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J. Cell. Mol. Med. Vol 10, No 1, 2006 pp. 132-144

SUMO-1 modification of MEF2A regulates its transcriptional activity

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Received: February 8, 2006; Accepted: February 20, 2006

Abstract

Myocyte enhancer factor 2 (MEF2) transcription factors are crucial regulators controlling muscle-specific and growth factor-inducible genes. Numerous studies have reported that the activity of these transcription factors is tightly modulated by posttranslational modifications such as activation by specific phosphorylation as well as repression by class II histone deacetylases (HDACs). We hypothesized that MEF2 could also be regulated by covalent modification by SUMO-1, a reversible posttranslational modification which has been shown to regulate key proteins involved in cell proliferation, differentiation and tumor suppression. In this study, we demonstrate that MEF2A undergoes sumoylation primarily at a single lysine residue (K395) both *in vitro* and *in vivo*. We also show that the nuclear E3 ligase, PIAS1, promotes sumoylation of MEF2A. Mutation of lysine 395 to arginine abolishes MEF2A sumoylation and the sumoylation incompetent mutant protein has enhanced transcriptional activity compared to the wild type protein. Our results suggest that protein sumoylation could play a pivotal role in controlling MEF2 transcriptional activity.

Keywords: MEF2A • SUMO • PIAS • sumoylation • transcription

Introduction

Four vertebrate *MEF2* genes encode nuclear phosphoproteins (MEF2A - MEF2D) belonging to the MADS (MCM1, Agamous, Deficiens, Serum response factor) superfamily of DNA-binding proteins [1]. The MEF proteins bind as homo- and heterodimers to a *cis* element containing the consensus (C/T)TA(A/T)$_4$TA(G/A). These transcriptional regulatory proteins have been characterized as key regulators of muscle-specific gene expression and also have been implicated as nuclear targets for signaling cascades in response to serum stimulation and some cellular stressors [2]. Despite considerable evidence supporting the transcriptional role of MEF2 proteins in the activation of muscle genes, the mechanism of regulation of their transcriptional activity by several cellular signaling pathways awaits unveiling. Indeed, the ubiquitous presence of MEF2-related RNAs must be reconciled with the correlation of the tissue-specific MEF2 activity.

Covalent posttranslational modifications of proteins are rapid mechanisms for reversibly altering protein function. Posttranslational modification by SUMO-1 is emerging as an important regulatory mechanism for controlling gene expression and the maintenance of genomic stability [3–5]. Conjugation of SUMO-1 requires an E1-activating enzyme, a heterodimer formed by Aos1/Uba2 [6], and an E2 SUMO-conjugating enzyme known as

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Ubc9 [3–5]. A growing list of E3 enzymes, including RanBP2, Pc2 and the PIAS family proteins [7–9], has been identified. Although they do not appear to be essential for SUMO conjugation, they usually enhance the rate and degree of sumoylation of the substrate. SUMO modification is a transient regulatory mechanism provided by a dynamic equilibrium between conjugation and deconjugation. The last process is driven by enzymes known as isopeptidases that can specifically remove SUMO [3, 10].

Most of the reported SUMO targets are predominantly nuclear residents including transcription factors, transcription co-activators, transcription co-repressors, nuclear pore complex proteins, nuclear body proteins and genome integrity proteins [11, 12]. In these cases, sumoylation may affect subnuclear localization, transcription activation capacity, and protein stability by interfering with ubiquitination [3–5].

In this study we investigated whether protein sumoylation plays a role in regulating MEF2A transcriptional activity. We found that the MEF2 family harbors a sumoylation consensus sequence and we demonstrate that MEF2A can be sumoylated *in vitro* and *in vivo* at a single lysine residue, K395. Our data also indicate that covalent SUMO modification of MEF2A could inhibit its transcriptional activity. These data point toward a role for sumoylation as a regulatory mechanism to control expression of MEF2-dependent target genes.

Materials and methods

Plasmids and mutagenesis

pCMV-Sport6_hMEF2A and pCMV-Sport6-mUbc9 were purchased from Open Biosystem. The mutant MEF2A K395R was generated by a QuikChangeXL mutagenesis kit (Stratagene). pET23a-hUbc9, pET11d-Uba2, and pET28a-Aos1 were kind gifts from Dr. Frauke Melchior. SUMO-1 (1-97) cDNA, kindly provided by Drs. Seeler and Dejean, was subcloned into the NdeI and NotI sites of pET23a. Dr. Stephan Müller kindly provided expression plasmids for GST-PIAS1 and GST-PIASxβ along with the mammalian expression vector pCMV-Tag2B-PIAS1. A mammalian expression plasmid for pcDNA3.1-SuPr-1 was a generous gift from Dr. Len Zon. C-terminal Flag or Myc-tagged hMEF2A were constructed by subcloning into pRK5-myc or pRK5-Flag (gifts from Ying Zhang and Rik Derynck, UCSF). cDNA for SUMO-2 and SUMO-3 was purchased from ATCC. Flag-Tev-His-SUMO-1, 2, and 3 were generated by subcloning into the BamH1 and Not1 sites of pREX-SP6-LIC-FTH-IRES-GFP (vector details available upon request).

Cell culture and transfection

10T1/2 mouse fibroblasts (CCL-226) and C2C12 mouse myoblasts (CRL-1772) were purchased from ATCC and maintained in DMEM supplemented with L-glutamine, penicillin, streptomycin, and 10% fetal bovine serum. Myogenic differentiation was induced by serum deprivation. Briefly, cells were washed with PBS and the culture media was replaced with DMEM supplemented with 5% horse serum. For luciferase assays, 10T1/2 cells were plated at 8000 cells/cm^2 in triplicate and 24h after were transfected with FuGENE6 transfection reagent (Roche) with the indicated plasmids and concentrations.

Western blotting

For western blotting, cells were lysed on ice 48h post transfection with a buffer containing a nonionic detergent (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 20mM N-ethylmaleimide, 5% glycerol), 1mM PMSF, and a complete cocktail of protease inhibitors (Roche), rotated for 30 min at 4°C, and spun down at 4°C for 30 min at 20,000xg. Samples were quantified by Bradford assay and equal amounts of protein were resolved on an 8% PAGE gel and transferred to nitrocellulose membrane.

Blotting was performed according to standard conditions with anti-MEF2 (#sc-313) at 1:500 dilution, anti-Flag (Stratagene) at 1:2500, or anti-MEF2A (gift of Dr. Ron Prywes) at 1:500 and membranes were developed in either Super Signal WestDura (Pierce) or ECL Plus (Amersham) reagent. Blots developed in ECL Plus reagent were visualized by scanning at 520 nm with a Typhoon 8600 Variable Mode Imager in addition to standard chemiluminescent methods.

Fluorescence labeling of SUMO-1

Alexa Fluor 555 carboxylic acid, succinimidyl ester (Molecular Probes A-20009) was used to label the N-terminal amine of recombinant SUMO-1 according to the manufacturer's protocol. Reactions involving fluorescent labeled

SUMO-1 were visualized by scanning at wavelength 555 nm with a Typhoon 8600 Variable Mode Imager.

