

PIAS1 Negatively Regulates Ubiquitination of Msx1 Homeoprotein Independent of Its SUMO Ligase Activity

Young Joon Song, and Hansol Lee*

Posttranslational modifications play key roles in many cellular processes including proliferation and differentiation by modulating the activities of target proteins. PIAS1, a member of PIAS family of protein, mediates the modification of protein by SUMO and thereby regulates the function of its interacting protein partners. Here we report that PIAS1 negatively regulates ubiquitination of Msx1 homeoprotein, a regulator of myogenic differentiation, in a SUMO-independent manner. We demonstrate that ubiquitination and SUMOylation of Msx1 are not mutually exclusive but require the same C-terminal PIAS1 interaction domain. In addition, deletion of C-terminal domain increases the steady-state protein level of Msx1, while mutations of SUMO acceptor sites have no significant effect on the stability of Msx1 proteins. Moreover, we find that forced expression of PIAS1 inhibits ubiquitination and thereby increases the stability of Msx1 protein regardless of its activity as a SUMO ligase. Furthermore, repressor activity of Msx1 in transcription is strengthened in the presence of PIAS1. Taken together, our studies uncover a new function of PIAS1, which is to control the stability of its interacting protein partner in a SUMO independent manner.

INTRODUCTION

Msx1 is a member of a diverged homeobox family related to the *Drosophila msh* (muscle segment homeobox) gene (Bendall and Abate-Shen, 2000). In vertebrate, *Msx1* is expressed in a variety of embryonic tissues including neural tube, the limb buds, and derivatives of the cranial neural crest (Bendall and Abate-Shen, 2000; Davidson, 1995). Although *Msx1* is expressed in unrelated and diverse tissues during embryonic development, a common feature is that expression of *Msx1* is temporally regulated and restricted to undifferentiated progenitor cells that are eventually differentiated into terminally differentiated cells (Bendall and Abate-Shen, 2000). For example, *Msx1* is transiently expressed in undifferentiated precursor cells but down-regulated upon terminal differentiation during skeletal muscle development (Bendall et al., 1999). Because of this inverse correlation between *Msx1* expression and cellular differentiation,

it is believed that a biological function of *Msx1* is to keep progenitor cells from differentiation until they receive proper signal from environment (Bendall et al., 1999). Indeed, numerous studies *in vivo* and *in vitro* have confirmed that *Msx1* inhibits the differentiation of various cell types including murine C2C12 myoblasts (Bendall et al., 1999; Hu et al., 2001; Song et al., 1992; Woloshin et al., 1995).

Functions of *Msx1* in cellular differentiation are believed to be mediated by its activity as a transcriptional repressor. It has been shown that forced expression of *Msx1* in C2C12 myoblast cells inhibits expression of *MyoD*, and this inhibition is correlated with binding of *Msx1* to *MyoD* core enhancer, an essential regulatory element directing embryonic *MyoD* expression in skeletal muscle (Bendall et al., 1999; Goldhamer et al., 1992; Lee et al., 2004; 2006). Activities of *Msx1* are also modulated by its protein partners. Linker histone H1b and Y-box protein YB1 cooperate with *Msx1* to inhibit myogenic differentiation while PIAS1 is thought to help proper localization of *Msx1* at the nuclear periphery where target genes of *Msx1* are positioned in C2C12 myoblast cells (Lee et al., 2004; 2006; Song and Lee, 2010).

Protein inhibitor of activated STAT1 (PIAS1) is a member of protein family that were initially identified as inhibitors of signal transducer and activator of transcription (Chung et al., 1997; Liu et al., 1998). Subsequent studies revealed that interactions of PIAS proteins are not limited to STAT, but they are associated with numerous protein partners, majority of those being nuclear proteins [reviewed in (Rytinki et al., 2009)]. PIAS proteins are known to modulate the function of its target *via* changes in nuclear localization, protein-DNA binding affinity and protein-protein interaction either by SUMOylation or by SUMO-independent manner. As is the case for other protein partners, interaction of PIAS1 with *Msx1* also accompanies the SUMOylation at two conserved lysine residues, K15 and K133 respectively. However, functions of SUMO modified *Msx1* are not clearly understood yet (Lee et al., 2006). PIAS1 is also thought to be involved in the stability of its interacting proteins, at least in part, by competing with ubiquitination machinery for the same lysine residues and thus antagonizing proteasome-mediated protein degradation (Desterro et al., 1998; Lin et al., 2003). Of note, it has been recently shown that a mutant form

