Sumoylation of the basic helix-loop-helix transcription factor Sharp-1 regulates recruitment of the histone methyltransferase G9a and function in myogenesis

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*** Running title**: Sumoylation of Sharp-1 regulates myogenic differentiation

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Key words: Dec2/Sharp-1, skeletal muscle, differentiation, sumoylation, G9a

Capsule

Background: Sharp-1 inhibits skeletal muscle differentiation.

Results: SUMO modification impacts of Sharp-1 mediated inhibition of myogenesis.

Conclusion: Sumoylation acts as a signal for recruitment of the chromatin modifier G9a.

Significance: These studies link sumoylation with chromatin structure and myogenic differentiation.

Summary

Sumoylation is an important post-translational modification that alters the activity of many transcription factors. However, the mechanisms which link sumoylation to alterations in chromatin structure that culminate in tissue specific gene expression are not fully understood. In this study, we demonstrate that SUMO modification of the basic helix-loop-helix transcription factor Sharp-1 is required for its full transcriptional repression activity and function as an inhibitor of skeletal muscle differentiation. Sharp-1 is modified by sumoylation at two conserved lysine residues 240 and 255. Mutation of these SUMO acceptor sites in Sharp-1 does not impact its sub-cellular localization, but attenuates its ability to act as a transcriptional repressor and inhibit myogenic differentiation. Consistently, co-expression of the SUMO protease SENP1 with wild type Sharp-1 abrogates Sharp-1 dependent inhibition of myogenesis. Interestingly, sumoylation acts as a signal for recruitment of the co-repressor G9a. Thus, enrichment of histone H3 lysine 9 dimethylation (H3K9me2), a signature of G9a activity, is dramatically reduced at muscle promoters in cells expressing sumoylation defective Sharp-1 compared to wild type Sharp-1. Our findings demonstrate how sumoylation of Sharp-1 exerts an impact on chromatin structure, and transcriptional repression of muscle gene expression through recruitment of G9a.

Introduction

Small Ubiquitin-like Modifier (SUMO) is one of the best-characterized members of ubiquitin-like proteins involved in regulation of transcription factors (1-3). There are four SUMO isoforms in mammals (SUMO-1, SUMO-2 SUMO-3 and SUMO-4). Like ubiquitin, SUMO is also ligated to lysine residues in target proteins, and the target lysine usually embedded within a canonical consensus ΨKXE, where Ψ is a hydrophobic amino acid, K is the acceptor lysine for covalent conjugation of SUMO, X is any amino acid and E is glutamic acid. SUMO is covalently attached to substrates through the activities of an enzyme cascade similar to the ubiquitination cycle: SUMO is activated by the E1 activation enzyme, transferred to the sole E2 enzyme Ubc9, which then conjugates to the substrate by a specific E3 ligase. Proteins from the PIAS (protein inhibitor of activated STAT) family, RanBP2 (Ran-binding protein 2) and Pc2 (Polycomb 2) have been identified as SUMO E3 ligases (3-5). Sumoylation is a highly dynamic and reversible modification with substrates undergoing rapid conjugation and de-conjugation. The removal of SUMO is catalysed by SUMO-specific isopeptidases of SENPs/ SUSP family (2,6). Despite the similarity between sumoylation and ubiquitination pathway, the functional consequences of these two modifications are quite different. Unlike ubiquitination which primarily facilitates the target protein for degradation, sumoylation has diverse effects including regulation of protein-protein interactions, sub-cellular localization, protein stability, and alteration of transcriptional activity of substrate proteins. Transcription factors are the largest group of target proteins whose functions are modified by sumoylation, and in most studies reported, sumoylation poses a negative effect on the activities of transcription factors (7-9).

Sharp-1 is a basic helix-loop-helix (bHLH)-Orange domain containing transcriptional repressor that is expressed in many cell types during embryonic development as well as in adult tissues (10-14). Sharp-1 binds to class B E-Box sites CACGTG with high affinity to repress transcription of target genes (15,16). Unlike related Hey and Hes sub-family

members which recruit the co-repressor transducin-like enhancer of split (TLE)/Groucho through a WRPW motif, Sharp-1 lacks the WRPW motif and associates with distinct co-repressors including histone deacetylase 1 (HDAC1) and the lysine methyltransferase G9a (12,17).

The myogenic regulatory factor MyoD plays a central role in differentiation of skeletal muscle precursor cells. MyoD heterodimerizes with ubiquitously expressed E proteins and binds to E-box sequences (CANNTG) present in promoters of muscle genes to turn on their expression. Sharp-1 expression is modulated during myogenesis, being high in undifferentiated cells and declines during differentiation. Both gain of function and loss of function studies have shown that Sharp-1 impairs myogenic differentiation through antagonism of MyoD (10,17). The mechanisms underlying Sharp-1 dependent inhibition of differentiation include dimerization with MyoD and E proteins. In addition, we have recently shown that Sharp-1 interacts with G9a, a lysine methyltransferase that mediates repressive histone H3 lysine 9 di-methylation (H3K9me2) marks and recruits it to MyoD target promoters. Consistent with the recruitment of G9a, enrichment in H3K9me2 is apparent at MyoD target promoters in Sharp-1 overexpressing cells. Moreover, MyoD methylation at lysine 104 (K104) is also enhanced by G9a (10,18). Thus inhibition of G9a expression or activity partially rescues Sharp-1-dependent repression of myogenesis (17). While these studies have implicated G9a as a mediator of Sharp-1-dependent inhibition of myogenesis, the molecular mechanisms that regulate its recruitment by Sharp-1 are unclear.

In this study, we provide evidence that SUMO modification of Sharp-1 serves as a platform for recruitment of the co-repressor G9a, and its ability to inhibit myogenesis. We demonstrate that Sharp-1 is sumoylated at two highly conserved lysine residues K240 and K255, which is further enhanced by PIAS3 and PIASxα. Mutation of these lysine acceptor sites in Sharp-1 (Sharp-1 2KR) abolishes sumoylation without any impact on its sub-cellular localization. However, in contrast to wild type Sharp-1 which inhibits MyoD transcriptional activity and myogenic differentiation, the sumoylation defective mutant Sharp-1 2KR is significantly less efficient at blocking MyoD and myogenesis. Interestingly, unlike wild type Sharp-1, Sharp-1 2KR exhibits a markedly reduced association with G9a, and is insensitive to inhibitors of G9a activity. Taken together, these studies reveal a key role for SUMO modification of Sharp-1 in the recruitment of G9a and inhibition of myogenic differentiation.

Experimental Procedures

Cell culture and differentiation assays. C2C12 cells were cultured and maintained in growth medium (GM) consisting of Dulbecco's modified Eagle's medium (DMEM) with 20% fetal bovine serum (FBS; Hyclone). HEK293 and C3H10T1/2 (10T1/2) cells were maintained in DMEM supplemented with 10% FBS (Gibco), and COS-7 cells in DMEM with 10% calf serum (Hyclone).

C2C12 cells were co-transfected with a 1:9 ratio of an expression vector for pCS2, Sharp-1 or Sharp-1 2KR and pBabe (which confers resistance to puromycin). To test the role of sumoylation, SENP1 was transfected as indicated. 48 h after transfection, cells were selected in medium containing 2 μg/ml puromycin for two days. Selected cells were differentiated in differentiation medium (DM), consisting of DMEM plus 2% horse serum (Hyclone). For myogenic conversion assays, 10T1/2 cells were transfected with equivalent levels of MyoD alone, or with Sharp-1 or Sharp-1 2KR. Undifferentiated (Day 0) and differentiated cells (Day 6) were harvested for analysis of protein lysates by western blot, and fixed for immunofluorescence assays. To quantify differentiation, myogenic index was calculated as the ratio of nuclei in MHC^{+} myotubes/total nuclei across 4 different microscopic fields. 700-1000 nuclei were counted.