In vitro sumoylation assay

Recombinant human His-Aos1/Uba2, Ubc9, and SUMO-1 were purified as described previously [8]. *In vitro* sumoylation assays were conducted as described previously [8]. Briefly, 150 ng E1, 50 ng E2, 15 ng SUMO1, an ATP regeneration buffer, and either 6 µl [^{35}S]-labeled MEF2 substrate or 250 ng GST-fused MEF2 substrate were incubated for the indicated times and quenched with Laemmli buffer. 100 ng GST-PIAS1 was added where indicated.

In vitro protein-protein binding assays

Protein-protein binding assays were performed as described previously [13].

Reporter gene assays

The reporter construct pGL3_MM (artificial promoter containing 2 MEF2 binding sites and the firefly luciferase gene) [14] was kindly provided by Dr. Aidong Han. Cells were co-transfected with the reporter gene and several vectors to overexpress MEF2A with different regulators as are indicated in each figure. Transfection efficiency was determined by western blot. All cells were lysed in Passive Lysis Buffer (Promega) and 50µL of lysate were analyzed with a Luciferase Assay System (Promega). A Dynex Microtiter Plate Luminometer was used for detection.

DNA-Affinity binding assay

Double stranded DNA oligos containing two tandem copies of the MEF2 binding site were generated by PCR amplification of the pGL3-MM reporter construct using a biotinylated oligonucleotide corresponding to the sequence upstream of the MEF2A binding site in the polylinker region of pGL3 (Promega). The sequences of oligonucleotides used are as follows: 5'-biotin-CATTTCTCTATCGATAGGTACC-3' and 5'-TGGCGTCTTCCATGGTGGC-3'. Cultured cells were lysed and extracted in high salt lysis buffer (10 mM Tris-HCl, pH7.5, 1 mM DTT 5 mM $MgCl_2$, 10% glycerol, 0.1% Nonidet P-40, 425 mM NaCl). Cell lysates were subjected to high-speed centrifugation (100,000xg on Beckman TL100) to remove cell debris. The supernatant was diluted with 2x volume of lysis buffer without salt. Soluble cell extracts were incubated with 1 µg of biotinylated double-strand DNA oligos loaded onto the streptavidin-beads (Dynal) at 4°C for 1h. The beads were then washed three times with low salt lysis buffer, eluted and subjected to immunoblotting analysis using an anti-MEF2A antibody.

Fluorescence microscopy

10T1/2 cells were grown on coverslips in six-well dishes. They were transfected with 3 µg total of DNA using FuGENE6. 48h post transfection, cells were washed 3 times with PBS, fixed for 15 minutes in 4% paraformaldehyde, and permeabilized with 0.5% Triton X-100 in PBS for 5 minutes. Indirect immunofluorescence analysis was performed by incubation with 1:2000 anti-myc (Cell Signaling). Cy3-conjugated affiniPure anti-mouse IgG (Jackson Immuno Research Laboratories) was used as secondary antibody. Coverslips were mounted with Vectashield mounting media containing DAPI (Vector laboratories). Visualization was then assessed using a Leica DMRXA deconvolution microscope with a 40x objective. Images were collected using the Slidebook software and electronically merged.

Results

MEF2A contains a consensus sumoylation site and can be SUMO modified *in vitro* and *in vivo*

Cells must be able to respond to rapid changes in their environments, and a particularly sensitive, rapid and reversible response to environmental stimuli is the posttranslational modification of specific proteins. Increasing numbers of cellular proteins, a large proportion of which are transcription factors, have been reported to be covalently conjugated to the small ubiquitin-related modifier-1 (SUMO-1) [4, 12]. One example of a transcription factor subject to sumoylation is Serum Response Factor (SRF), which is a member of the MADS box superfamily [15]. These results are

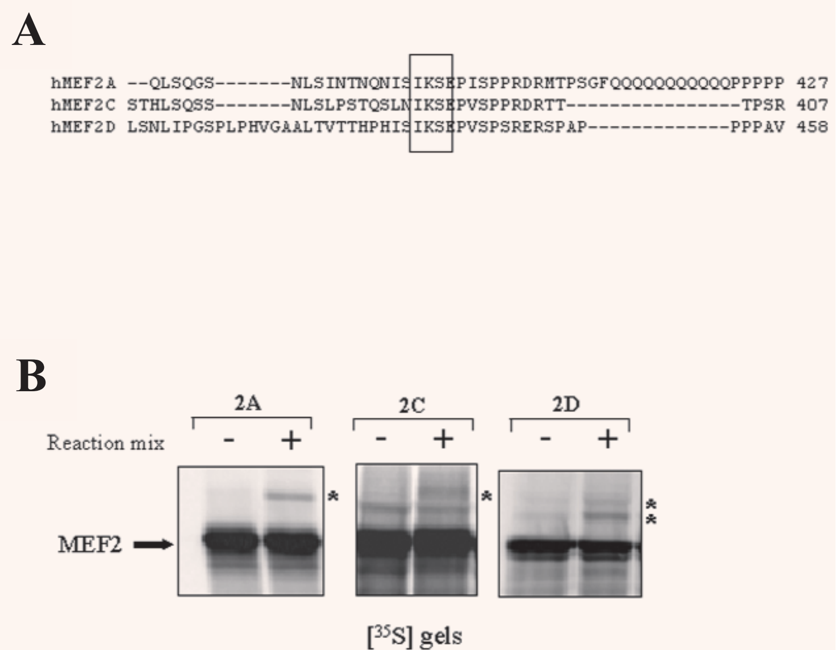


Fig. 1 MEF2A, MEF2C, and MEF2D contain a sumoylation consensus site and can be sumoylated *in vitro*. (**A**) ClustalW alignment of 3 human MEF2 isoforms. The aligned region containing the sumoylation consensus motif (ψKXD/E) is shown: MEF2A, MEF2C, and MEF2D all harbor IKSE. (**B**) MEF2A, MEF2C, and MEF2D are sumoylated *in vitro*. *In vitro* translated [^{35}S]-Met-labeled MEF2 proteins were incubated without or with the sumoylation reaction mix (150 ng E1, 50 ng E2, 15 ng SUMO-1, Energy Regeneration System) for 1 hr at 37°C. Samples were resolved on an 8% gel, and phosphorimaging reveals higher migrating bands (indicated by asterisks) upon addition of reaction mix.

suggestive of the possibility that other MADS box transcription factors could also be sumoylated. Therefore, the sequences of transcription factors in the MEF2 family (a subset of the MADS box superfamily) were inspected for the presence of the sumoylation consensus site, ψKxD/E, to explore the possibility that a member (or members) of this family could be subject to covalent modification by SUMO. Three members of the family (MEF2A, MEF2C, and MEF2D) were positive for this criterion as they contain the conserved sequence IKSE. Fig. 1A shows an alignment of the consensus sequence found among three MEF2 isoforms.