Department of Biological Sciences, College of Natural Science, Inha University, Incheon 402-751, Korea
*Correspondence: hlee@inha.ac.kr

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of Msx2, which is a member of Msx family proteins and associated with boston-type craniosynostosis, displays increased susceptibility to ubiquitination *in vivo*, suggesting that posttranslational modifications by ubiquitin plays important roles in activities of Msx family of proteins (Sasaki et al., 2002; Yoon et al., 2008).

In this study, we have found that PIAS1 regulates ubiquitination of Msx1 homeoprotein in a SUMO-independent manner. Both wild type Msx1 and a SUMO-defective mutant were effectively ubiquitinated in cells. However, experiments with both Msx1 and PIAS1 mutants revealed that deletion of the C-terminal PIAS1 interacting domain of Msx1 abrogated ubiquitination and increased the stability of Msx1. Moreover, forced expression of PIAS1 prevented Msx1 from being ubiquitinated in cells. Taken together, unlike other previously known functions, our data highlight a new role of PIAS1 in ubiquitination, which is to control the stability of its protein partner in a SUMO independent manner.

MATERIALS AND METHODS

Plasmids

Plasmids encoding Flag-tagged Msx1, Msx1A [Msx1 (K174A, R176A, F179A)], and the truncated Flag-Msx1 derivatives as well as HA-tagged PIAS1 were previously described (Lee et al., 2004; 2006). Plasmids expressing mutant Msx1 in which lysine residue(s) were replaced with arginine were generated using overlapping polymerase chain reaction (PCR) method. V5-tagged mouse ubiquitin was generated from genomic DNA using PCR with a forward primer that introduced a *Bam*HI restriction site and a V5 epitope tag at the N terminus and a reverse primer that introduced a *Xho*I restriction site at the C terminus. Amplified products were then digested with *Bam*HI and *Xho*I restriction enzymes and cloned into pcDNA3 mammalian expression vector (Invitrogen). It should be noted that genomic DNA was used as a template for PCR as ubiquitin gene is composed of single exon. The complete sequences of all PCR-amplified constructs were confirmed.

Cell cultures and transfection

Human 293T cells and murine C2C12 myoblast cells were maintained in DMEM (WelGENE) supplemented with 10% fetal bovine serum (WelGENE) and antibiotics in humidified atmosphere with 5% CO₂ at 37°C. Cells were transfected using WelfectEX™ PLUS Reagent (WelGENE) following manufacturer's recommendation.

Reporter assay

C2C12 cells were grown to 30% confluency in 6-well plates in DMEM with 10% fetal bovine serum and transfected with 0.5 µg of pMyoD-Luciferase containing F3/-2.5 fragment and MyoD promoter and indicated amounts of Msx1 expression vector and/or HA-PIAS1 expression vector (Lee et al., 2004; 2006). The total amount of plasmid DNA was adjusted to 1.5 µg by adding empty vector (pcDNA3). Cells were harvested at 24 h after transfection and analyzed for luciferase activity following manufacturer's protocol (Promega).

Ubiquitination assay

To detect ubiquitinated Msx1, cells were cotransfected with relevant plasmids using WelfectEX™ PLUS reagent (WelGENE). Transfected cells were harvested after 36 h and initially lysed in 1% sodium dodecyl sulfate (SDS) solution, which would disrupt non-covalent protein-protein interaction and thus avoid the detection of ubiquitinated proteins interacting with Msx1. Lysed

