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# Modification of Msx1 by SUMO-1

Vandana Gupta, Marianna Bei *

*Cutaneous Biology Research Center, Massachusetts General Hospital and Harvard Medical School, 149, 13th Street, Charlestown, MA 02129, USA*

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**Abstract**

The small ubiquitin-related modifier SUMO reversibly modifies many proteins, including promoter-specific transcription factors. Genetic studies in both humans and mice indicate that the Msx1 transcription factor is associated with specific disorders, including cleft palate. We show that Msx1 conjugation to SUMO-1 in vivo is enhanced by an E3 SUMO ligase, PIAS1, suggesting that sumoylation of Msx1 is a new mechanism for modulating the molecular function of Msx1 during organogenesis.


*Keywords:* Msx1; Sumoylation; Post-translational modifications

Post-translational modification of proteins by the small ubiquitin-related modifier (SUMO) is increasingly recognized as an important regulatory mechanism. The conjugation of SUMO to target proteins (SUMOylation) leads to the formation of an isopeptide bond between the C-terminus of SUMO and an ε-amino group of a lysine residue in the target protein [1]. In most cases, the lysine residue is embedded in a consensus sequence composed of a characteristic ΨKXE motif, where Ψ is a large hydrophobic amino acid, X is any residue, and K (lysine) is the site of SUMO-conjugation [1–3]. The SUMO pathway mechanistically resembles that of ubiquitination, but the enzymes involved in the two processes are distinct. SUMOylation utilizes the heterodimeric E1 SUMO-activating enzyme SAE1/SAE2, the E2 SUMO conjugating enzyme Ubc9, and recently identified E3 ligases for substrate selection and reaction specificity [4–6]. Among the ligases is the family of PIAS (protein inhibitors of activated STATs) nuclear proteins that function as SUMO ligases for STAT and for other proteins [6]. As a dynamic process, SUMOylation is readily reversed by a family of SUMO specific proteases (SENP family members in mammals), thus providing a control mechanism for the modified protein by the cell [7].

The proteins targeted by SUMO fall into different categories such as signal transducers, enzymes, viral proteins, regulators of chromatin structure, DNA repair proteins, and transcription factors [1,2]. Sumoylation of transcription factors has been shown to affect their stability, localization, and activity as activators or repressors, by altering protein–protein interactions to favor recruitment of co-repressors, by regulating their sub-nuclear localization, by inducing conformational changes in the structure of the transcription factor or by competing with other post-translational modifications for lysine residues [8–11]. Msx1 is a member of the Msx transcription factors that play key roles in development by repressing gene expression through interactions with components of the core transcription complex as well as with other homeoprotein [12,13]. Mutations in both human and mice indicate that Msx1 is associated with specific human craniofacial disorders including cleft palate and anodontia [12]. Here, we show that Msx1 is sumoylated in vivo and we propose that Msx1 sumoylation constitutes a new regulatory mechanism modulating Msx1 function during organogenesis.

## Materials and methods

*Computational studies.* Putative sumoylation sites in Msx1 (NCBI Accession No: BC016426) were identified using SUMOPLOT (Abgent, Inc., CA). Multiple sequence clustering for phylogenetic analysis was

* Corresponding author. Fax: +1 617 726 4453.
*E-mail address:* marianna.bei@cbrc2.mgh.harvard.edu (M. Bei).

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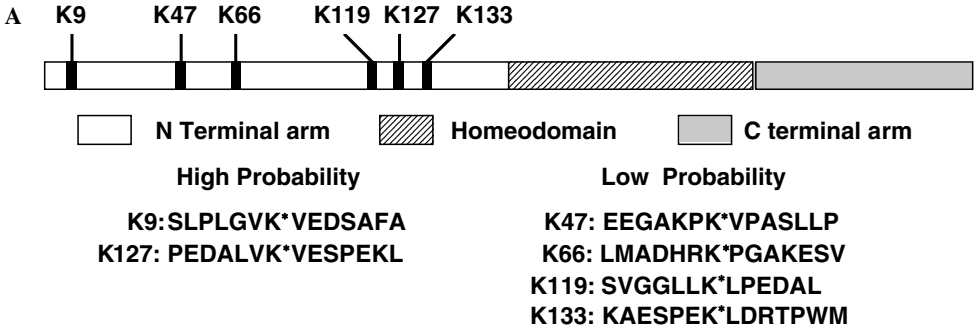
carried out with ClustalW. The sequences used for the phylogenetic analysis were *Danio rerio* (NM_131273.1), *Xenopus laevis* (BC081101), *Gallus gallus* (BAA01209.1), *Mus musculus* (BC016426), and *Homo sapiens* (AAA52683).