In order to test the possibility that members of this family could be regulated by sumoylation, we first investigated whether MEF2 proteins could be sumoylated in a reconstituted *in vitro* system. To this end, we purified all of the proteins that are involved in catalyzing SUMO transfer *in vitro*, namely E1 (Aos1/Uba2 heterodimer), E2 (Ubc9) and SUMO-1. It is well known that Ubc9 tightly binds to all identified SUMO targets and that sumoylation can occur in the absence of any E3 in a totally reconstituted *in vitro* system [16–18]. We therefore reconstituted a system based upon previously published assays [8, 19] using these components and in vitro translated [^{35}S]-Met-labeled human MEF2A, MEF2C and MEF2D as substrates. Fig. 1B show that upon addition of reaction components, a distinct band migrating about 15 kD above unmodified MEF2 proteins (asterisks) appeared. The size of this band is consistent with SUMO-modified MEF2 protein.

As this experiment strongly implicated MEF2 as a SUMO target, we proceeded to further characterize sumoylation of MEF2A. Thus, we analyzed this modification with a quantitative and direct visualization assay that monitors *in vitro* sumoylation reactions. In accordance with an assay we previously developed [20], we N-terminally labeled purified recombinant human SUMO-1 with the fluorescent dye Alexa Fluor 555 succinimidyl ester, carboxylic acid (hereafter referred to as SUMO-1^{555}) and used this as the source of SUMO in the *in vitro* reconstituted assay. The gel was then directly scanned at 555 nm. Fig. 2A shows a sumoylation timecourse of an enzymatic reaction identical to Fig. 1B in terms of reaction conditions but this time using purified recombinant GST-MEF2A as substrate and SUMO-1^{555} as the source of SUMO-1. In this experiment, an autofluorescence scan reveals that a distinct band (MEF2A-S) increases in intensity with time; this species is specific to SUMO-1 as this is the only protein fluorescently labeled in the system. As further confirmation, this band is clearly Ubc9- and ATP-dependent, as reaction mixes missing either

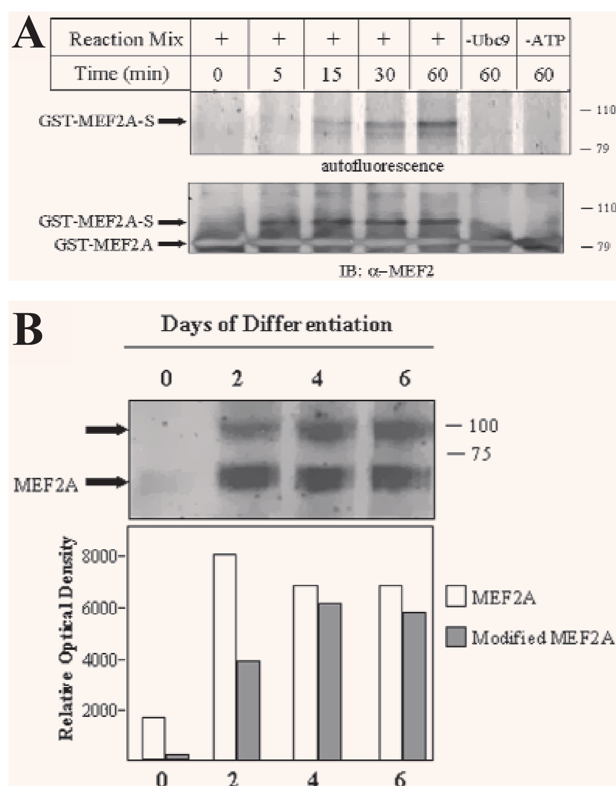


Fig. 2 MEF2A is sumoylated *in vitro* and *in vivo*. (**A**) *In vitro* sumoylation of recombinant GST-MEF2A. Recombinant GST-MEF2A was incubated with a reaction mixture containing E1, E2, and SUMO-1[555]. Controls lacking Ubc9 or ATP were run in parallel. Reactions were quenched at the indicated time points with sample buffer and resolved on an 8% SDS-polyacrylamide gel. The top panel: fluorescence scan of the gel with a Typhoon Scanner set to a wavelength of 555 nm. The bottom panel depicts a western blot for MEF2 of the same gel. (**B**) MEF2A modification in a differentiation timecourse. Equal protein amounts from lysates of a C2C12 differentiation timecourse were analyzed with a MEF2A immunoblot, which revealed unmodified MEF2A and a second modified form of MEF2A migrating 15 kD above unmodified. The quantification of each lane (performed with ImageJ) is shown below the western blot.

of these components failed to produce this product. To confirm that this is also specific to MEF2A, the same gel was analyzed *via* western blot with an antibody against MEF2. The lower panel of Fig. 2A clearly shows that the slower migrating band is recognized by MEF2 antibodies. Taken together, these data clearly indicate that MEF2A can be sumoylated *in vitro*.

To assess whether MEF2A can be sumoylated *in vivo* over the course of myogenesis in C2C12 cells, we took timepoints at Days 0 (growing myoblasts), 2, 4, and 6 and resolved equal amounts of protein by SDS-PAGE (Fig. 2B). Immunoblotting for MEF2A revealed the induction of MEF2A expression with the onset of differentiation as well as the appearance of a slower migrating band approximately 15 kD above the basal level. This is highly indicative of sumoylation as it is generally too large a shift to be due to phosphorylation (more likely to be the upper band of the tight doublet observed here), which is the most well known modification of MEF2A.

Lysine 395 is the SUMO acceptor site in MEF2A

Although SUMO-1 modification frequently occurs within the consensus sequence ψKXD/E, there are exceptions to this rule [4, 12, 21]. The presence of a consensus site does not guarantee sumoylation at that location, as there are several examples of non-consensus lysine residues serving as the acceptor site for SUMO. Therefore, it was important to determine in this case whether consensus lysine 395 is indeed the major sumoylation acceptor site in MEF2A as predicted. In this vein, we constructed a MEF2A mutant, MEF2A K395R, by mutating lysine 395 to another positively charged residue, arginine. *In vitro* translated [^{35}S]-Met-labeled MEF2A and MEF2A K395R were subjected to the same sumoylation reaction used in Fig. 1B. As shown in Fig. 3A, while the wild type MEF2A is sumoylated, judging by the appearance of a slower migrating band about 15 kD larger than the unmodified MEFA, the K395R mutation abrogates the appearance of this band, suggesting that lysine 395 is likely to be the acceptor for SUMO-1 *in vitro*.