Immunofluorescence assays: For differentiation assays, cells were washed with PBS and fixed in ice cold 4% paraformaldehyde. After permeabilization, cells were incubated with anti-MHC (MY32) (Sigma) antibody and detected with secondary antibody coupled with Alexa Fluor (Molecular Probes). Slides were mounted in Vectashield mounting medium (Vector laboratories) containing DAPI (4′, 6′-

diamidino-2-phenylindole) to stain nuclei. Images were captured using a Nikon Eclipse TE 2000-U fluorescence microscope using MetaMorph software (version 7.0r3). COS-7 cells were used to examine subcellular localization of Myc-tagged Sharp-1 and Sharp-1 2KR. Cells were seeded at a density of $1x10^4$ cells/well in 6-well plates. 24 h later, cells were transfected with Myc-Sharp-1 and Myc-Sharp-1 2KR, or additionally co-transfected with SUMO-1. 48 h after transfection, cells were fixed, incubated with mouse anti-Myc antibody and detected with Texas-red (Invitrogen) coupled secondary antibody. Cells were visualized on a Zeiss LSM 510 META confocal laser-scanning microscope.

Plasmids and mutagenesis: Flag-mPIAS1, Flag-mPIAS3 Flag-mPIASxα, Flag-mPIASy, SUMO-1, SENP1 were kindly provided by Martin Lee (19). pCS2-Myc-Sharp-1 has been described (17). To mutate potential sumoylation residues from lysine (K) to arginine (R) in Sharp-1, QuickChange™ site-directed mutagenesis kit (Stratagene) was used. The primers for generating pCS2-Myc-Sharp-1 K240R are: 5'-CGC GCG GCC GTC CGA CAG GAG CCA CCC-3' and 5'-GGG TGG CTC CTG TCG GAC GGC CGC GCG-3'; primers for Sharp-1 2KR are: 5'-CCC AAG AGG CCG CGA CTG GAG GCG CGC-3' and 5'-GCG CGC CTC CAG TCG CGG CCT CTT GGG-3'. The cDNA was sequenced entirely to confirm the presence of directed mutations.

Immunoprecipitation and western blotting: To detect sumoylation, expression vectors were transfected using Lipofectamine™2000 as described (20). Cells were collected 48 h after transfection and lysed in presence of 20 mM NEM (N-ethylmaleimide (Sigma) using an ice-cold lysis buffer (50mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.5 mM PMSF and protease inhibitors (Roche). Lysates were analyzed by western blotting using the following antibodies: anti-SUMO-1, anti-Myc, anti-Flag and anti-β-actin. For co-immunoprecipitation assays, Myc-tagged Sharp-1 and Sharp-1 2KR were transfected into C2C12 cells. Lysates were immunoprecipitated using Myc-agarose beads and analyzed for association with endogenous MyoD by western blotting using anti-MyoD (Santa Cruz) or endogenous G9a using anti-G9a antibody (Cell Signaling).

Chromatin Immunoprecipitation assays (ChIP): C2C12 cells were transfected with vector alone, Sharp-1 or Sharp-1 2KR, and SENP-1. ChIP assays were performed using a kit as described by the manufacturer (Upstate) with 2 µg H3K9me2 antibody (Millipore), 10µl of G9a antibody (Abcam), 2 µg anti-Sharp-1 antibody (Santa Cruz). DNA was amplified with primers specific to myogenin promoter as described previously (17).

Luciferase assays: HEK293T cells were transfected with 9E-TK-luc reporter along with Sharp-1 or Sharp-1 2KR. 10T1/2 cells were transfected with pMyogLuc promoter reporter, MyoD, Sharp-1 and Sharp-1 2KR as indicated in the figures together with 5 ng of Renilla luciferase. 24 h after transfection, UNC0638 (Sigma) was added for 24 h, after which cells were lysed and assayed using the dual-luciferase reporter assay system (Promega). Each transfection was performed in triplicates, and repeated at least twice. Values were reported as means with standard deviation (shown as error bars).

Statistical analysis: Error bars indicate mean ± standard deviation (S.D.). Statistical analysis was performed using Student's t-test and p values <0.05 were considered statistically significant.

Results

Sharp-1 is SUMO modified

We have recently reported that Sharp-1 interacts with the co-repressor G9a to inhibit differentiation of skeletal muscle precursor cells (17). The interaction mapped to amino acid residues 173-265 in Sharp-1. Further examination of this region revealed two highly conserved lysine residues that perfectly matched the consensus sumoylation motif: lysine (K) 240 in the sumoylation motif VKQE; and K255 in the sequence PKLE (Fig. 1*A*). To determine whether Sharp-1 undergoes sumoylation, we transiently co-transfected HEK293 cells with Myc-Sharp-1 and SUMO-1. Cells were lysed in the presence of N-ethylmaleimide (NEM), an inhibitor of SUMO hydrolases, and lysates were immunoprecipitated with Myc-agarose beads followed by western blotting with anti-SUMO-1 antibody. Interestingly, in the presence of

SUMO-1, Sharp-1 appeared to be sumoylated (Fig. 1*B*). Furthermore, in presence of co-transfected SENP1 (Sentrin-specific protease1), which is able to remove SUMO conjugates from target proteins, Sharp-1 sumoylation was almost abolished. These results confirmed that Sharp-1 is SUMO conjugated in cells.

To validate that K240 and K255 are bonafide sumoylation sites, we generated point mutants changing the putative target lysine residues to arginine with site-directed mutagenesis. These mutants as well as wild type Sharp-1 were co-expressed with SUMO-1 in cells. Immunoprecipitation and western blotting analysis revealed that mutation of K240 alone (K240R), or both K240 and K255 (2KR) to arginine abrogated sumoylation even in the presence of SUMO-1 indicating that K240 is the major site for SUMO modification of Sharp-1 (Fig. 1*C*).

Most sumoylation reactions are enhanced by specific SUMO E3 ligases, of which the PIAS family proteins have been well-characterized (5). To determine whether PIAS proteins regulate Sharp-1 sumoylation, we co-transfected cells with Myc-Sharp-1, SUMO-1 and Flag-PIAS1, PIAS3, PIASxα, and PIASy. Cell lysates were immunoprecipitated with Myc-agarose beads, followed by western blotting with anti-SUMO-1 antibody. Overexpression of PIAS3 and PIASxα enhanced sumoylation of Sharp-1, whereas the presence of PIAS1 and PIASy had a minimal impact, suggesting that PIAS3 and PIASxα act as E3 SUMO ligases for Sharp-1 (Fig. 1*D*). To examine whether sumoylation of Sharp-1 is physiologically relevant in myogenesis, we first sought to determine whether Sharp-1 is SUMO conjugated in muscle cells. C2C12 myoblasts were co-transfected with Myc-Sharp-1 and SUMO-1. Cell lysates from undifferentiated cells and 24 h after induction of differentiation were immunoprecipitated with Myc-agarose beads followed by western blotting with anti-SUMO-1 antibody. Sumoylation of Sharp-1 was detected in undifferentiated C2C12 cells, and was significantly reduced upon differentiation (Fig. 1*E*). To further validate this finding, endogenous Sharp-1 was immunoprecipitated from undifferentiated and differentiated C2C12 cells. Consistent with the previous results, endogenous Sharp-1 was SUMO conjugated to higher levels in undifferentiated cells compared to differentiated cells (Fig. 1*F*).

Sub-cellular localization of Sharp-1 is not altered by SUMO modification

Since sumoylation has been shown to affect sub-cellular distribution of a number of target proteins, we analyzed localization of Sharp-1 and Sharp-1 2KR. Cells were transfected with Myc-Sharp-1 or Myc-Sharp-1 2KR in the absence and presence of SUMO-1, and visualized by confocal microscopy. Both proteins showed almost identical patterns of localization (Fig. 2*A*), suggesting that the nuclear localization of Sharp-1 is independent of its sumoylation status. To test the impact of sumoylation in Sharp-1 mediated transcriptional repression, cells were transfected with 9E-TK-Luc, a reporter harbouring Sharp-1 binding sites (21) . Consistent with previous reports (17), overexpression of wild type Sharp-1 significantly repressed reporter activity. In contrast, Sharp-1 2KR was considerably less potent in mediating transcriptional repression of the reporter (Fig. 2*B*).