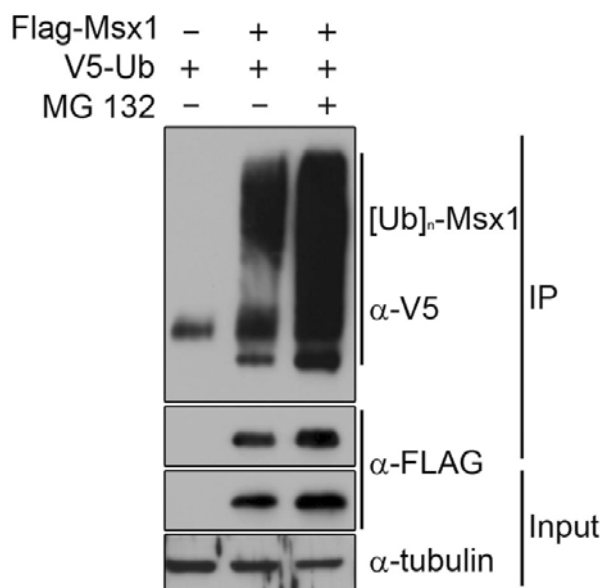


Fig. 1. Polyubiquitination of Msx1 in cell extracts. Human 293T cells were transfected with plasmids encoding V5-ubiquitin along with Flag-Msx1 or empty vector (pcDNA3). After 36 h, cells were lysed in 1% SDS and total extract (denatured) were sonicated for 3 min and clarified by centrifugation. Proteins were immunoprecipitated with anti-Flag M2 agarose (Sigma) and precipitated proteins were resolved on 10% SDS-PAGE. Ubiquitinated Flag-Msx1 proteins were detected using anti-V5 antibody (top panel) and unmodified Flag-Msx1 proteins were detected using anti-Flag antibody (middle panel). Levels of tubulin proteins were monitored to assure that equal amount of total proteins were used for the experiment (bottom panel). Note that 5 µM of MG 132, a 20S proteasome inhibitor, was added to confirm that ubiquitination of Msx1 directs the proteasome mediated degradation.

proteins were sonicated for 3 min at 100 watts, clarified by centrifugation, and then diluted with 10 volumes of dilution buffer containing 16.7 mM of Tris-Cl (pH 8.0), 1.1 mM of EDTA (pH 8.0), 167 mM of NaCl, 1.1% of Triton X-100, 0.01% of SDS and protease inhibitor cocktail (Roche). Proteins were immunoprecipitated with Flag M2 agarose (Sigma-Aldrich) for 16 h at 4°C and immunoprecipitated proteins were analyzed by western blot using ECL plus Western blotting detection system (Amersham) as described in the manufacturer's instruction. Antibodies used were monoclonal anti-Flag M2, monoclonal anti-V5 (both from Sigma-Aldrich), and monoclonal anti-HA (Cell Signaling Technologies).

Protein stability assay

C2C12 myoblast cells transfected with relevant plasmids were starved for 2 h in methionine/cysteine-free medium (Invitrogen) and labeled for 50 min with 400 µCi/ml of [³⁵S] Met/Cys (NEN). Labeled cells were washed 5 times and incubated in pre-warmed complete medium. Total cell extracts were prepared from labeled cells at the indicated time points and immunoprecipitated with Flag M2 agarose. Proteins were subjected to SDS-PAGE. Labeled proteins were visualized and quantitatively analyzed using a phosphorimager (Molecular Dynamics). Alternatively, human 293T cells transfected with relevant plasmids were split into four 60 mm plates and treated with cycloheximide to inhibit new protein synthesis. Cells were harvested