Additionally, SUMO modification of MEF2A at lysine 395 can be recapitulated *in vivo*. To this end, 10T1/2 cells were transiently transfected with MEF2A-myc either alone or in combination with expression plasmids for SUMO-1, SUMO-2, or SUMO-3 (Fig. 3B). An α-MEF2 western blot was performed on equal amounts of lysate normalized for protein level. MEF2A-myc alone (lane 2) produces a band migrating about 15 kD above the major MEF2A species, but when co-transfected with SUMO-1, this band significantly increases in intensity (lane 3). SUMO-3 co-transfection also leads to a greater fraction of this modified species (lane 5)

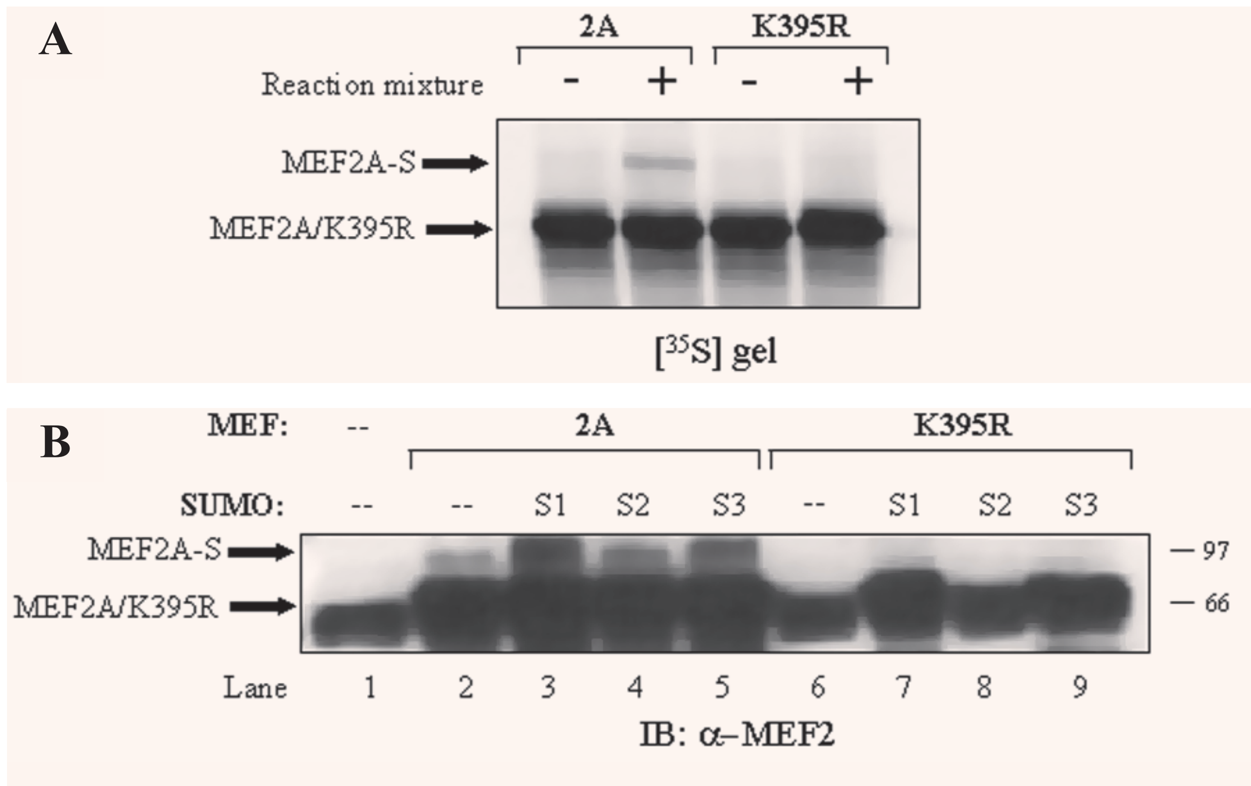


Fig. 3 Lysine 395 is the SUMO acceptor site in MEF2A (**A**) K395 is the major SUMO acceptor site. Sumoylation assays were performed using either [^{35}S]-labeled wild type MEF2A or a MEF2A K395R mutant as substrate. While wild type MEF2A displays a clear band migrating about 15 kD above unmodified in the presence of the sumoylation reaction mixture (MEF2A-S), the corresponding band is absent in the mutant. (**B**) MEF2A is sumoylated at K395 *in vivo*. 10T1/2 cells were transfected with either control empty vector (lane 1), wild type MEF2A-myc (lane 2), wild type MEF2A-myc with Flag-Tev-His-SUMO-1, 2, or 3 (lanes 3, 4, and 5 respectively), MEF2A K395R-myc (lane 6), or MEF2A K395R-myc with Flag-Tev-His-SUMO-1, 2, or 3 (lanes 7, 8, and 9 respectively). Western blotting for MEF2 reveals a slower migrating band associated with wild type MEF2A that increases in intensity upon co-transfection with SUMO. This band is not present in experiments with mutant MEF2A.

while SUMO-2 only modestly enhances it (lane 4). Concurrently, the same battery of experiments was run using sumoylation deficient MEF2A K395R. As lanes 6–9 clearly demonstrate, the slower migrating band no longer appears, even upon co-transfection with SUMO species, strongly suggesting that sumoylation has been interrupted and that K395 is the principal SUMO acceptor residue in MEF2A.

PIAS1 acts in an E3 ligase fashion to promote sumoylation of MEF2A at K395

A number of proteins have been identified as E3 ligases for sumoylation in that they promote the rate and degree of substrate sumoylation [7–9]. Therefore, several of these E3-like proteins were tested against MEF2A, including RanBP2, PIAS1, and PIASxβ (Fig. 4A) [8, 22, 23]. Of these, GST-RanBP2ΔFG had no effect, and perhaps even competed SUMO away from MEF2A, while GST fusions of both PIAS1 and PIASxβ (which is a PIASx splice variant) increased the degree of MEF2A sumoylation. To confirm the identity of the higher molecular weight band, Fig. 4B shows the effect of GST-PIAS1 addition as assayed by autofluorescence of SUMO-1^{555} (left panel) and by immunoblotting the same gel with an antibody against MEF2 (right panel). All identified SUMO E3s display cis-sumoylation in absence of substrate, but the MEF2 western blot confirms that the bands seen in the autofluorescence are specific to MEF2A modification (asterisks) rather than any residual GST-