*Antibodies.* Mouse monoclonal anti-FLAG antibody M2 and mouse monoclonal anti-HA, mouse monoclonal anti-Msx1 and mouse monoclonal anti-GMP-1 (SUMO-1) were purchased from Sigma, Covance and Zymed, respectively. Horseradish peroxidase-conjugated anti-mouse IgG was purchased from Amersham Biosciences.

*Expression cloning.* The plasmid pCMV-FLAG-Msx1 encoding wild-type full-length Msx1 tagged with FLAG epitope was constructed as follows: The Msx1 ORF was amplified by PCR using 5′-CGCGGATCCA TGACTTCTTTGCCACTCGG-3′ as the forward primer and 5′-CCAA GCTTCTAAGTCAGGTGGTACATGCTGTAT as the reverse primer. The PCR product was digested with *Bam*HI and *Hin*dIII, and cloned in the corresponding *Bam*HI- and *Hin*dIII-restricted pCMV-FLAG plasmid (Stratagene, La Jolla, CA). Point mutations K9R, K47R, K66R, K119R, K127R, and K133R in pCMV-Msx1-Flag were generated by PCR amplification using mutated oligonucleotides employing GeneTailor Site-Directed Mutagenesis system (Invitrogen, Carlsbad, CA). The following oligonucleotide primers were used: sense K9R: 5′-CACTCGGTGTCA GAGTGGAGGACTC-3′; antisense K9R: 5′-GAGTCCTCCACTCTGA CACCGAGTG-3′; sense K47R: 5′-AGGGGGCCAAGCCCAGAGTG CCCGCT-3′; antisense K47R: 5′-GTCTACTCCTCCCCCGGTTCGGG T-3′; sense K66R: 5′-CCGATCAC AGGAGGCCCGGGGCCA AG-3′; antisense K66R 5′-CTTGGCCCCGGGCCTCCTGTGATCGG-3′; sense K119R: 5′-GGAGGACTCCTCAGGCTGCCAGAAGATG-3′; antisense K119R: 5′-CATCTTCTGGCAGCCTGAGGAGTCCTCC-3′; sense K127R: 5′-GATGCTCTGGTGAGGGCCGAAAGCCC-3′; antisense 5′-GGGCTTTCGGCCCTCACCAGAGCATC-3′; sense K133R: 5′-CCGA AAGCCCCGAGAGACTAGATCGGAC-3′; antisense K133R: 5′-GAC CACTTCCGGCTTTCGGGGCTCT-3′. Wild-type SUMO-1-GFP and mutant SUMO1-GFP were kindly provided, by Dr. Palvimo (University of Helsinki). PIAS1 was provided by Dr. Shuai (University of California, LA). HA-tagged SUMO-2 and SUMO-3 were provided by Dr. R. Hay (University of St. Andrews, Scotland) and Senp1 was obtained from Dr. E. Yeh (The University of Texas-M.D. Anderson Cancer Center).

*Cell culture and transfections.* C3H10T1/2 cells were obtained from American Type Culture Collection and cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 10 U/ml penicillin, 10 μg/ml streptomycin, and 25 ng/ml amphotericin B (Invitrogen). $3 \times 10^5$ cells/well were plated in 6-well plates and next day transient transfections were performed with Lipofectamine (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Each transfection was repeated at least three times.

*Preparation of cell lysates and Western blotting.* Cells were harvested after 36 h and lysed in a modified RIPA buffer containing 50 mM Tris (pH 7.5), 0.15 M NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5% Nonident P-40, and 0.1% sodium deoxycholate with complete protease inhibitors (Roche Molecular Biochemicals), and 10 mM *N*-ethylmaleimide (NEM). One hundred micrograms of protein was loaded (per lane) for Western blotting experiments. Proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membrane. Blots were blocked in Tris-buffered saline with 0.1% Tween 20 (TBST) containing 5% nonfat dried milk for 2 h at room temperature. Primary antibodies were incubated overnight at 4 °C and washed three times each in TBST. Horseradish peroxidase-conjugated IgG secondary antibodies (Amersham Lifesciences) were added to TBST and membranes were incubated for 1 h in secondary antibody followed by three washes in TBST (10 min each). Western blots were incubated with ECL Western blotting reagent (Amersham Biosciences), exposed to X-ray film, and developed.