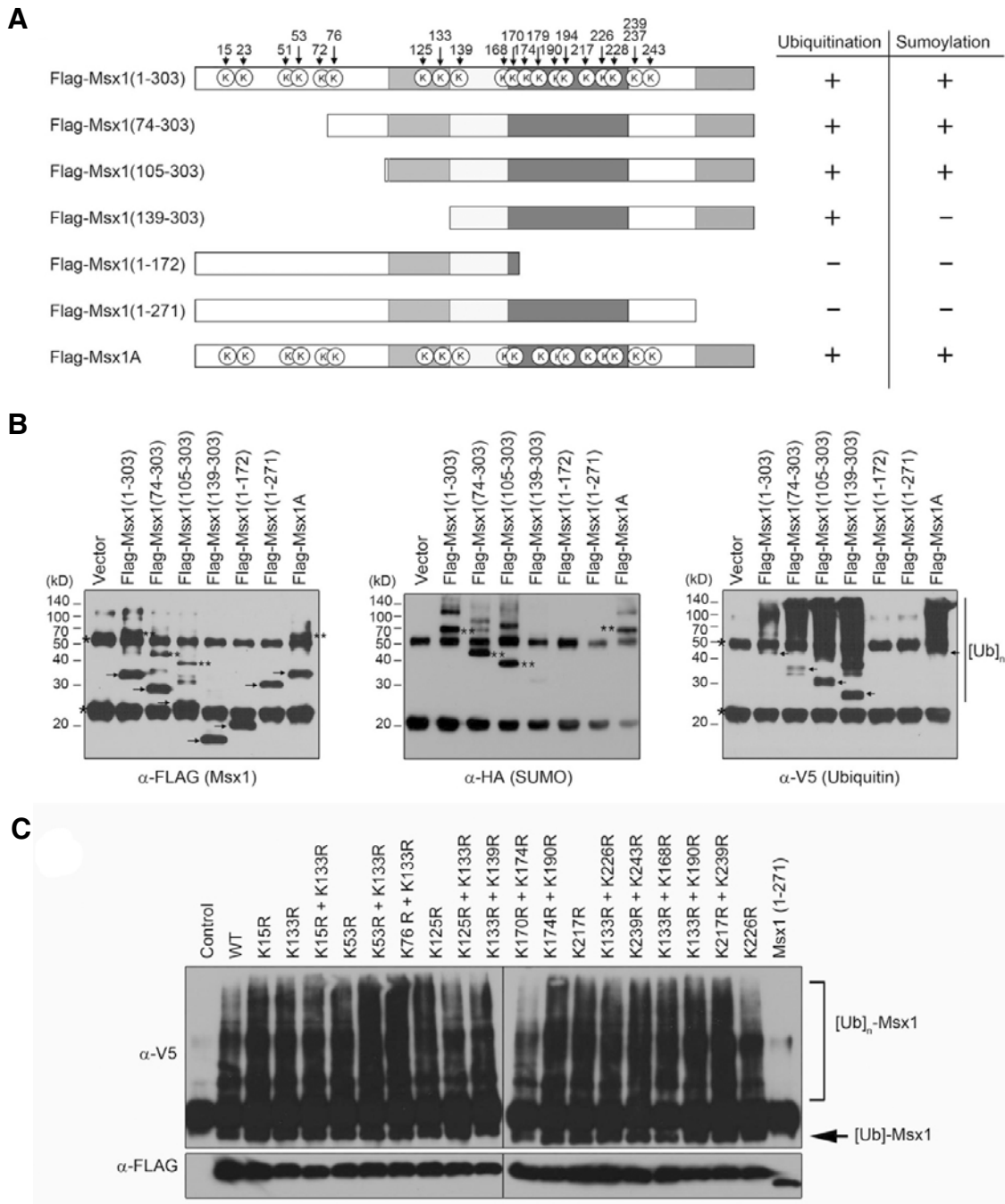


Fig. 2. Ubiquitination of Msx1 is not mutually exclusive with SUMOylation but requires C-terminal PIAS1 interaction domain. (A) Schematic representation of Flag-tagged full-length Msx1 [Flag-Msx1 (1-303)] and its derivatives with shaded areas indicating domains required for (i) interaction with linker histone (LHID), (ii) interaction with PIAS1 (PID), and (iii) the homeodomain (HD) (left) Positions of all lysine residues that can serve as ubiquitin acceptor site(s) were shown with number. Flag-Msx1A contains three point mutations in the N-terminal arm of homeodomain (K174A, R176A, F179A). Results from ubiquitination and SUMOylation assays were summarized in right panel. (B) Human 293T cells were transfected with plasmid expressing Flag-Msx1 or the indicated Flag-Msx1 derivatives along with plasmid encoding either V5-ubiquitin or HA-SUMO2. Denatured total extracts were prepared as described in Materials and methods and immunoprecipitated using anti-Flag M2 agarose (Sigma-Aldrich). Immunoprecipitated proteins were run on 12% SDS-PAGE and detected with anti-Flag (left panel), anti-HA (middle panel), or anti-V5 antibody (right panels). Single asterisk (*) shown in left and right panels indicates non-specific immunoglobulin proteins. In the left and middle panels, bands with double asterisk (**) represent SUMOylated Flag-Msx1 and its derivatives. Unmodified Flag-Msx1 proteins were shown with arrows (left panel). In the right panel, monoubiquitinated Flag-Msx1 and its derivatives were shown with arrows. (C) Human 293T cells were transfected with plasmid expressing Flag-Msx1 and its derivatives in which one or two lysine residues were replaced with arginines. Denatured total extracts were immunoprecipitated as described in “Materials and Methods”. Immunoprecipitated proteins were run on 10% SDS-PAGE and visualized with ECL reagent.