Sumoylation of Sharp-1 is required to inhibit myogenic differentiation

We have previously found that Sharp-1 inhibits the differentiation of skeletal muscle precursor cells (10,17). To investigate the biological relevance of Sharp-1 sumoylation, we tested the possibility that sumoylation is involved in this process. C2C12 cells were co-transfected with Myc-tagged Sharp-1, Sharp-1 2KR together with the puromycin resistance vector pBabe. Both Sharp-1 and Sharp-1 2KR were expressed at similar levels (Fig. 3*A*). After selection, cells were analyzed for their ability to differentiate relative to control vector expressing cells. Consistent with our previous reports (10, 17), overexpression of Sharp-1 resulted in significant inhibition in myogenic differentiation as evidenced by a reduced number of terminally differentiated cells expressing myosin heavy chain (MHC) compared with vector-transfected cells (Fig. 3*B, C*). Interestingly, in contrast to wild type Sharp-1, the SUMO-defective mutant Sharp-1 2KR did not inhibit differentiation as efficiently as Sharp-1 (Fig. 3*B*, *C*). To confirm this finding, the expression of Troponin T, a differentiation marker was examined.

Consistently, Troponin T levels were reduced in Sharp-1 expressing cells, while Sharp-1 2KR expressing cells did not show a significant change in the expression of this marker at late stages of differentiation (Fig. 3*D*).

To further confirm that Sharp-1-mediated muscle differentiation inhibition is SUMO-dependent, we investigated whether de-sumoylation of Sharp-1 resulted in a phenotype similar to Sharp-1 2KR expressing cells. The SUMO protease SENP1 was co-transfected with equivalent levels of Sharp-1 and Sharp-1 2KR in C2C12 cells (Fig. 4*A*). Consistent with a role for sumoylation, inhibition of myogenesis by Sharp-1 was partially reversed with the addition of SENP1 (Fig. 4*B*, *C*). On the other hand, expression of SENP1 had no impact on Sharp-1 2KR. We then investigated the molecular mechanisms underlying the differential impact on differentiation between Sharp-1 and Sharp-1 2KR. We have recently demonstrated that Sharp-1 recruits G9a, and correspondingly H3K9me2, is enhanced in Sharp-1 overexpressing cells (17). We therefore analyzed H3K9me2 in cells expressing Sharp-1 and Sharp-1 2KR. Consistent with our recent report, H3K9me2 was enriched in C2C12 cells expressing Sharp-1 compared to control cells. Interestingly however, no enrichment was observed in cells expressing equivalent levels of Sharp-1 2KR and Sharp-1 co-transfected with SENP1 (Fig. 4*D*). Corresponding with enrichment of H3K9me2 marks, G9a occupancy was higher in Sharp-1 overexpressing cells relative to Sharp-1 2KR cells. However, no significant differences were apparent in Sharp-1 recruitment between the two cells lines (Fig. 4*E,F*). Together, these results demonstrate that sumoylation of Sharp-1 is important for its ability to inhibit myogenic differentiation and for enrichment of G9a-dependent H3K9me2 marks at the myogenin promoter. These results also suggest that sumoylation may be important for recruitment of G9a.

Sharp-1 sumoylation is essential for suppression of MyoD transcriptional activity and function

Since our previous studies have shown that Sharp-1 inhibits MyoD function (17), we examined the impact of Sharp-1 sumoylation specifically on MyoD transcriptional activity and MyoD-dependent myogenic conversion. 10T1/2 cells were transfected with MyoD alone, or with equivalent amount of Sharp-1 and Sharp-1 2KR (Fig. 5*A*). MyoD dependent myogenic conversion was inhibited in cells expressing MyoD and Sharp-1. On the other hand, Sharp-1 2KR expressing cells differentiated better and exhibited a higher percentage of MHC^+ cells (Fig. 5*B*) and increased myogenic index (Fig. 5*C*), indicating that Sharp-1 2KR was less potent in the repression of MyoD function. Consistent with the effect on myogenic differentiation, Troponin T expression was reduced to a greater extent in Sharp-1 overexpressing cells relative to control cells but no such reduction was seen in cells expressing Sharp-1 2KR (Fig. 5*D*). We then tested the impact of Sharp-1 sumoylation on MyoD transcriptional activity which is inhibited by Sharp-1 (10,18). 10T1/2 cells were transfected with the myogenin promoter reporter pMyog-Luc (22) along with MyoD alone, or together with Sharp-1 or Sharp-1 2KR. Cell extracts were analyzed for luciferase activity. As reported previously, Sharp-1 significantly repressed the myogenin promoter, whereas the 2KR mutant was less effective in repression (Fig. 5*E*). To examine whether reduced transcriptional repression by Sharp-1 2KR may be a consequence of impaired association with MyoD, we examined interaction of wild type and sumoylation defective Sharp-1 with endogenous MyoD. C2C12 were transfected with equivalent levels of Myc-tagged Sharp-1 and Sharp-1 2KR. Lysates were immunoprecipitated and examined for association with MyoD (Fig. 5*F*). Consistent with our previous reports Sharp-1 interacted with MyoD (10), and no significant difference was apparent in the ability of Sharp-1 2KR to interact with MyoD. These data indicate that sumoylation is not involved in the association of Sharp-1 and MyoD, and support the notion that sumoylation impacts Sharp-1 dependent transcriptional repression.

SUMO modification of Sharp-1 is essential for its interaction with G9a

We have recently demonstrated that Sharp-1 interacts with the co-repressor G9a through the region spanning the sumoylation motifs (17). Several reports suggest that protein-protein interactions are SUMO modification-dependent. A SUMO Binding Motif (SBM)

consensus sequence V/I-X-V/I-V/I present in many proteins is known to be important for their recruitment by SUMO modified transcription factors (23). To investigate the mechanisms that render non-SUMO modified Sharp-1 a less efficient suppressor of myogenesis, we examined the cDNA sequence of G9a. Interestingly, we identified a SBM consensus ID/EVI which is conserved in G9a from various species (Fig. 6*A*). Moreover, G9a SBM is within its ANK repeats sequence which is essential for G9a interaction with Sharp-1 (17). To examine if SUMO modification of Sharp-1 is essential for its interaction with G9a, co-immunoprecipitation assays were performed. 293 cells were co-transfected with Flag-G9a, Myc-Sharp-1 and Myc-Sharp-1 2KR. Consistent with previous studies (17), immunoprecipitation of Sharp-1 revealed its association with G9a (Fig. 6*B*). Interestingly, the association of Sharp-1 2KR with G9a was greatly reduced. To validate these findings in muscle cells, we examined the interaction of Sharp-1 and Sharp-1 2KR with endogenous G9a in C2C12 cells. Similar to the impact seen in 293 cells, Sharp-1 2KR exhibited reduced interaction with endogenous G9a in C2C12 cells compared to wild type Sharp-1 (Fig. 6*C*). To corroborate the findings that sumoylation regulates the interaction between G9a and Sharp-1, we examined the impact of SENP1 on the association of Sharp-1 with G9a. Consistent with the reduced interaction of G9a with Sharp-1 2KR, expression of SENP1 reduced association of wild type Sharp-1 and G9a (Fig. 6*D*). We then tested whether inhibition of G9a impacts SUMO-dependent transcriptional repression mediated by Sharp-1. To address this, the myogenin promoter reporter pMyog-Luc was transfected in 10T1/2 cells along with MyoD, Sharp-1 and Sharp-1 2KR. 24 h later, 0.25 μM UNC0638, a pharmacological inhibitor of G9a methyltransferase activity, was added. In presence of UNC0638, transcriptional repression mediated by Sharp-1 was reversed. On the other hand, Sharp-1 2KR-mediated repression was not significantly impacted by UNC0638 (Fig. 6*E*). Taken together, these results demonstrate that recruitment of G9a is sumoylation dependent and impacts the ability of Sharp-1 to mediate transcriptional repression of MyoD transcriptional activity and myogenic differentiation.