B

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Danio rerio      ----------MAPVVTMSSQAAEED----AGVMLAGQ---------TEMQTRVTVQEDAQ 37
Xenopus laevis   SQLGSLCMAPALLMASYQPGVKIEE---RPLNRMQQTG----------VKPSLGED---- 57
Gallus gallus    -------MAPAADMTTAPTGVRSDEPPASAFSKPGGG---------LPVAAAMGGEEESD 44
Bos taurus       -------------MTSLPLGVKVED---PPFGKPAGGGGGQTPSTTAATAAAMGADAEGA 44
Mus musculus     -------------MTSLPLGVKVED---SAFAKPAGGGVGQAPGAAAATATAMGTDEEGA 44
Homo sapiens     EGGARLCMAPAADMTSLPLGVKVED---SAFGKPAGGGAGQAPSAAAATAAAMGADEEGA 117

Danio rerio      EDAQRPKILPFSVEALMAD-RRPNRGS---------------------------------- 59
Xenopus laevis   KPKVP-GILPFSVEALMAD-RKPG-RDRDLSSPTGSPLAGTSHSPR---VGSIAAG--ET 109
Gallus gallus    KPKVSPSPLPFSVEALMADRRKP---------PGGRDGPEGSGPPLGSARANLGALTTEA 95
Bos taurus       KPKVSPSLLPFSVEALMADHRKPGAKKSVLAASEGAQAAGGSAKPLGARPGSLAAP--DA 102
Mus musculus     KPKVPASLLPFSVEALMADHRKPGAKESVLVASEGAQAAGGSVQHLGTRPGSLGAP--DA 102
Homo sapiens     KPKVSPSLLPFSVEALMADHRKPGAKESALAPSEGVQAAGGSAQPLGVPPGSLGAP--DA 175

Danio rerio      PDADARLG----FSVEVLQLP----VKAESPE---RSTWVPRARFSPS---RLSPPACPL 105
Xenopus laevis   PNSPISLGNRY-PVGAIMQLPEETLLKPESPE---RSSWIQSPSFSPSPTRRMSPPACPL 165
Gallus gallus    PTSPLPLGGHFPSVGALGKLPEDALLKAESPEKPERSPWMQSPRFSPPPPRRLSPPACTL 155
Bos taurus       PSSPRPLG-HF-SVGGLLKLPEDALVKAESPEKPERTPWMQNPRFSPPPSRRLSPPACTL 160
Mus musculus     PSSPRPLG-HF-SVGGLLKLPEDALVKAESPEKLDRTPWMQSPRFSPPPARRLSPPACTL 160
Homo sapiens     PSSPRPLG-HF-SVGGLLKLPEDALVKAESPEKPERTPWMQSPRFSPPPARRLSPPACTL 233
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Fig. 1. Msx1 sumoylation sites. (A) Schematic representation of putative sumoylation sites in Msx1. (B) Phylogenetic sequence comparison of the sumoylation sites (in box) of Msx1. Asterisks represent the position of lysines in murine Msx1.

## Results and discussion

Analysis of the mouse Msx1 sequence revealed the presence of six consensus motifs for SUMO-1 conjugation, two high probability sumoylation motifs at K9 and K127 and four low probability motifs at K47, K66, K119, and K133 (Fig. 1A). Phylogenetic analysis showed significant conservation of these sumoylation motifs at all positions except K119 and K133 (Fig. 1B).

To determine whether Msx1 is sumoylated in vivo, expression vectors encoding FLAG-tagged wild-type mouse Msx1 (WT Msx1-FLAG), wild-type GFP-tagged SUMO-1 (SUMO1-GFP-WT) or mutant GFP-tagged SUMO-1, a conjugation defective form of SUMO-1 (SUMO1-GFP-MT) were transiently transfected into C3H10T1/2 cells. Cells were lysed under conditions that preserve SUMO modification by inclusion of the SUMO-1 specific isopeptidase inhibitor NEM. Western blot of total cellular proteins using anti-FLAG (Msx1) or anti-SUMO-1 antibodies revealed that FLAG-tagged Msx1 (unmodified Msx1) migrates as a major species of 34 kDa (Fig. 2A, lane 1). In the presence of exogenous SUMO-1, two slower migrating forms of Msx1 were observed with an increased apparent molecular mass corresponding to 82 and 130 kDa, respectively (Fig. 2A, lane 2). These two slower migrating Msx1 species disappeared when mutant SUMO1-GFP was co-transfected with FLAG-tagged Msx1 (Fig. 2A, lane 3). Antibodies to SUMO-1 further confirmed that these protein species were the result of Msx1-GFP-SUMO1 conjugation (Fig. 2B). We did not see any modification of Msx1 in the presence of exogenous SUMO-2 and/or SUMO-3 (Fig. 2C).