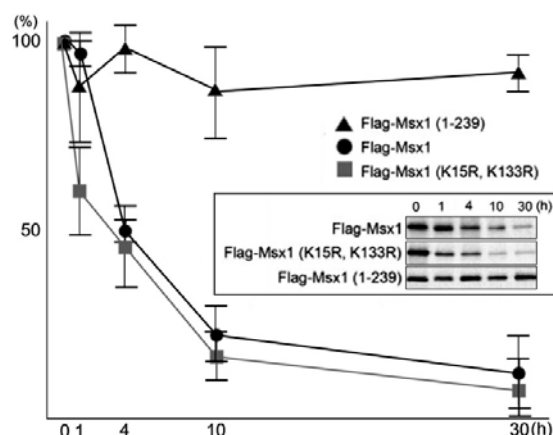


Fig. 3. Deletion of C-terminal domain increases the stability of Msx1 in cells. C2C12 myoblast cells transfected with relevant plasmids were labeled with [³⁵S] Met/Cys (NEN). Labeled cells were harvested at indicated time points and total extracts were immunoprecipitated with Flag M2 agarose. Precipitated proteins were run on 12% SDS-PAGE and analyzed using a phosphorimager. Inset shows representative data of three independent experiments. Error bars indicate standard deviation.

at indicated time points and levels of proteins were analyzed by immunoblotting.

RESULTS

Msx1 is poly-ubiquitinated in cells

Previous studies have shown that PIAS1 specifically interacts with Msx1 and promotes SUMOylation at two lysine residues in the N-terminal region, K15 and K133 respectively, and mutations of both lysine residues had modest effect on the activities of Msx1 in transcriptional repression as well as in inhibition of cellular differentiation (Lee et al., 2006). These slight but statistically discernible differences observed in SUMOylation-deficient Msx1 prompted us to test whether SUMOylation of Msx1 antagonizes ubiquitination, which often directs proteasome dependent degradation of protein, and thereby affects overall activities of Msx1 by increasing the steady-state protein level. We first examined the ubiquitination of Msx1 in human 293T cells transfected with plasmids expressing V5-tagged ubiquitin and/or Flag-Msx1. Transfected cells were initially disrupted with 1% SDS-containing buffer to dissociate noncovalent protein-protein interactions, which avoid the detection of ubiquitinated proteins interacting with Msx1. As shown in Fig. 1, immunoprecipitation followed by Western blotting analysis revealed that Flag-Msx1 can be ubiquitinated in human 293T cells, which is

