H) Overexpression of SoxN K439R results in strong defects in embryonic CNS. Whole mount immunostainings for HRP (D–F) and Fasciclin II (G and H) of stage 16 embryos of the following genotypes: (D) Oregon R, (E, G) da-GAL4; UAS SoxN, and (F, H) da-GAL4; UAS SoxN K439R. (I–K) SoxN immunostaining in wild-type stage 9 embryos (I), da-GAL4; UAS SoxN K439R stage 10 (J), and da-GAL4; UAS SoxN stage 9 (K).

Table 1. Potential SUMOylation sites in human and *Drosophila* Sox proteins

Species ^a	Gene	Group ^b	ΨKXE motif ^c	SC motif consensus ^d	Accession no.
H.s	Sry	A	No		NM_003140
H.s	Sox1	B1	K318		N_005986
H.s	Sox2	B1	K245		N_003106
H.s	Sox3	B1	K375	P-X(4)-VKSE-X(0)-P	N_005634
D.m	SoxN	B1	K439	P-X(4)-IKSE-X(0)-P	SPTREMBL:Q9U1H5
H.s	Sox14	B2	No		N_004189
H.s	Sox21	B2	No		N_007084
D.m	Dichaete	B2	No		SWP:Q24533
D.m	SoxB2-2	B2	K368		SPTREMBL:Q9VUD3
D.m	SoxB2-3	B2	No		SPTREMBL:Q9VUD1
H.s	Sox4	C	No		N_003107
H.s	Sox11	C	K240		N_003108
H.s	Sox12	C	No		N_006943
D.m	SoxC	C	K336		
H.s	Sox5	D	K156, K723		N_006940
H.s	Sox6	D	K404	P-X(2)-IKNE-X(5)-P	N_033326
			K417	P-X(3)-VKDE-X(4)-P	
H.s	Sox13	D	K25		N_005686
D.m	Sox D	D	No		SPTREMBL:Q8STH7
H.s	Sox8	E	K245	P-X(1)-VKSE-X(0)-P	N_014587
			K339	P-X(1)-IKTE-X(0)-P	
H.s	Sox9	E	K61		N_000346
			K254	P-X(4)-LKRE-X(2)-P	
			K398		
H.s	Sox10	E	K55, K357		N_006941
D.m	Sox100B	E	K28		SPTREMBL:Q9VA16
H.s	Sox7	F	No		N_031439
H.s	Sox17	F	K45		N_022454
H.s	Sox18	F	No		N_018419
D.m	SoxF	F	No		SWP:P40657
H.s	Sox15	G	No		N_006942
H.s	Sox30	H	K74		N_007017
			K167	P-X(5)-VKLE-X(1)-P	
			K213		
			K307	P-X(1)-VKIE-X(4)-P	

^a D.m, *Drosophila melanogaster*; H.s, *Homo sapiens*.^b Sox groups as determined in Bowles *et al.* (2000).^c Amino acid position of the acceptor lysine.^d See Komatsu *et al.* (2004). Assigned here as P-X(0-5)-ΨKXE-X(0-5)-P.

pressing either wild-type SoxN or the K439R SUMO deficient form during *Drosophila* development. For this purpose, we used the UAS-GAL4 system to drive the expression of both proteins in larval imaginal discs. Although overexpressing wild-type SoxN results in severe defects in the formation of adult structures (including defects in wings, thorax, and legs), it was impossible to obtain adults from the SoxN K439R-overexpressing larvae, because this overexpression was lethal during larval and/or early pupal periods (our unpublished data). When SoxN K439R expression was driven in the eye imaginal discs by using ey-GAL4, it was nevertheless possible to collect few adult escapers from the pupal case, showing a headless phenotype (Figure 6A). By contrast, overexpression of wild-type SoxN with ey-GAL4 led to a much milder phenotype, with reduction or complete absence of the eye structures (Figure 6, B and C). These phenotypes could result from interference of SoxN ectopic expression with the development pathway initiated by eyeless in the eye imaginal discs, a phenomenon already observed when driving the expression of several transcription factors in the developing eye and called developmental pathway interference (Jiao *et al.*, 2001). These results sug-