Discussion

In this study, we report that Sharp-1 undergoes SUMO-1 dependent sumoylation at conserved lysine residues K240 and K255. The E3 SUMO ligases PIAS3 and PIASxα enhance Sharp-1 sumoylation. Mutation of the SUMO acceptor lysine residues does not impact the sub-cellular localization of Sharp-1; however it does attenuate its transcriptional repression capacity, abrogate interaction with the chromatin modifier G9a, and impacts its function as a repressor of myogenic differentiation.

Like other members of the bHLH-O subfamily, Sharp-1 is a potent transcriptional repressor (10,12,17). However, the mechanisms underlying transcriptional regulation by Sharp-1 remain unclear. Our previous studies linked the transcriptional repression activity of Sharp-1 to its role as an inhibitor of cellular differentiation along the skeletal muscle as well as the adipocytic lineage (10,17,24). The transcriptional repression of myogenic differentiation by Sharp-1 relies on inhibition of MyoD transcriptional activity. This is achieved, in part, through dimerization with MyoD and E-proteins. In addition, Sharp-1 recruits the co-repressor G9a through a C-terminal region spanning amino acid residues 173-265. In support of this, disruption of the interaction with G9a results in reduced transcriptional repression of MyoD by Sharp-1. Moreover, overexpression of Sharp-1 results in enrichment of H3K9me2 marks that are mediated by G9a at MyoD target promoters. Conversely, loss of G9a expression or activity attenuates the ability of Sharp-1 to repress myogenic differentiation with a concomitant reduction of H3K9me2 occupancy. Collectively, these data demonstrate that G9a mediates transcriptional repression and inhibition of myogenesis by Sharp-1.

Sumoylation plays an important role in the regulation of transcription factor activity and function which includes an impact on protein stability, cellular localization, DNA-binding, and protein-protein interactions. An emerging theme among these is the role of sumoylation in transcriptional repression by facilitating assembly of complexes that regulate chromatin

accessibility and gene expression. Consistent with this notion, SUMO modification at K240 and K255 in Sharp-1 is essential for its full transcriptional repression activity. This is evidenced by the attenuated ability of the SUMO defective mutant Sharp-1 2KR in mediating repression of MyoD transcriptional activity and MyoD-dependent myogenic conversion of fibroblast cells compared to wild type Sharp-1. Similarly, co-expression of SENP1 with wild type Sharp-1 mimics the phenotype of Sharp-1 2KR cells. Interestingly, sumoylation of Sharp-1 at K240 and K255 creates an interface for recruitment of G9a, and possibly assembly of other chromatin modifiers/remodelling complexes. Thus loss of Sharp-1 sumoylation correlates with reduced G9a dependent H3K9me2 marks and repression of muscle promoters. These findings are in line with other reports that have demonstrated a link between SUMO modification of transcription factors and transcriptional repression via recruitment of co-repressors. Several studies have linked sumoylation with HDACs. For instance, SBM containing HDAC6 and HDAC2 are recruited by sumoylated p300 and Elk-1 respectively to target promoters to repress transcription (25, 26). Similarly, sumoylation of Stra13, a bHLH factor related to Sharp-1 facilitates interaction with HDAC1 (20). In addition to G9a, a previous study has demonstrated that Sharp-1 also interacts with HDAC1 and Sirt1 (12). However, in the case of Sharp-1, the interaction with HDAC1 and Sirt1 maps to a different region that include the C-terminus encompassing amino acids 265-410 for HDAC1, and the bHLH domain for Sirt1. Thus, the association and recruitment of HDAC1 and Sirt1 is not likely to be dependent on sumoylation of Sharp-1.

While many studies have shown the role of sumoylation in regulation of transcription factor activity, the role of sumoylation in control of cellular differentiation is largely unclear. During myogenic differentiation, the overall levels of SUMO modified proteins decline (27). While a few transcription factors such as the homeoprotein Msx1 and Myocyte enhancer factor (MEF2) are known to be sumoylated, the role of SUMO modification of these transcription factors in regulation of muscle gene expression is unclear. For instance, Msx1 is SUMO modified, and was recently shown to repress myogenesis by recruiting G9a (28,29). However the functional relevance of SUMO modification of Msx1 in recruitment of G9a, or in the control of myogenesis has not been reported. Similarly, the MEF2 transcription factors which play a key role during myogenesis by activating muscle specific genes are also known to be sumoylated. MEF2A is modified by SUMO-1, and MEF2C and MEF2D are modified by SUMO-2 and SUMO-3 (30,31). Sumoylation of MEF2 proteins represses their transcriptional activity, but whether it impacts their function in myogenesis remains to be clarified. A recent study demonstrated that Pax7 is SUMO modified, and sumoylation at K85 is important for Pax7 mediated repression of myogenesis and transactivation of selective target genes (32). However, the mechanisms by which sumoylation of Pax7 regulates its transcriptional activity remain to be investigated. In this regard, our findings identify a novel regulatory axis that links sumoylation to the control of skeletal muscle differentiation by regulation of G9a recruitment and consequently the transcriptional repression activity of Sharp-1.

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Figure Legends

Figure 1. Sharp-1 is SUMO modified. *A,* The domain structure Sharp-1 is shown with the basic, helix-loop-helix (HLH), and the Orange domains (upper panel). Potential sumoylation sites at K240 and K255 are indicated. Alignment of the two SUMO consensus motifs in Sharp-1 cDNA from human, mouse, rat, dog and xenopus showed high conservation across species. *B*, Cells were co-transfected with constructs encoding Myc-Sharp-1, SUMO-1 and SENP1 as indicated. Lysates were subjected to immunoprecipitation with Myc-agarose beads followed by immunoblotting with anti-SUMO-1 antibody. Anti-Myc antibody was used to detect expression of Sharp-1. β-actin served as a loading control. *C*, Cells were co-transfected with Myc-Sharp-1, Sharp-1 K240R, and Sharp-1 2KR along with SUMO-1. Lysates were immunoprecipitated with Myc-agarose beads followed by western blotting with anti-SUMO-1 antibody. *D*, Flag-tagged PIAS1, PIAS3, PIASxa and PIASy were co-transfected with Myc-Sharp-1 and SUMO-1. Cell lysates were immunoprecipitated with Myc-agarose beads and immunoprecipitates were subjected to western blotting with anti-SUMO-1 antibody. Sharp-1 and PIAS proteins were detected with anti-Myc and anti-Flag antibodies respectively. *E*, C2C12 cells were co-transfected with Myc-Sharp-1 and SUMO-1. Cells were harvested as undifferentiated cells (D0) and one day after differentiation (D1), immunoprecipitated with Myc-agarose beads followed by western blotting with anti-SUMO-1 and anti-Myc antibodies. *F*, C2C12 cells were transfected with SUMO-1. Endogenous Sharp-1was immunoprecipitated from D0 and D1 lysates using anti-DEC2 (Sharp-1) antibody followed by western blotting with anti-SUMO-1 antibody.

Figure 2. Sumoylation impacts Sharp-1 mediated transcriptional repression but not its sub-cellular localization. *A*, COS-7 cells were transfected with Sharp-1 and Sharp-1 2KR alone or together with SUMO-1. 48 h later, cells were fixed and stained with anti-Myc antibody. DAPI was used to stain and visualize nuclei. *B*, 293T cells were transfected with 9E-TK-Luc reporter (100ng) along with Sharp-1 (50ng) or Sharp-1 2KR (50ng) as indicated. 48 h later, cells were harvested and luciferase activity was measured. Error bars indicate mean±SD.