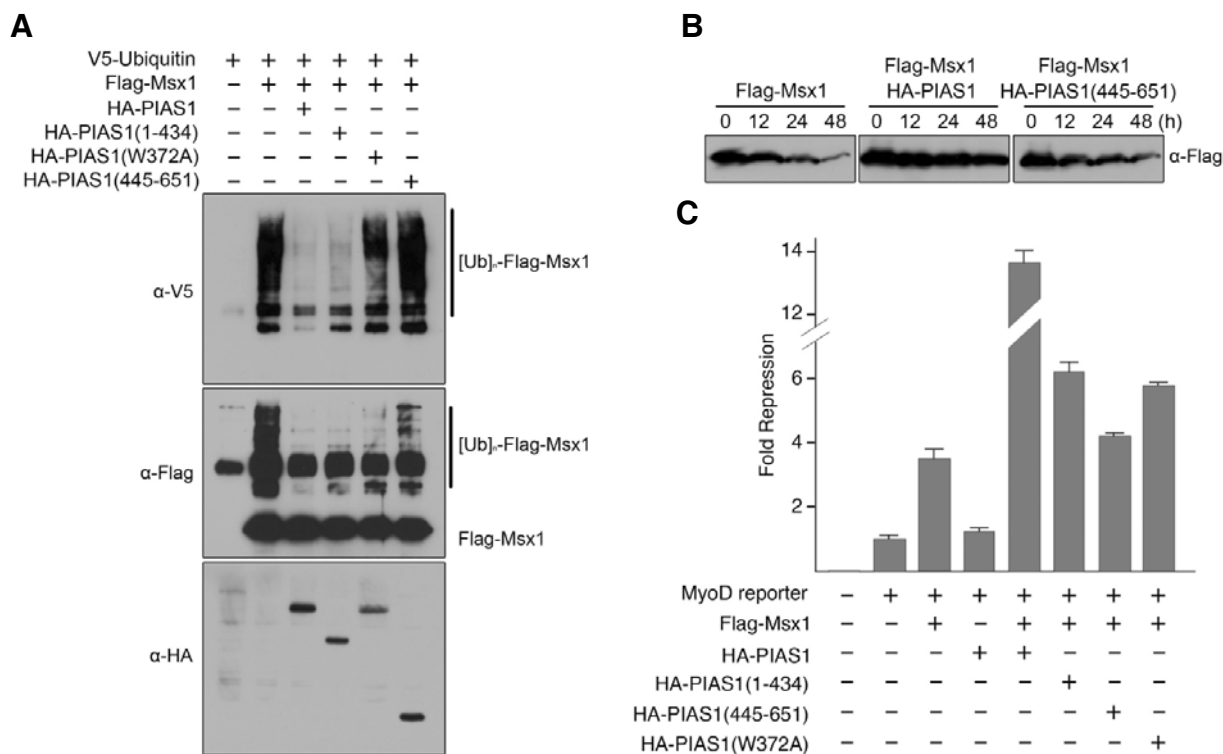


Fig. 4. PIAS1 increases the protein stability of Msx1 by preventing it from ubiquitinated in cells. (A) Cells were transfected with Flag-Msx1 and V5-ubiquitin expressing plasmids along with plasmid expressing indicated PIAS1 or its derivatives. After 36 h, denatured total extracts were prepared as described in "Materials and Methods" and immunoprecipitated with Flag M2 agarose. Proteins were resolved on 10% (for anti-Flag and anti-V5) or 12% (For anti-HA) SDS-PAGE and detected with anti-V5 (top panel), anti-Flag (middle panel) or anti-HA (bottom panel) antibodies. Shown is representative data of three independent experiments. (B) Transfected human 293T cells were treated with cycloheximide to inhibit new protein synthesis. Cells were harvested at indicated time points and levels of Msx1 proteins were analyzed by immunoblotting. (C) Cells were transfected with Msx1 expression vector and the indicated PIAS1 derivatives along with a *MyoD* luciferase reporter plasmid. Data are expressed as fold repression relative to the control. Shown is a representative data of four independent experiments done in triplicate; error bars indicate standard deviation.

evident by high molecular weight bands/smear corresponding to poly-ubiquitinated Msx1 as well as mono-ubiquitinated Msx1. In addition, ubiquitination of Msx1 increased in the presence of a proteasome inhibitor MG132, suggesting that ubiquitination of Msx1 may direct the 26S proteasome mediated degradation in cells.