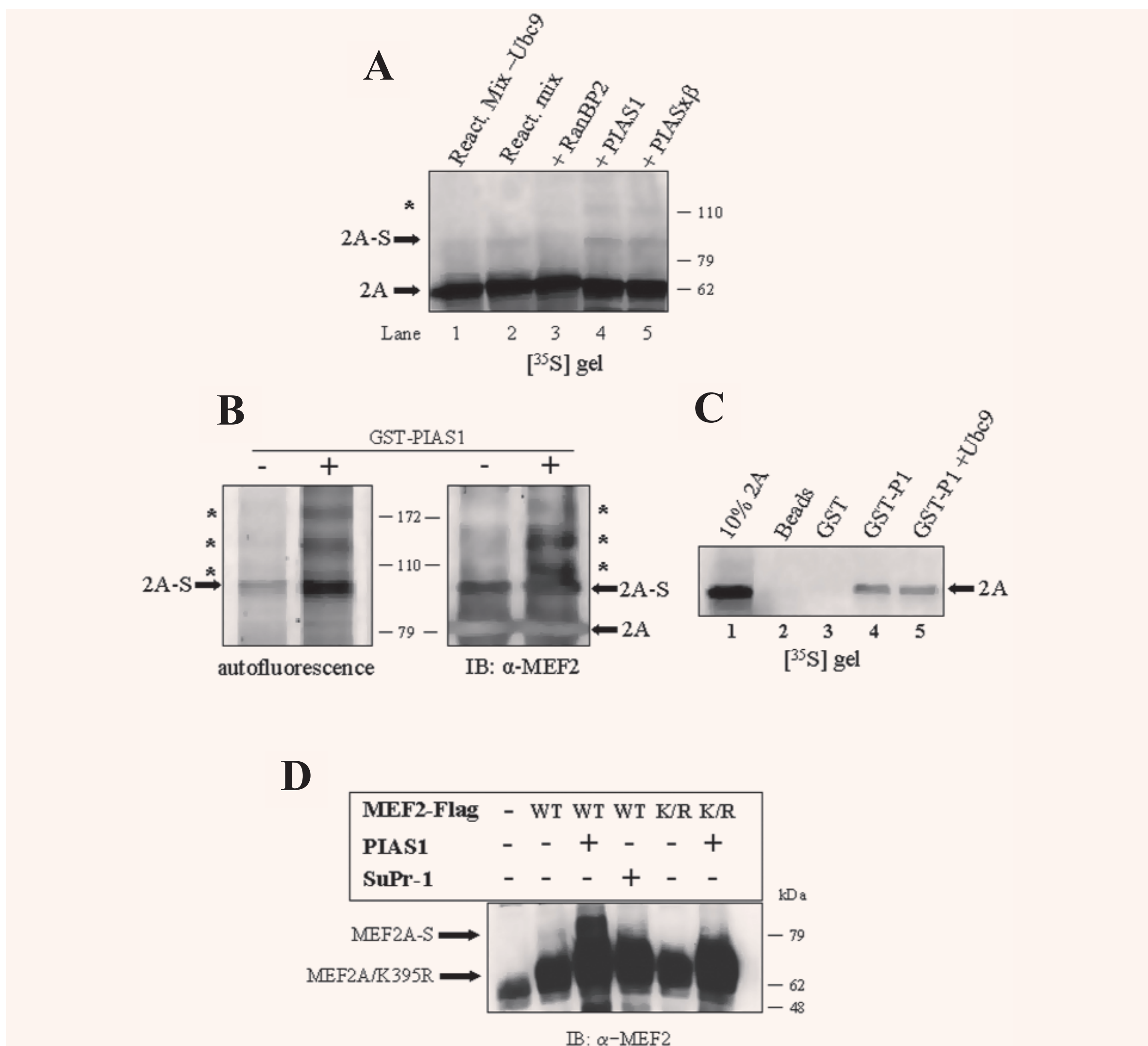


Fig. 4 PIAS1 binds MEF2A and enhances its sumoylation *in vitro* and *in vivo.* (**A**) PIAS1 stimulates MEF2A sumoylation *in vitro*. MEF2A 100% input (lane 1) or MEF2A sumoylation carried out in the absence of E3 (lane 2), in the presence of GST-RanBP2ΔFG (lane 3), GST-PIAS1 (lane 4) or GST-PIASxβ (lane 5). 100 ng of each recombinant E3 was added to the standard sumoylation reaction mixture. The asterisk denotes sumoylated species specific to incubation with GST-PIAS1 or GST-PIASxβ. (**B**) PIAS1-stimulated MEF2A sumoylation is also supported by autofluorescence and immunoblotting. Left Panel: A 60-minute sumoylation of GST-MEF2A using standard reaction mixture but with SUMO-1^{555} as the SUMO-1 source. Reactions were incubated either without (lane 1) or with (lane 2) 100 ng PIAS1. The gel was directly scanned with a laser set at 555 nm to detect sumoylated species. Right Panel: Same gel as left panel but transferred to nitrocellulose membrane. Anti-MEF2 western blot indicates increased sumoylation of MEF2A upon addition of recombinant GST-PIAS1. Asterisk denotes higher molecular weight products, which could represent sumoylation directed to secondary lysines or polysumoylation branching from the major acceptor lysine. (**C**) GST-PIAS1 binds MEF2A *in vitro*. MEF2A was labeled with [^{35}S]-methionine by *in vitro* translation. Binding assays were performed as described in Materials and methods. Lane 1: 10% MEF2A input; Lane 2: Glutathione beads alone; Lane 3: GST protein control; Lane 4: GST-PIAS1; Lane 5: GST-PIAS1 and Ubc9. (**D**) Sumoylation of transiently transfected MEF2A in cultured cells. 10T1/2 cells were transiently transfected with 2.5 µg each of the indicated plasmids for a total of 5 µg DNA per plate (the difference being made up for by empty pCDNA3 vector). Forty-eight hours post transfection, cells were lysed and equal protein amounts were resolved on an 8% SDS-polyacrylamide gel. The proteins were analyzed by western blotting with a polyclonal rabbit anti-MEF2 antibody. The arrows indicate the presence of the unmodified and modified MEF2A.

PIAS1 autosumoylation. Although the higher molecular weight bands are specific to MEF2A modification, we cannot discriminate whether these bands are modifications directed to secondary lysines or polysumoylation branching from the major acceptor lysine (Fig. 4B, asterisks). To further support the E3 ligase activity of PIAS1 towards MEF2A, it can be shown that GST-PIAS1 can specifically bind *in vitro* translated MEF2A. Ubc9 addition does not affect this binding (Fig. 4C). Overall, these data reveal strong E3 ligase activity of PIAS towards MEF2A *in vitro*.

PIAS1-mediated sumoylation of MEF2A at lysine 395 *in vivo*

Once MEF2A was established as an *in vitro* sumoylation substrate and PIAS1 identified as a putative E3 ligase, it was important to confirm the relevance of this modification in a cellular context. Therefore, 10T1/2 cells were transiently transfected with C-terminally Flag-tagged MEF2A or MEF2A K395R either alone or in combination with PIAS1 or the SUMO-specific isopeptidase, SuPr-1 [24]. Direct western blotting of the lysates with a MEF2 antibody reveals a slower migrating band about 15 kD above unmodified MEF2A (Fig. 4D). As we expected based on the *in vitro* experiments, the intensity of this band increases significantly when cells are co-transfected with PIAS1. In addition, the slower migrating band completely disappears upon co-transfection of the isopeptidase, SuPr-1, strongly suggesting that this is a specific modification by SUMO. In support of our previous results, this upper band is not present when MEF2A K395R is transfected as the MEF2 source. Thus, MEF2A is sumoylated at K395 in 10T1/2 cells, and this modification can be PIAS1-directed and suppressed by isopeptidase SuPr-1.