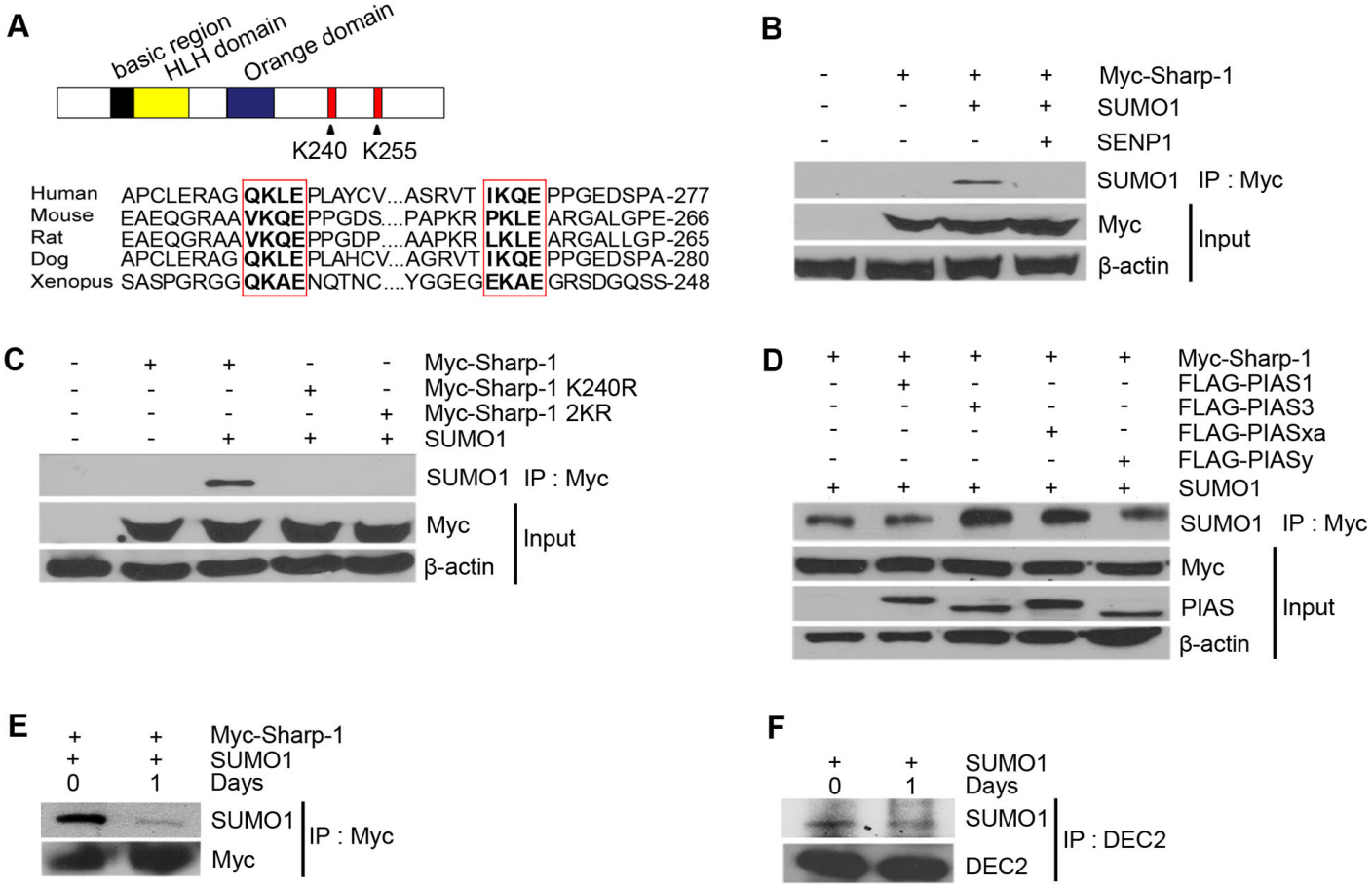
Figure 3. Mutation of sumoylation sites abrogates the ability of Sharp-1 to suppress myogenesis. *A*, C2C12 cells were co-transfected with Sharp-1 or Sharp-1 2KR along with a puromycin resistance vector. Empty vector (pCS2) was transfected in control cells. Sharp-1 and Sharp-1 2KR expression was determined by western blotting with anti-Myc antibody. *B-C*, MHC staining (*B*) and myogenic index (*C*) was assessed in Sharp-1 and 2KR expressing cells compared to control cells at Day 2 of differentiation. *D*, Cell lysates at Day 0, 1 and 2 of differentiation from control, Sharp-1 and Sharp-1 2KR cells were analyzed by western blotting using Troponin T antibody. Error bars indicate mean±SD.

Figure 4. SENP1 rescues Sharp-1 mediated suppression of myogenesis. *A,* C2C12 cells were transfected with vector alone (Control), Myc-Sharp-1, or Sharp-1 2KR individually or together with SENP1. Lysates were immunoblotted with anti-Myc antibody. *B-C,* After selection, differentiation was analyzed at Day 2 with anti-MHC antibody. Nuclei were stained with DAPI (*B*). Myogenic index was determined. *D*, ChIP assays were performed to determine H3K9me2 enrichment at the myogenin promoter in cells expressing vector, Sharp-1, Sharp-1 2KR and Sharp-1 along with SENP1.*E-F*, ChIP assay was performed to determine occupancy of G9a (E), and Sharp-1 (F) at the myogenin promoter in cells expressing vector alone, Sharp-1 and Sharp-1 2KR. Error bars indicate mean±SD.

Figure 5. SUMO modified Sharp-1 inhibits MyoD transcriptional activity and function. *A,* 10T1/2 cells transfected with MyoD, Myc-Sharp-1 and Myc-Sharp-1 2KR were analyzed for expression of MyoD

and Sharp-1 by western blotting. *B-D,* Myogenic conversion assays were performed in cells transfected with MyoD, MyoD and Sharp-1, or MyoD and Sharp-1 2KR. 6 days later, differentiated cells were stained with anti-MHC antibody (red). Nuclei were stained with DAPI (*B*). Differentiation was quantified by plotting myogenic index (*C*). Troponin T expression was assessed by western blotting (*D*). *E,* 10T1/2 cells were transfected with pMyog-Luc promoter (100ng) together with MyoD (50ng), Sharp-1 (25ng and 50ng) or Sharp-1 2KR (50ng) as indicated. 48 h later, cells were harvested and assayed for luciferase activity. Error bars indicate mean±SD. *F*, C2C12 cells were transfected with Myc-tagged Sharp-1 and Sharp-1 2KR. 24 hr later, lysates were immunoprecipitated and interaction with endogenous MyoD was analysed by western blotting with anti-MyoD antibody. Lysates (input) were immunoblotted with anti-Myc and anti-MyoD antibodies to detect Sharp-1and MyoD expression.