gested that wild-type and SUMO-deficient SoxN behave differently when overexpressed in *Drosophila* and prompted us to examine the effects of their overexpression on embryonic CNS development. For this purpose, the da-GAL4 driver line was used, which results in ubiquitous expression starting at embryonic stage 8. Ubiquitous K439R SoxN expression resulted in lethality at the end of embryogenesis, with severe defects in the CNS as observed with HRP (Figure 6, D–F) and Fasciclin II stainings (Figure 6, G and H). Indeed, defects included fusion or absence of commissures (Figure 6F), reduction or absence of longitudinal axon tracts (Figure 6F) and strong perturbation of the regular axonal fasciculation pattern as seen with FasII staining (Figure 6H). In marked contrast, overexpressing the wild-type SoxN form had no detectable effect on embryonic CNS development (Figure 6, E and G). Immunostaining with anti-SoxN antibodies in these embryos revealed that both proteins were expressed at similar levels, and both localized in the nuclei (Figure 6, I–K). Collectively, these data show that SUMO modification is important for regulating the activity of SoxN in vivo and strongly suggest that SUMO modification of SoxN is involved during CNS development.

DISCUSSION

In this report, we show that the *Drosophila* SoxN transcription factor and its human counterpart Sox3, both involved in CNS development, are SUMO modified *in vivo*. We searched for potential SUMOylation sites (Ψ KXE motif) in all mammalian and *Drosophila* Sox proteins (Table 1). One or several Ψ KXE motifs are present in some but not all Sox genes, these motifs being usually conserved within a given subgroup between *Drosophila* and humans. These include group B1 (H.s Sox1/2/3 and D.m SoxN), group C (H.s Sox11 and D.m SoxC), group D (H.s Sox5/6/13), group E (H.s Sox8/9/10 and D.m Sox100B), group F (H.s Sox17), and group H (H.s Sox30). Recently, Sox9 was shown to be SUMO modified, and SUMO modification was associated with transcriptional repression (Komatsu *et al.*, 2004). In all the other groups (B2, F, and G), no Ψ KXE motif is present (except *Drosophila* SoxB2-2, human group C Sox11 and group F Sox17), suggesting that these proteins are not SUMO modified. To confirm this, we used the same SUMOylation assay as described in this report for SoxN and Sox3, and were unable to detect SUMO modified human Sox7, mouse Sox15 and *Drosophila* Dichaete (respectively, group F, G, and B) (our unpublished data). Thus, based on our data and that of Komatsu *et al.* (2004) and the presence of Ψ KXE motif in various Sox, one can postulate that SUMO modification might be used to regulate several Sox group genes.

Our results show that SUMO modification of the CNS-specific group B1 SoxN and Sox3 proteins was conserved during evolution to regulate their transcriptional capacity. Based on the presence of Ψ KXE motif in group B1 proteins (SoxN in *Drosophila* and Sox1/2/3 in humans), and its absence in group B2 (Dichaete in *Drosophila* and Sox14/21 in humans), it is tempting to speculate that these two subgroups differ in their ability to be regulated by SUMOylation. This is particularly interesting because in *Drosophila*, SoxN and Dichaete were shown to partially overlap in their expression and function within the neuroectoderm, suggesting that these genes are to some extent functionally redundant in the developing CNS but that there must exist molecular mechanisms responsible for their specificity of action in restricted areas of the CNS (interactions with specific partners? posttranslational modifications?) (Buescher *et al.*, 2002; Overton *et al.*, 2002; Gomez-Skarmeta *et al.*, 2003). Furthermore, it was shown in chick that group B2 Sox14/21 could bind and differentially regulate δ 1-crystallin gene regulatory sequences, known to be regulated by group B1 Sox1/2/3 factors *in vivo* (Uchikawa *et al.*, 1999). These observations suggested that target of group B genes might be regulated by the counterbalance of activating and repressing Sox proteins in restricted sites of the developing CNS. In light of our results, SUMOylation might be one of the mechanisms used for this purpose.