The C-terminal domain of Msx1 is required for the poly-ubiquitination

We next attempted to determine the major ubiquitination site or sites by using derivatives of Msx1 in which either different domain of Msx1 was deleted or specific lysine residue(s) were replaced with arginine (Fig. 2). Mouse Msx1 protein possesses 21 lysines throughout its 303 amino acids and most of them are found in the N-terminal region and the homeodomain (Fig. 2A). In our immunoprecipitation assay, we observed two different types of modified Msx1, one of which corresponds to SUMOylated Msx1 (Fig. 2B, left and middle panels) and the other type is believed to be ubiquitinated Msx1 (Fig. 2B, left and right panels). Deletions up to N-terminal 104 amino acids, which still maintain a major SUMOylation site (K133), had little or no significant effect on both posttranslational modifications [Fig. 2B, see *Flag-Msx1 (74-303)* and *Flag-Msx1 (105-303)*]. However, *Flag-Msx1 (139-303)*, in which both SUMO acceptor sites (K15 and K133) are not available and thus resistant to modification by SUMO, was found to be ubiquitinated in cells (Fig. 2B, right panel), suggesting that ubiquitination of Msx1 is not mutually exclusive with SUMOylation. Of particular interest, experiments with mutants lacking the C-terminal PIAS1 interaction domain of Msx1 [Fig. 2B, see *Flag-Msx1 (1-271)* and *Flag-Msx1 (1-172)*] revealed inhibition of poly-ubiquitination in Msx1 homeoprotein (Fig. 2B, right panel). Considering that *Flag-Msx1 (1-271)* still has all 21 possible ubiquitin acceptor sites, these results suggest that C-terminal domain of Msx1 might be required for the interaction with cellular protein that mediates poly-ubiquitination of Msx1. In contrast to the effect of deletion of C-terminal PIAS1 interaction domain, mutation of single lysine residue did not affect the ubiquitination of Msx1, raising a possibility that either there are more than two ubiquitin acceptor sites in Msx1 or ubiquitination may occur to other cryptic sites when preferred sites are unavailable (Fig. 2C).

Deletion of C-terminal domain resulted in increased stability compared with Wild-type Msx1

Since deletion of C-terminal domain rendered Msx1 protein from being ubiquitinated, we next asked whether the same domain is involved in the control of steady-state protein level of Msx1. To address this question, we performed pulse-chase experiments to determine relative stabilities of wild-type Msx1 [*Flag-Msx1 (1-303)*] and mutant Msx1 proteins in which either C-terminal domain is deleted [*Flag-Msx1 (1-239)*] or two SUMOylation sites were replaced with arginine [*Flag-Msx1 (K15R, K133R)*] (Fig. 3). Wild-type Msx1 showed a reduction of about 50% after 4 h of chase and more than 80% in steady-state level after 30 h. Consistent with the previous data showing that mutant Msx1 lacking SUMO acceptor sites can be ubiquitinated in cells (Fig. 2C), *Flag-Msx1 (K15R, K133R)* displayed similar stability in cells. In contrast, Msx1 lacking C-terminal domain appeared to have much longer half-life in cells since less than 20% of reduction in protein level was observed even after 30 h (Fig. 3).

Interaction with PIAS1 decreases the ubiquitylation of Msx1

While our previous study showed that SUMOylation of Msx1

was not mutually exclusive with ubiquitination and therefore had no significant effect on protein stability, C-terminal domain of Msx1 that mediates the interaction with PIAS1 appeared to be critical for ubiquitination. We next investigated whether PIAS1 can regulate the ubiquitination of Msx1. As shown in Fig. 4A, we found that forced expression of HA-PIAS1 can efficiently prevent ubiquitination of Msx1 in human 293T cells. Consistently, expression of HA-PIAS1 (W372A) that maintains an ability to interact with Msx1 but lacks its SUMO ligase activity inhibited the ubiquitination of Msx1. In addition, an experiment with HA-PIAS1 (1-434) that also interacts with Msx1 but lacks C-terminal SUMO interacting motif (Lee et al., 2006), resulted in decreased ubiquitination of Msx1. However, expression of HA-PIAS1 (445-651), which lacks SAP domain and therefore does not interact with Msx1 (Lee et al., 2006), had no effect on the formation of ubiquitin chain in Msx1 homeoprotein. Moreover, forced expression of HA-PIAS1 increased the stability of *Flag-Msx1* protein but HA-PIAS1 (445-651) appeared to have little effect on the half-life of *Flag-Msx1* protein in cells (Fig. 4B). Finally, we asked whether increased stability is correlated with the activity of Msx1 as a transcriptional repressor using *MyoD* promoter luciferase assay (Fig. 4C). Indeed, we found increased repressor activity of Msx1 in the presence of HA-PIAS1 in *MyoD* promoter driven transcription, while HA-PIAS1 alone did not affect transcription in our reporter assay. Notably, two mutants [HA-PIAS1 (W372A) and HA-PIAS1 (1-434)] that are defective of SUMO ligase activity but capable of interacting with Msx1 modestly increased the activity of Msx1 as a transcriptional repressor but HA-PIAS1 (445-651) failed to do so, suggesting that increased stability of Msx1 through interaction with PIAS1 is correlated, at least in part, with its transcriptional repressor activity.