Figure 6. SUMO modification of Sharp-1 is critical for interaction with G9a. *A*, Alignment of G9a cDNA sequence from human, mouse, rat, and zebrafish revealed a conserved SBM (highlighted). *B*, Cells transfected with Flag-G9a, Myc-Sharp-1 or Myc-Sharp-1 2KR, were immunoprecipitated with Myc-agarose beads, and immunoblotted with anti-Flag and anti-Myc antibodies. Input (lysates) were analysed for G9a, and Sharp-1 expression. *C*, C2C12 cells were transfected with Myc-Sharp-1 or Myc-Sharp-1 2KR, immunoprecipitated with Myc-agarose beads and endogenous G9a interaction was analysed by immunobloting with anti-G9a antibody. Lysates (input) were immunoblotted with anti-G9a and anti-Myc antibodies to detect G9a and Sharp-1 expression. *D,* Cells were transfected with Myc-Sharp-1, Flag-G9a, alone or together with SENP1. Lysates were immunoprecipitated and immunoblotted with anti-Flag and anti-Myc antibodies. *E*, 10T1/2 cells were transfected with pMyog-Luc promoter (100ng) together with MyoD (50ng), Sharp-1 (50ng), Sharp-1 2KR (50ng) as indicated. 24 h after transfection, UNC0638 was added to cells expressing Sharp-1 and Sharp-1 2KR. 24 h later, cells were harvested and analyzed for luciferase activity. Error bars indicate mean±SD.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HLH domain
Orange domain
K240
K255
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Dog APCLERAG QKLE PLAHCV...AGRVT IKQE PPGEDSPA-280
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B
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β-actin
Input
C
Myc-Sharp-1
Myc-Sharp-1 K240R
Myc-Sharp-1 2KR
SUMO1
SUMO1 IP : Myc
Myc
β-actin
Input
D
Myc-Sharp-1
FLAG-PIAS1
FLAG-PIAS3
FLAG-PIASxa
FLAG-PIASy
SUMO1
SUMO1 IP : Myc
Myc
PIAS
β-actin
Input
E
Myc-Sharp-1
SUMO1
Days
SUMO1
Myc
IP : Myc
F
SUMO1
Days
SUMO1
DEC2
IP : DEC2

FIGURE 1

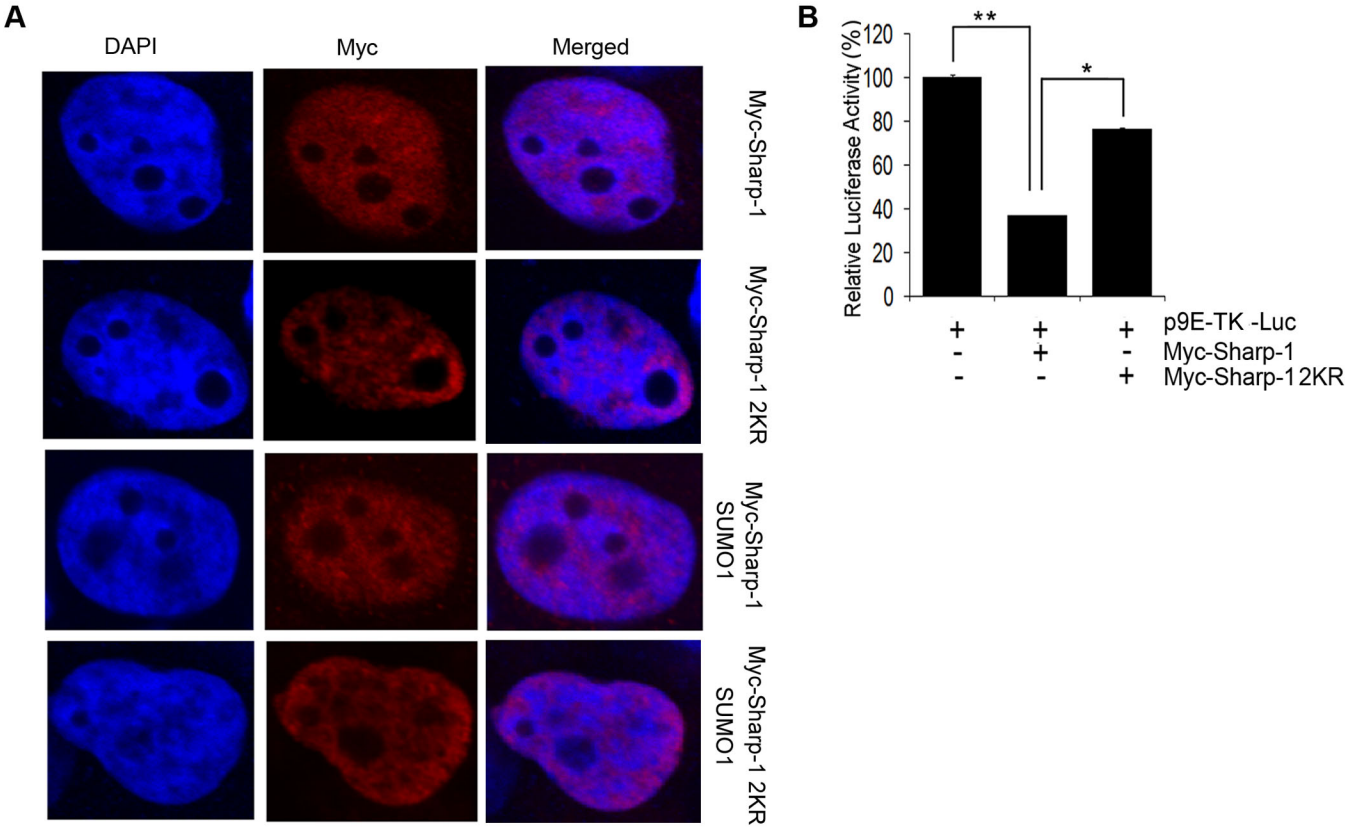

A
DAPI
Myc
Merged
Myc-Sharp-1
Myc-Sharp-1 2KR
Myc-Sharp-1 SUMO1
Myc-Sharp-1 2KR SUMO1
B
Relative Luciferase Activity (%)
0
20
40
60
80
100
120
**
*
+
-
-
+
+
-
+
-
+
p9E-TK -Luc
Myc-Sharp-1
Myc-Sharp-1 2KR