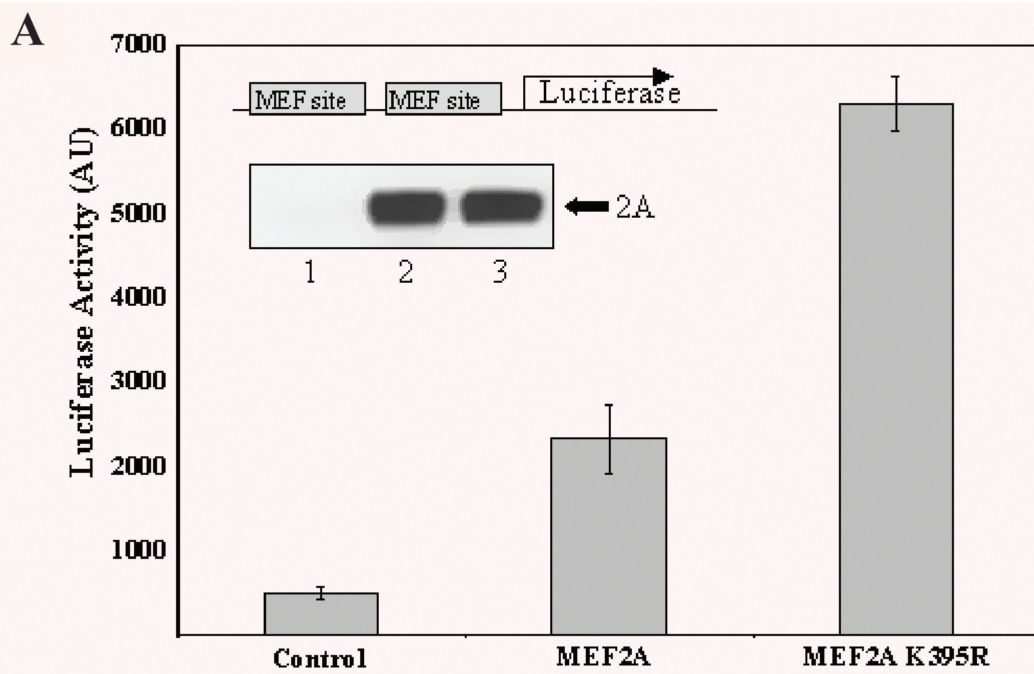
MEF2A K395R has stronger transcriptional activity than wild type MEF2A

After establishing SUMO as a covalent modifier of MEF2A, it was important to explore what effect this might have on regulating MEF2A. Sumoylation has been hypothesized to play a role in regulating the subcellular localization of a variety of nuclear proteins [25]. To determine whether MEF2A sumoylation affects its nuclear localization, we expressed MEF2A and MEF2A K395R in 10T1/2 cells by transient transfection. As shown in Fig. 5B (right panels), both the wild type and the K395R mutant display strong and diffuse nuclear localization as judged by immunofluorescence. Therefore, modification of K395 of MEF2A does not appear to affect subcellular localization.

To determine whether SUMO modification exerts a regulatory effect on MEF2A activity *in vivo*, 10T1/2 cells were co-transfected with either C-terminally myc tagged wild type MEF2A or MEF2A K395R and a reporter construct containing two tandem MEF2 binding sites followed by the firefly luciferase gene [14]. As shown in Fig. 5A, MEF2A K395R exhibits a greater transcriptional activation than wild type MEF2A (approximately 2.7 times greater activity). To verify that this difference could not be a result of different expression levels of MEF2A and MEF2A K395R, a western blot against MEF2 was performed against the lysates used in the luciferase assay. As the inset panel of Fig. 5A demonstrates, the expression levels are essentially equivalent.

Furthermore, this effect cannot be attributed to a mislocalization event as it is shown in Fig. 5B that protein distribution does not change upon mutation of K395 (right panel). It is also not due to a difference in expression level between the wildtype and the mutant as revealed by the inset panel in Fig. 5A. Moreover, this difference in activity is also not due to differential DNA binding affinity as evidenced by the DNA affinity precipitation assay presented in Fig. 5B (left panel). When a biotin-labeled double stranded DNA probe containing two tandem MEF binding sites was used to pull down MEF2A from whole cell lysate, it was found that there is no difference in DNA binding capacity between MEF2A and MEF2A K395R (compare lanes 2 and 3) judging by the amount of MEF2A precipitated. Lanes 4, 5 and 6 show the levels of expression in the 10% input cell lysate, where lane 4 represents the lysate of the untransfected cells, lane 5 the lysate of the MEF2A-transfected cells and lane 6 the sumoylation incompetent point mutant MEF2A-transfected cells. This result strongly suggests that SUMO modification could act as an inhibitory event towards inherent MEF2A transcriptional activity.