DISCUSSION

Properly controlled cellular differentiation is achieved by coordinated actions of cell type specific transcription factors as well as ubiquitously expressed coregulators. Among those are Msx homeoproteins that act as a transcription repressor and regulate the differentiation of a variety of progenitor cells including skeletal muscle progenitor cells by suppressing the expression of target genes. Expression studies on Msx1 have clearly shown its inverse correlation with cellular differentiation and forced expression of it attenuates differentiation of many cell types, supporting the idea of Msx1 being associated with proliferative undifferentiated progenitor cells. However, despite of considerable effort devoted to understand the spatiotemporal variations in Msx1 expression, underlying mechanisms by which protein levels are regulated post-transcriptionally have not yet been defined. In this study, we demonstrated an important role of PIAS1 in regulating poly-ubiquitination of Msx1, which is thought to direct the proteasome-mediated degradation of Msx1 homeoprotein. Involvement of E3 SUMO ligases in the regulation of ubiquitination has been implicated in several previous studies and thus its role in protein stability control is not unprecedented (Desterro et al., 1998; Lin et al., 2003). However, in contrast with previous reports showing that effect of SUMO ligase like PIAS1 in protein ubiquitination is largely indirect and mediated by SUMOylation that competes for the same acceptor lysine residues (Desterro et al., 1998; Lin et al., 2003), our current study is of particular interest in that inhibition of ubiquitination by PIAS1 does not require its E3 SUMO ligase activity and is not mutually exclusive with post-translational modification by SUMO.

While our ubiquitination assay using Msx1 derivatives with deletion or point mutations revealed that C-terminal domain of

Msx1 is crucial for ubiquitination, we were unable to determine the preferred acceptor sites for ubiquitination. This can be partly attributed to the previous notion that other lysine residues can be an alternative cryptic target for ubiquitination when bona fide *in vivo* ubiquitin acceptor sites are not available (Fung et al., 2005).

Different domains of PIAS proteins are known to contribute to the interactions and functions of their protein partners. The N-terminal SAP domain mediates protein-protein interactions with p53, Msx1, and nuclear receptors (Lee et al., 2006; Schmidt and Muller, 2002; Sentis et al., 2005). It also binds to nuclear matrix attachment region (MAR) DNA, playing a role in nuclear architecture (Sachdev et al., 2001). On the other hand, SP-RING domain located in the central part of PIAS proteins mediates SUMOylation of its interacting proteins (Rytinki et al., 2009). Our results along with previous studies highlight the important roles of SAP domain for the functions of Msx1 homeoprotein (Lee et al., 2006), which include proper localization of Msx1 at nuclear periphery, selective finding of target genes and sites *in vivo*, and steady-state protein levels.

Expression patterns of both Msx1 and PIAS1 are largely overlapped during development. Moreover, they are transiently expressed in undifferentiated C2C12 myoblast cells and their expressions decreased as C2C12 myoblast cells undergo terminal differentiation into myotube (Lee et al., 2006). Although we do not yet fully understand biological functions of PIAS1-mediated regulation of protein ubiquitination, especially during myogenic differentiation, we speculate that inhibition of myogenic differentiation by Msx1 is controlled by PIAS1 at multiple levels. In undifferentiated C2C12 myoblast cells, expression of PIAS1 protects Msx1 from ubiquitination, and thereby prevents protein degradation, which would allow C2C12 myoblast cells maintain undifferentiated state. In differentiating myoblast cells where expression of PIAS1 is decreased, however, Msx1 can no longer be protected from ubiquitination. Finally, as shown in a recent study that provided evidences for possible association of boston-type craniosynostosis with a mutation that affect ubiquitination of Msx2 proteins, our results suggest that aberrant ubiquitination of Msx1 might be involved in human congenital diseases such as cleft palate and hypodontia, which can be attributed by at least in part abnormal level of Msx1 protein.

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