FIGURE 2

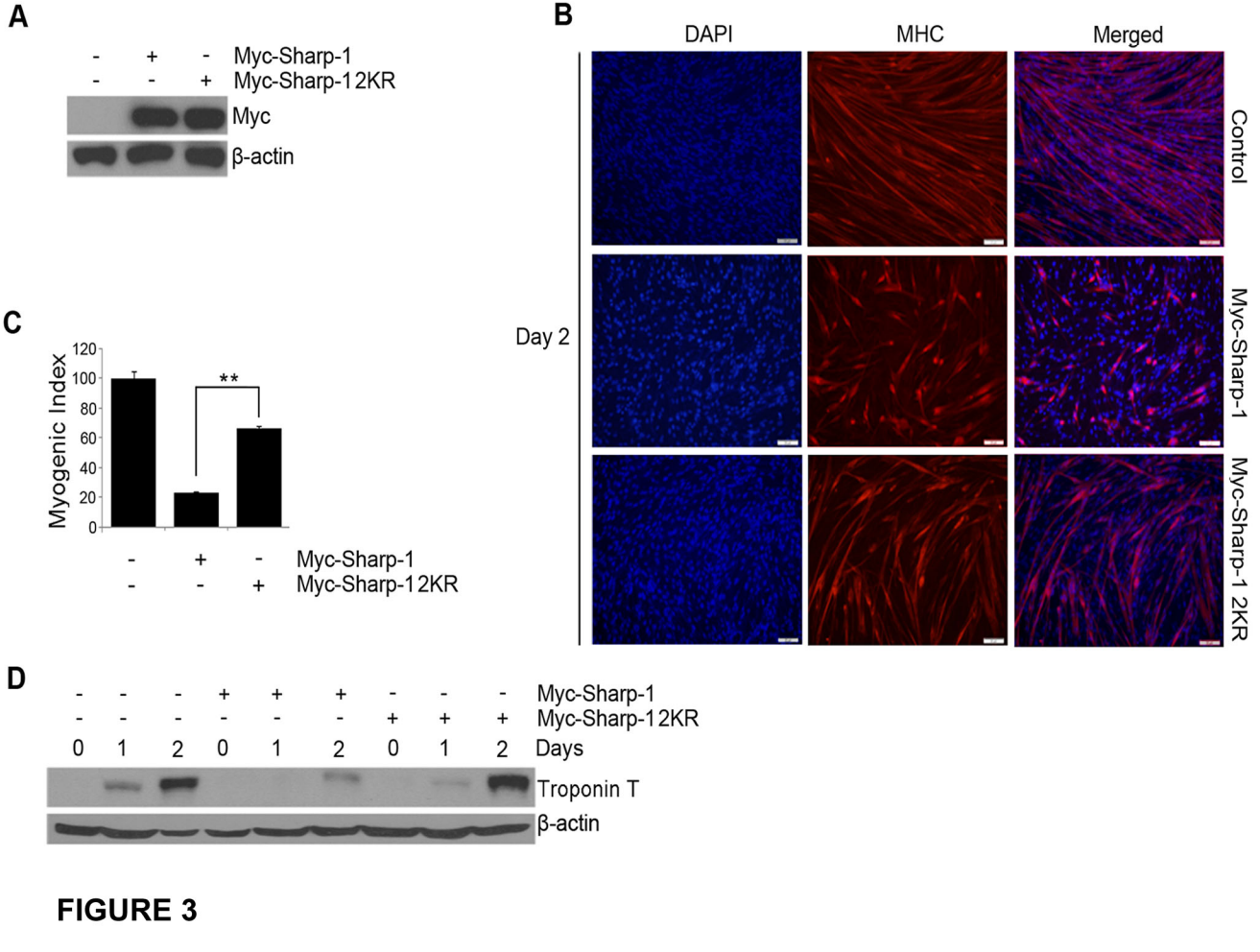

A
- + - Myc-Sharp-1
- - + Myc-Sharp-1 2KR
Myc
β-actin
B
DAPI
MHC
Merged
Day 2
Control
Myc-Sharp-1
Myc-Sharp-1 2KR
C
Myogenic Index
120
100
80
60
40
20
0
**
- + - Myc-Sharp-1
- - + Myc-Sharp-1 2KR
D
- - - + + + - - - Myc-Sharp-1
- - - - - - + + + Myc-Sharp-1 2KR
0 1 2 0 1 2 0 1 2 Days
Troponin T
β-actin

FIGURE 3

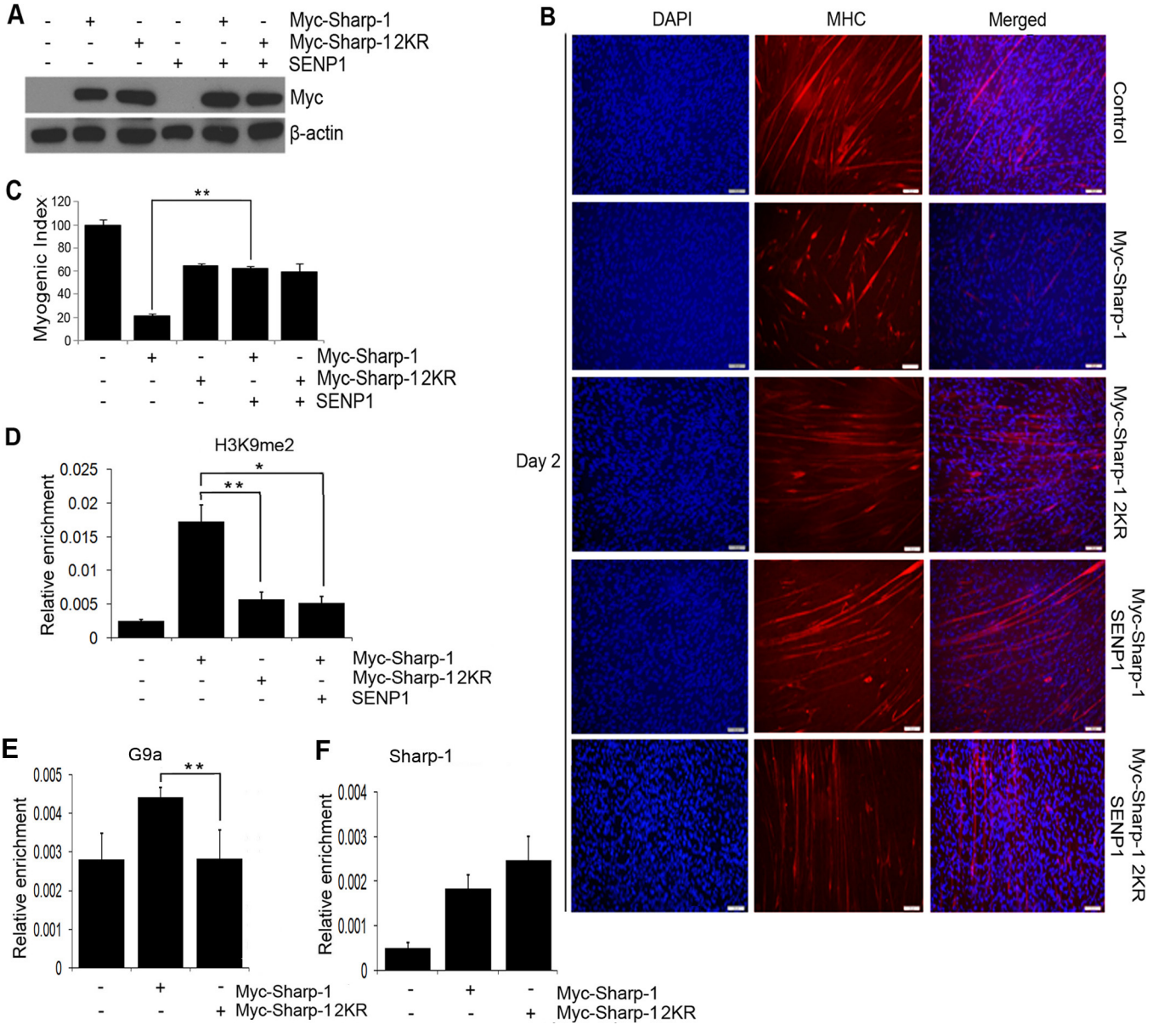

A
Myc-Sharp-1
Myc-Sharp-12KR
SENP1
Myc
β-actin
B
DAPI
MHC
Merged
Control
Myc-Sharp-1
Myc-Sharp-12KR
Myc-Sharp-1 SENP1
Myc-Sharp-12KR SENP1
Day 2
C
Myogenic Index
D
H3K9me2
Relative enrichment
E
G9a
F
Sharp-1