To verify that the transcriptional effect of the K395R mutant versus the wild type MEF2A is due to a loss of sumoylation, a luciferase assay was performed using the same reporter gene construct but also co-transfecting the previously mentioned isopep-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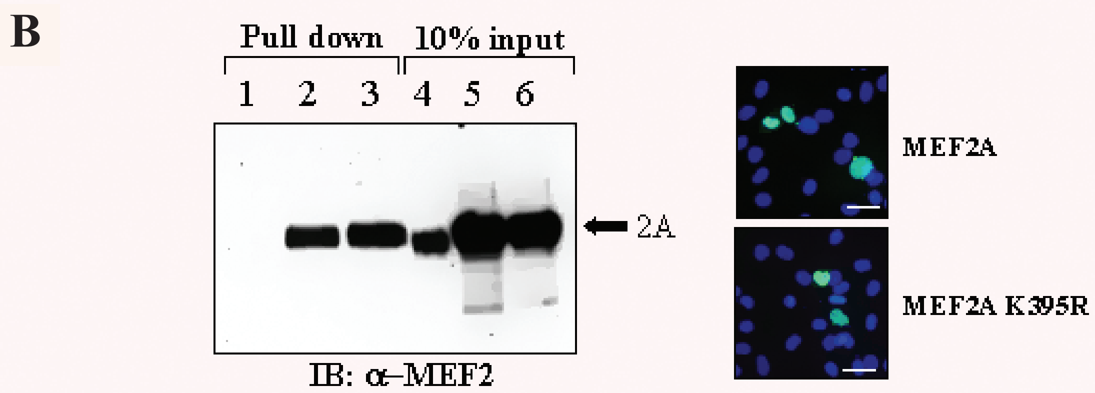
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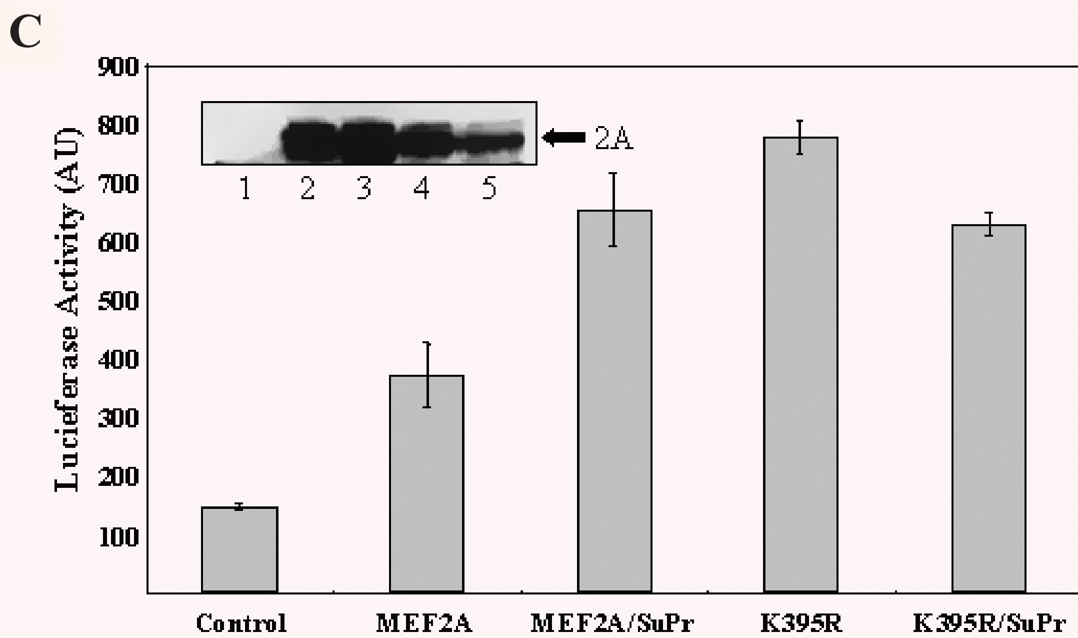
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tidase SuPr-1. If the mutant effect is due solely to loss of sumoylation, then a SUMO-specific isopeptidase transfected with the wild type should yield a fold activation of the mutant that is similar to wild type. Fig. 5C shows that this is in fact true as SuPr-1 co-transfection with wild type MEF2A enhances MEF2A transactivation to the extent of MEF2A K395R. Importantly, SuPr-1 transfection does not enhance the activity of MEF2A K395R. As in Fig. 5A, a western blot against MEF2 was performed on the lysate used for the luciferase assay (Fig. 5C inset). The immunoblot reveals that, although the activity of MEF2A K395R is slightly lower in the presence of SuPr-1, the expression level of MEF2A K395R (lane 5) is also lower than the experiment run in the absence of SuPr-1 (lane 4). Therefore, SuPr-1 expression is not likely to have had any effect on the activity of the MEF2A mutant. Thus, in light of the effect observed with MEF2A K395R, these results indicate that MEF2A desumoylation enhances its ability to activate transcription. Taken together, our results demonstrate that SUMO-1 modification of MEF2A inhibits its ability to activate transcription.

Discussion

The significance of SUMO modification to the control of the activity of several nuclear proteins is becoming increasingly apparent. Here, we identified MEF2A, a key factor for muscle-specific gene expression, as a target for SUMO modification. Furthermore, we demonstrated that sumoylation has a clear effect on regulating MEF2A transcriptional activity, and as *in vitro* experiments presented in Fig. 1 reveal that MEF2A, 2C, and 2D are all possible sumoylation targets, this could be a general regulatory mechanism for all three isoforms. In support of this, recent reports indicate that MEF2D can be sumoylated through the E3 ligase activity of HDAC4 and that this can regulate its transcriptional activity [26, 27]. Moreover, it has been reported that MEF2A purification yields two distinct bands, indicating that a fraction of it had been posttranslationally modified [28]. This mobility shift is in agreement with previous detection of MEF2A from HeLa [29] and muscle cell cultures [30]. This phenomenon could be explained by the fact that MEF2A can be covalently modified by SUMO. We demonstrated that MEF2A is indeed sumoylated at K395 *in vitro* and in an overexpression *in vivo* system, which is evidenced by a slower migrating band sensitive to the activities of both PIAS and SuPr-1. Preliminary experiments suggest that MEF2A can be sumoylated at endogenous levels (data not shown) although the efficiency of the modification appears to be low. However, this is a challenging experiment to perform due to the combined effects of notoriously low steady-state levels of the sumoylated forms of most identified sumoylation targets and the difficultly

Fig. 4 Mutation of K395 to arginine in MEF2A augments the transcriptional activity of MEF2A. (**A**) MEF2A K395R is a more potent transcriptional activator than the wild type. 10T1/2 cells were transiently transfected with 0.2 µg of either wild type MEF2A or MEF2A K395R along with 0.5 µg of a reporter construct where the luciferase gene is under the control of a promoter containing two MEF binding sites (MM reporter). Forty-eight hours post transfection, luciferase activity was measured. Five independent experiments, each in triplicate, were performed and the figure shows a representative result. Inset panel: Immunoblotting for MEF2 shows equal expression levels of wild type MEF2A and MEF2A K395R. Lane 1: control; Lane 2: wild type MEF2A; Lane 3: MEF2A K395R. (**B**) MEF2A and MEF2A K395R exhibit similar DNA binding affinity. A biotinylated DNA probe harboring 2 MEF binding sites was used to pull down whole cell extract of C2C12 cells that were either untransfected (lane 1), transfected with wild type MEF2A (lane 2) or transfected with MEF2A K395R (lane 3). Samples were analyzed for co-precipitation of MEF2A with a polyclonal antibody against MEF2. Lanes 4–6, 10% input of the lysate used in the precipitation. The right panels show that sumoylation does not affect MEF2A expression levels and subcellular localization. Myc-tagged MEF2A or MEF2A K395R were transfected into 10T1/2 cells. Forty-eight hours post transfection cells were analyzed with anti-myc antibody (green signal) and DAPI stained to reveal the position of the nucleus (blue signal). The bar represents 10 µm. (**C**) 10T1/2 cells were transiently transfected with 0.2 µg wild type MEF2A in the presence or absence of 0.1 µg of a construct expressing the isopeptidase SuPr-1, along with 0.5 µg of a MM reporter gene. Forty-eight hours post transfection, luciferase activity was measured. A representative experiment from three independent experiments, each in triplicate, is shown in graphical form. Inset panel: Immunoblotting for MEF2 shows expression levels of wild type MEF2A-myc and MEF2A K395R-myc. Lane 1: untransfected; Lane 2: wild type MEF2A-myc; Lane 3: MEF2A-myc + SuPr-1; Lane 4: MEF2A K395R-myc; Lane 5: MEF2A K395R-myc + SuPr-1. Arrow indicates MEF2A-myc migration.

in immunoprecipitating modified species, perhaps due to a decrease in solubility of the sumoylated protein or a loss of antibody recognition upon sumoylation.