To investigate whether the residues identified in Msx1 sequence are in vivo acceptor sites for SUMO-1, we constructed a series of lysine to non-sumoylatable arginine mutants (Fig. 2D). Sumoylation of these mutants was compared with that of wild-type Msx1 following transient transfections of the expression vectors encoding the different point mutants together with the expression plasmid for SUMO1-GFP. Total cell extracts were analyzed by Western blot using anti-FLAG (Msx1) and anti-SUMO1 antibodies (Fig. 2D and data not shown). As shown, Msx1

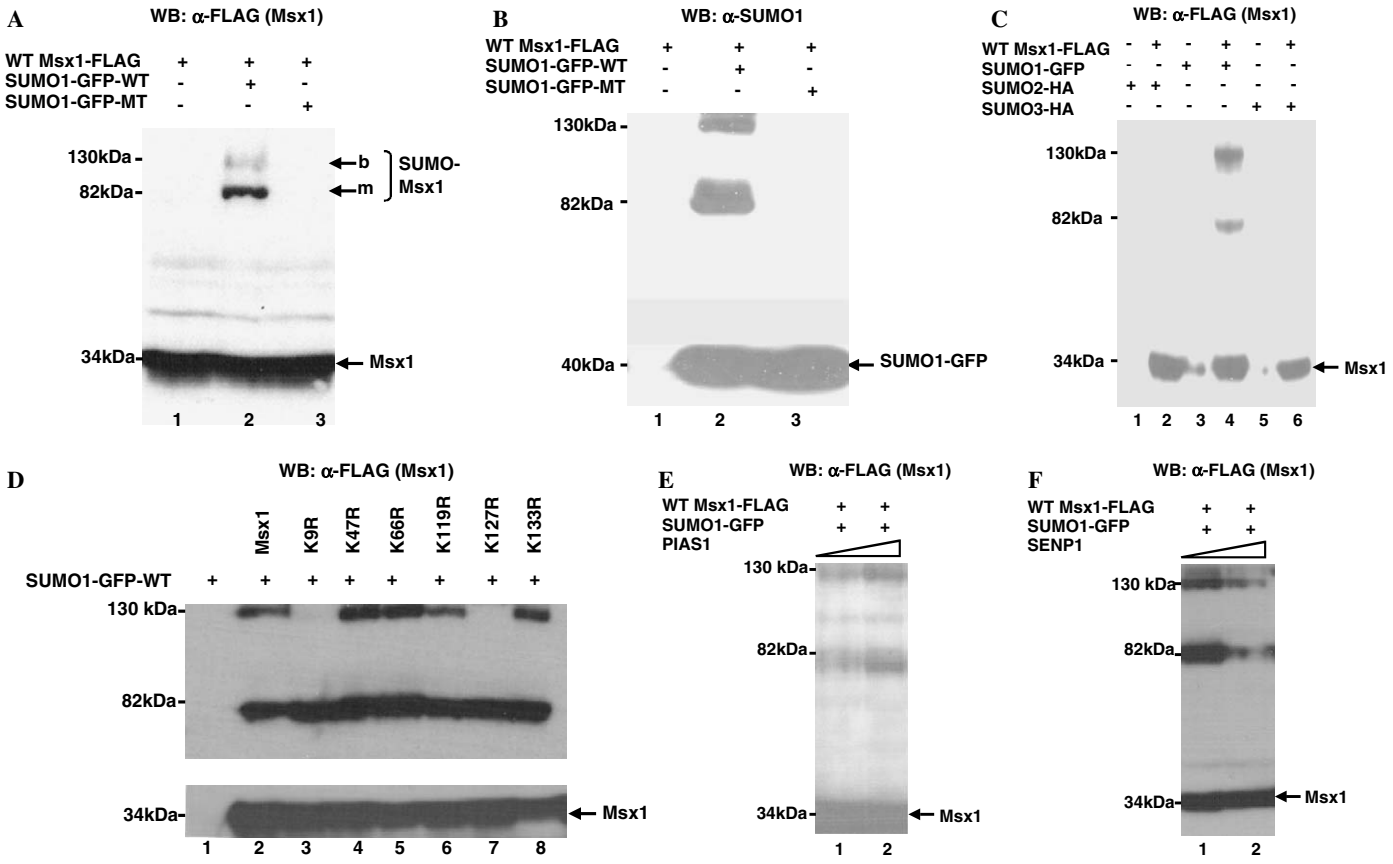


Fig. 2. Msx1 is sumoylated in vivo. (A,B) C3H10T1/2 cells were transfected with Msx1-FLAG alone (lane 1), or with Msx1-FLAG and wild-type SUMO-1-GFP (lane 2) or with Msx1-FLAG and mutant SUMO-1-GFP (lane 3). Cell extracts were subjected to Western blot analysis with anti-Msx1 antibody (A) or SUMO-1 antibody (B). (C) Cells were co-transfected with SUMO1, SUMO2 or SUMO3 or with Msx1-FLAG. (D) SUMO-1-GFP, WT Msx1-FLAG, K9RMsx1-FLAG, K47RMsx1-FLAG, K66RMsx1-FLAG, K119RMsx1-FLAG, K127RMsx1-FLAG, and K133RMsx1-FLAG co-expressed in C3H10T1/2 cells. (Upper panel) Modified slow migrating forms of Msx1, (lower panel) unmodified Msx1. (E,F) C3H10T1/2 cells were transfected with Msx1-FLAG, SUMO-1-GFP, and increasing concentrations of PIAS1 or Senp1. PIAS1 protein enhances the sumoylation of Msx1 (E) and Senp1 decreases the sumoylation of Msx1 (F). Arrows indicate the sumoylated forms of Msx1 (b, bi-sumoylated form; m, mono-sumoylated form).

K47R, K66R, K119R, and K133R were modified as efficiently as wild-type Msx1. In contrast, mutation of $Lys^{9}$ and $Lys^{127}$ in Msx1 K9R and K127R, respectively, abolished the formation of the higher molecular mass sumoylated form of Msx1 (Fig. 2D, lanes 3 and 7).

Taken together, these results show that Msx1 is sumoylated in vivo at two lysine residues ($Lys^{9}$ and $Lys^{127}$) located in the N-terminal domain of the Msx1 protein, that SUMO-1 serves as a substrate for this modification and that the higher-molecular weight species observed represent sumoylated forms of Msx1.