FIGURE 4

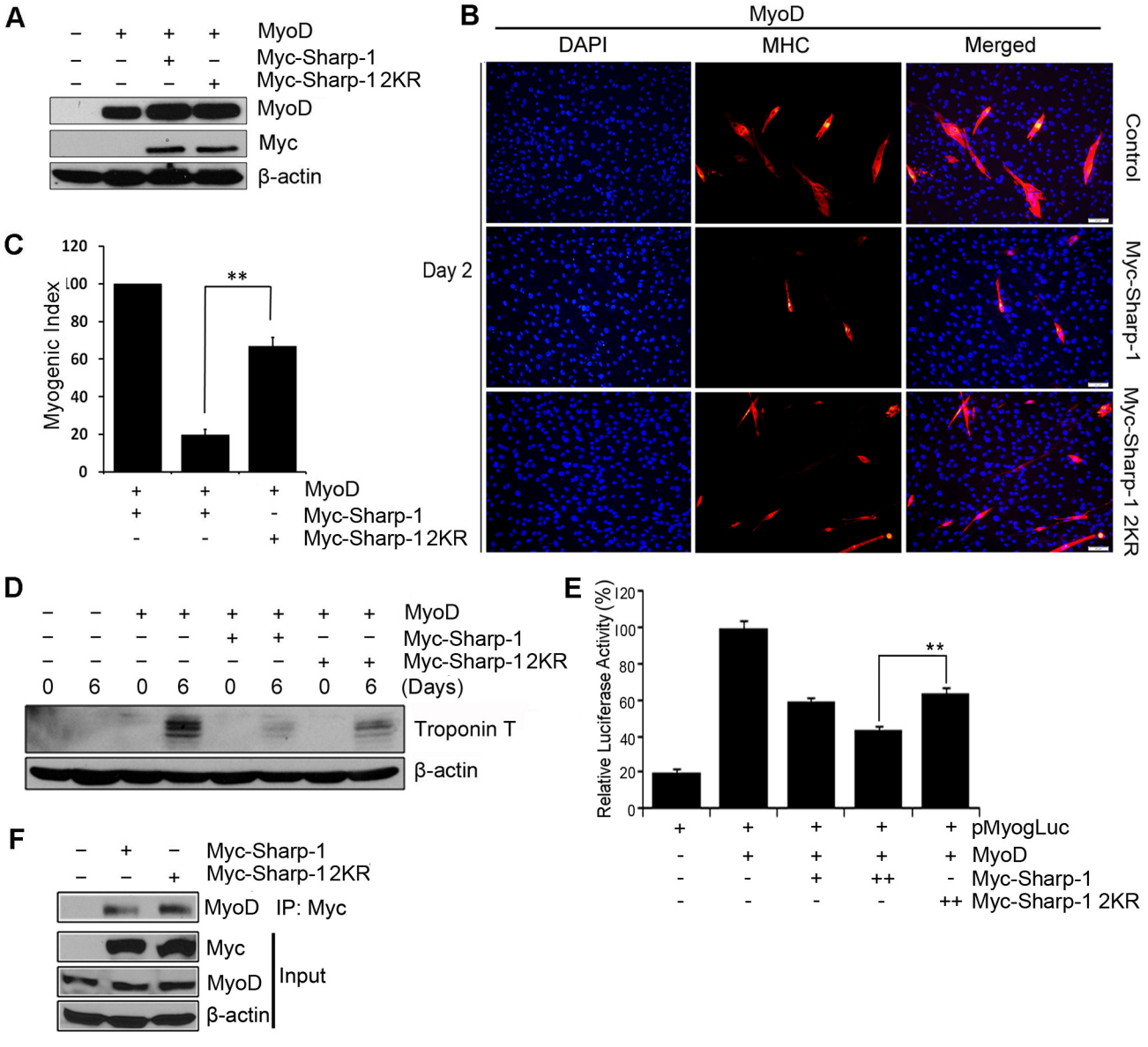
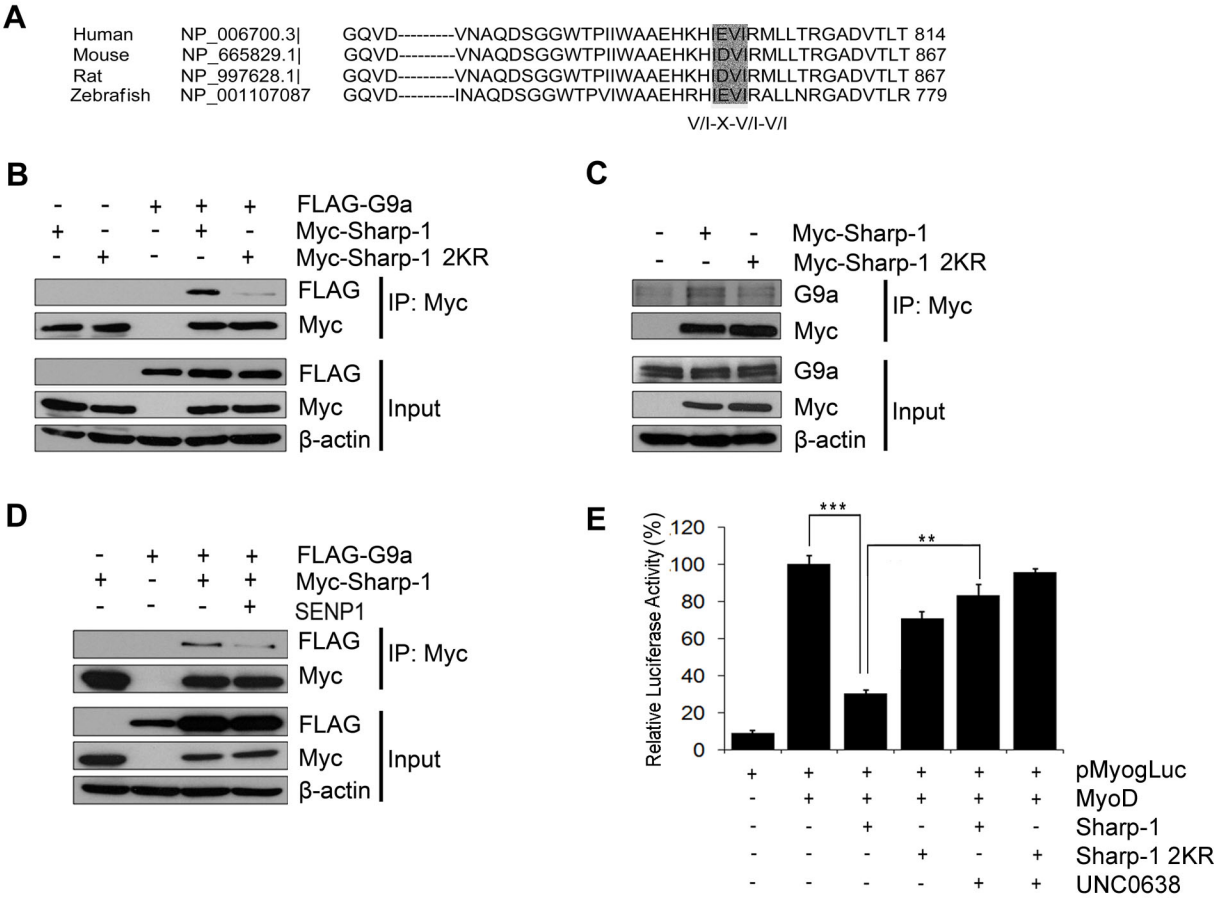

A
MyoD
Myc-Sharp-1
Myc-Sharp-1 2KR
MyoD
Myc
β-actin
B
MyoD
DAPI
MHC
Merged
Control
Day 2
Myc-Sharp-1
Myc-Sharp-1 2KR
C
Myogenic Index
120
100
80
60
40
20
0
**
MyoD
Myc-Sharp-1
Myc-Sharp-1 2KR
D
MyoD
Myc-Sharp-1
Myc-Sharp-1 2KR
0 6 0 6 0 6 0 6 (Days)
Troponin T
β-actin
E
Relative Luciferase Activity (%)
120
100
80
60
40
20
0
**
pMyogLuc
MyoD
Myc-Sharp-1
Myc-Sharp-1 2KR
F
Myc-Sharp-1
Myc-Sharp-1 2KR
MyoD IP: Myc
Myc
MyoD
β-actin
Input

FIGURE 5


A
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Mouse NP_665829.1| GQVD---------VNAQDSGGWTPIIWAAEHKHIDVIRMLLTRGADVTLT 867
Rat NP_997628.1| GQVD---------VNAQDSGGWTPIIWAAEHKHIDVIRMLLTRGADVTLT 867
Zebrafish NP_001107087 GQVD---------INAQDSGGWTPVIWAAEHRHIEVIRALLNRGADVTLR 779
V/I-X-V/I-V/I
B
FLAG-G9a
Myc-Sharp-1
Myc-Sharp-1 2KR
FLAG
Myc
IP: Myc
FLAG
Myc
β-actin
Input
C
Myc-Sharp-1
Myc-Sharp-1 2KR
G9a
Myc
IP: Myc
G9a
Myc
β-actin
Input
D
FLAG-G9a
Myc-Sharp-1
SENP1
FLAG
Myc
IP: Myc
FLAG
Myc
β-actin
Input
E
Relative Luciferase Activity (%)
120
100
80
60
40
20
0
***
**
pMyogLuc
MyoD
Sharp-1
Sharp-1 2KR
UNC0638

FIGURE 6