As shown here, substitution of lysine 439 to arginine within SoxN IKSE motif impaired SoxN SUMO modification in both transfected HeLa and S2 cells. SoxN transcriptional activity was dramatically enhanced in three conditions: in the substitution mutant K439R, in the deletion mutants where the IKSE motif was deleted, and when the dominant negative form of Ubc9 was used to interfere with the endogenous SUMO machinery. This correlation between transcriptional repression and the ability of SoxN to be SUMOylated strongly suggests that SUMO conjugation to SoxN results in transcriptional repression. Similar results were obtained for its human counterpart Sox3. Many of the SUMO-modified proteins identified to date are transcription factors, and in most cases, SUMO modification has been associated with

transcriptional repression. Nevertheless, the molecular mechanisms underlying this repression are still a matter of debate. In some cases, SUMO modification was associated with the relocalization of the targeted factor to specialized repressive subnuclear structures such as PML bodies (for review, see Gill, 2003, 2004; Girdwood *et al.*, 2004). In SoxN and Sox3, our data in HeLa and S2 cells suggest that SUMOylation is apparently not associated with major changes in the nuclear localization of these proteins (Figure 5). This was also evident *in vivo*, because the wild-type and K439R SoxN forms both localized similarly in the nuclei (Figure 6).

In both SoxN and Sox3, we found that the Ψ KXE motif is targeted for SUMOylation, and constitutes an inhibitory domain able to affect the activity of adjacent TADs. Interestingly, this motif is surrounded by conserved proline residues, reminiscent of the SC synergy domain (consensus P-X0-4- Ψ KXE-X0-3-P) found in several transcription factors, including SP3, c-myb, C/EBP, and Sox9 (Komatsu *et al.*, 2004, and references therein). Potential SC motifs also are found in other Sox: H.s Sox6, H.s Sox8, and H.s Sox30 (Table 1). SC motif is both necessary and sufficient to limit transcriptional synergy, because its disruption selectively enhances synergistic activation at compound response elements without altering the activity driven from a single site (Iniguez-Lluhi and Pearce, 2000). Thus, SUMOylation of the SC domain is believed to modulate higher order interactions among transcriptional regulators. This motif in Sox proteins might behave as SC domain, because these factors are known to pair off with specific partners to exert full and synergistic activity in a context dependent manner (Kamachi *et al.*, 2000). Because SUMO modification is believed to modulate protein-protein interactions, it will be of interest to examine whether Sox SUMOylation is able to interfere with their ability to interact with their partners.

Using transgenic *Drosophila* lines, we obtained strong evidence that SUMOylation regulates the activity of SoxN *in vivo*. Indeed, overexpressing the SUMO-deficient K439R SoxN form resulted in strong defects in embryonic CNS. Because the GAL4 driver used for embryonic overexpression is ubiquitous, we interpret these results as the capacity of the nonSUMOylable form to interfere with endogenous SoxN in the cells where SoxN is expressed (neuroblasts and neurons). In addition, our experiments where the wild-type and K439R SoxN proteins were overexpressed in larvae clearly showed that the two forms display different activity *in vivo*, further demonstrating the functional relevance of SoxN SUMOylation *in vivo*. Because the K439R form is a strong transcriptional activator as observed in our luciferase assays in transfected cells, we can postulate that the repressing activity of SoxN is important for the proper development of embryonic CNS. Further work will be required to demonstrate whether SUMOylation regulates SoxN activity in all the different cell types where the protein is expressed (embryonic, larval and adult CNS, larval and adult eyes, and larval leg imaginal discs).

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