Sumoylation is a process controlled by opposing enzymatic activities. To further establish the Msx1 SUMO-1 modification, Msx1 sumoylation was examined in the presence of different amounts of the SUMO E3 ligase PIAS1, that preferentially promotes SUMO-1 conjugation, and in the presence of Senp1, a mammalian SUMO-specific isopeptidase that hydrolyzes the peptide bond between the SUMO-1 and lysine of the modified proteins (Figs. 2E and F). An increase in SUMO E3 ligase PIAS1 significantly enhanced the formation of sumoylated forms of Msx1 (Fig. 2E). This result indicates that Msx1 sumoylation can be regulated by the PIAS1 E3 ligase. Conversely, when Msx1 sumoylation was examined in the presence of increased levels of the isopeptidase SenP1, sumoylated forms of Msx1 decreased, implying that Senp1 is a specific isopeptidase that also regulates the sumoylation of Msx1.

Here, we report that Msx1 is sumoylated in vivo at $Lys^{9}$ and $Lys^{127}$, and that this modification can be mediated by the SUMO E3 ligase PIAS1. These residues are conserved across several species, suggesting that this modification is likely to be conserved throughout mammalian evolution. Interestingly, both target lysines reside in the N-terminal domain of Msx1 that is involved in transcriptional repression [13]. Transcriptional repression by Msx1 is mediated by protein–protein interactions, implying that SUMO-1 conjugation may play an important role in controlling the ability of Msx1 to interact with distinct transcriptional co-factors to regulate transcriptional output. Consistent with such an idea, *SUMO1* haploinsufficiency leads to cleft lip and palate; in addition, Eya1 and Pax9, which like Msx1 function in palatogenesis, are substrates for sumoylation in vivo [14]. Taken together, these results unify these proteins in a molecular and genetic framework regulating organogenesis. It is possible that sumoylation of cooperating factors is important in regulating their transcriptional synergy in a promoter and cell-specific manner.

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