We also report here that the E3 ligase PIAS1 can enhance the degree of MEF2A sumoylation. Although incubation with PIAS1 produces multiple bands corresponding to sumoylated MEF2A, we cannot positively distinguish whether these are due to branched chains from K395 or sumoylation directed to secondary acceptor lysines. However, preliminary data suggest that all sumoylation is directed at K395, as incubating MEF2A K395R with PIAS1 cannot recapitulate sumoylation at any other lysines (data not shown). There is precedence for SUMO modification of other members of the MADS box family. Serum Response Factor (SRF) can be modified by SUMO-1 at lysine 147 within the MADS domain and, consistent with our results, the modification negatively controls SRF-mediated transcription [15].

Protein sumoylation has been shown to be important for modulating protein-protein interactions, for targeting modified proteins to distinct subcellular locations, or for antagonizing ubiquitination [3, 25]. None of the MEF2 family members has been shown to be modified by ubiquitin. Neither can we detect any significant ubiquitination of MEF2A. Thus, it is unlikely that the primary function of MEF2A sumoylation is to antagonize its ubiquitination in order to increase metabolic stability of the protein.

In addition to antagonizing protein ubiquitination, protein sumoylation can also affect the redistribution of the target protein within the cell. Histone acetylases (HATs) such as p300 and GRIP1 are transcriptional activators known to associate with MEF2 family members [31]. Specifically, studies performed inducing the ectopic expression of GRIP1 in myoblast cells demonstrated that GRIP1 co-activates MEF2C-mediated transcription and interacts with the transcription factor through its MADS domain [32]. Co-transfection of GRIP1 in 10T1/2 cells can both potentiate the transcriptional activity of MEF2C and induce localization to punctate nuclear structures [31]. Typically, these nuclear structures are associated to nuclear bodies (NBs) which consist of an aggregation of several proteins, such as PML and Sp100 [33]. Notably, numerous proteins that transiently associate with the NBs (for example, p53, Daxx and Lef-1) are also sumoylated. This is also the case for GRIP1, which was reported to be modified by SUMO-1 [34]. It would not be surprising to find that MEF2C is also sumoylated *in vivo* and that this alteration affects its subcellular localization. Thus, it is also possible that SUMO-modified MEF2A could target it to nuclear bodies. However, our immunostaining analysis of overexpressed MEF2A or the MEF2A K395R mutant indicated that they are distributed uniformly in the nucleus. However, this does not preclude the possibility that endogenous MEF2A localizes to nuclear bodies upon SUMO modification.

SUMO modification regulates MEF2A transcriptional activity

MEF2 proteins, like most transcription factors, have a modular structure consisting of a nuclear localization domain, a DNA binding domain, a dimerization domain and a transcriptional activation domain (TAD). Specifically, the DNA binding and dimerization domains lie in the N-terminal region and the TAD and nuclear localization domains lie in the C-terminal region [35]. Each of these domains could be subject to regulation and a potentially important mechanism to regulate MEF2 activity lies in the control of its transcriptional activity [36]. In addition, a network of transcriptional co-activators and repressors modulates MEF2 function. We demonstrated here that the major sumoylation site (K395) of MEF2A is located within the TAD domain. We characterized the transcriptional activity of both wild type and the MEF2A K395R mutant and we found that the mutant has an increased transcriptional activity when both are cotransfected with a reporter gene containing 2 MEF2 binding sites. This result implies that the K395R mutation is unlikely to have altered the overall structure of MEF2A and suggests that sumoylation exerts an inhibitory effect on MEF2A activity. Sumoylation of MEF2A in this region could alter the conformation of the TAD domain or impede MEF2A interactions with transcription coactivators to activate transcription. Alternatively, MEF2A sumoylation could result in the recruitment of histone deacetylase (HDAC) activity to promoters similar to what has been demonstrated recently for the ETS domain transcription factor Elk-1 [37]. MEF2 is required for positive regulation of the transcription of downstream muscle-specific differentiation genes in collaboration with MyoD. Desumoylation of MEF2A in theory could facilitate its transition from transcriptional repression to transcriptional activation. In this scenario, MEF2A sumoylation may regulate the timing of MEF2A activity. Preliminary experiments overex-

pressing MEF2A and MEF2A K395R in C2C12 do not show a clear phenotype in regards to the myogenic activity of this transcription factor and the effect that sumoylation may have on this activity (data not shown). However, the data must be considered in light of the fact that the mutant overexpression is in the background of endogenous wild type MEF2A and the possibility that the other MEF2 isoforms could compensate for any alteration in MEF2A function. Therefore, the possibility exists that the functional effect of MEF2A sumoylation is more significant than reflected by the data observed.

This report provides the first evidence implicating sumoylation as a posttranslational modification controlling MEF2A activity; however there are several considerations that should be addressed in the future. For instance, MEF2 family members have a number of potential phosphorylation sites that clearly regulate their activity [28]. It is necessary to determine the effect of these phosphorylations on MEF2 sumoylation and finally the regulation of its transcriptional activity. In addition, the relevance of MEF2 as an essential transcription factor has been revealed by several reports in which perturbation of MEF2 expression levels induced dramatic effects on several physiological processes [38–41]. Since MEF2 proteins have a critical role in multiple processes, understanding the mechanism that controls their activities will help to shed light on the molecular pathways regulated by this transcription factor.

Finally, this leads to the broader question of whether sumoylation could be a more general mechanism regulating skeletal muscle differentiation. Although several proteins known to modulate MEF2-mediated gene expression and to directly interact with MEF2 have also been shown to be sumoylated (including the transcription factor GATA-1 [42], the HATs GRIP1 [34] and p300 [43], and the class IIA HDACs [27]), a global assessment of the role of sumoylation in myogenesis has not been reported. However, the accumulation of data linking sumoylation to various muscle-related transcription factors and their partners suggests that sumoylation could play a rather significant role in regulating myogenesis. For instance, the dynamic nature of sumoylation would fit a scenario in which modification by SUMO regulates the timing of activation of factors involved in the myogenic transcriptional program. More specifically, sumoylation could prevent precocious activation of a given transcription factor and could also represent a means of turning off the E3 ligase activity of HDAC4, but HDAC4 itself is subject to sumoylation, which actually attenuates its ligase activity [27]. This apparent hierarchy of events hints at a built-in temporal regulatory mechanism governing the transcriptional events of myogenesis. It would be interesting to further explore what effects perturbation of the sumoylation pathway, for instance, at the level of the E1 or the E2, would have on skeletal muscle differentiation.

Acknowledgements

We thank Drs. Melchior, Seeler, Dejean, Müller, Suske and Zon for the generous supply of the cDNA clones and reagents used in this study; Dr. Ron Prywes for the kind gift of anti-MEF2A. We also want to thank Dr. Brad Olwin, Dr. Hugo Olguin and Dr. Lin Chen for helpful discussions and Drs. Natalie Ahn, Jim Goodrich and Ding Xue for critically reading the manuscript. This work is supported by grants from the National Institutes of Health (CA107098-01) and Colorado Tobacco Research Program (2R-45) to Xuedong Liu. K.B. was supported by a predoctoral training grant from NIGMS (T32GM08759).

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