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Acetylation of Myocardin Is Required for the Activation of Cardiac and Smooth Muscle Genes*

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Background: Myocardin is a cardiac- and smooth muscle-specific transcription factor.
Results: Myocardin is directly acetylated by p300.
Conclusion: Acetylation of myocardin is critical for myocardin to activate cardiac and smooth muscle target gene expression.
Significance: This study provides a molecular mechanism to explain how chromatin-remodeling enzymes participate in the regulation of tissue-specific gene expression by directly modulating key transcription factors.

Myocardin belongs to the SAF-A/B, Acinus, PIAS (SAP) domain family of transcription factors and is specifically expressed in cardiac and smooth muscle. Myocardin functions as a transcriptional coactivator of SRF and is sufficient and necessary for smooth muscle gene expression. We have previously found that myocardin induces the acetylation of nucleosomal histones surrounding SRF-binding sites in the control regions of cardiac and smooth muscle genes through recruiting chromatin-modifying enzyme p300, yet no studies have determined whether myocardin itself is similarly modified. In this study, we show that myocardin is a direct target for p300-mediated acetylation. p300 acetylates lysine residues at the N terminus of the myocardin protein. Interestingly, a direct interaction between p300 and myocardin, which is mediated by the C terminus of myocardin, is required for the acetylation event. Acetylation of myocardin by p300 enhances the association of myocardin and SRF as well as the formation of the myocardin-SRF-CArG box ternary complex. Conversely, acetylation of myocardin decreases the binding of histone deacetylase 5 (HDAC5) to myocardin. Acetylation of myocardin is required for myocardin to activate smooth muscle genes. Our study demonstrates that acetylation plays a key role in modulating myocardin function in controlling cardiac and smooth muscle gene expression.

Myocardin and myocardin-related transcription factors (MRTFs)[3] belong to a family of SAF-A/B, Acinus, PIAS (SAP) domain transcription factors (1–3). Unlike many other transcription factors, which bind to the conserved DNA sequences on the regulatory regions of their target gene, myocardin and MRTFs do not directly bind DNA. Instead, they form a ternary complex with the serum-response factor (SRF) bound to the DNA consensus sequence $CC(A/T)_6GG$, known as a CArG box. Such recruitment of myocardin and MRTFs brings the powerful transcription activation domain of myocardin and MRTFs to SRF-dependent target genes with resulting transcriptional activation (1, 4). Whereas MRTFs are broadly expressed in multiple tissue and cell types, myocardin expression is restricted to muscle lineages (2, 5, 6). It has been demonstrated that myocardin transactivates the cardiac-specific gene atrial natriuretic factor (*ANF*) and the smooth muscle-specific gene *SM22*, both known targets for SRF (1, 7, 8). In addition, myocardin is a key component of a molecular switch that regulates the ability of SRF to mediate cellular proliferation and muscle cell differentiation (4, 9). Genetic studies revealed that myocardin and MRTFs play critical roles in a variety of biological processes, including vascular smooth muscle development, aortic vessel patterning, mammary myoepithelium formation, and others (10–14).

Chromatin modification events, which include histone acetylation, methylation, phosphorylation, and ubiquitination, are central to the regulation of gene expression (15, 16). Acetylation introduces an acetyl functional group into the lysine residues of a peptide, whereas deacetylation is the removal of the acetyl group. Acetylation and deacetylation reactions are catalyzed by enzymes with histone acetyltransferase (HAT) or histone deacetylase (HDAC) activity, respectively. Acetylation and deacetylation are most commonly found in the modification of histones as part of gene regulation (17, 18). It is generally believed that histone acetylation leads to the decondensation of chromatin thus allowing for physical accessibility of the RNA polymerase and transcriptional activation (17, 18). We have previously found that the transcriptional activity of myocardin is positively and negatively modulated by p300, a HAT, and HDAC4 and HDAC5. p300 interacts with myocardin at its C-terminal transactivation domain to enhance the transactivity

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[3] The abbreviations used are: MRTF, myocardin-related transcription factor; SRF, serum-response factor; ANF, atrial natriuretic factor; HDAC, histone deacetylase; HAT, histone acetyltransferase; Co-IP, coimmunoprecipitation; SM, smooth muscle; EMSA, electrophoretic mobility shift assay.

of myocardin in activating cardiac and smooth muscle gene expression (19). We have further demonstrated that myocardin induces the acetylation of nucleosomal histones surrounding SRF-binding sites in the control regions of smooth muscle genes through recruitment of p300 (19). Interestingly, histone is not the only substrate acetylated by p300. p300 has been shown to acetylate transcription factors, including p53, MyoD, and others (20–24), thereby changing the DNA-binding affinity of transcription factors (25) or disrupting the ability of transcription factors to recruit additional cofactors (26). Previously, we proposed three putative mechanisms for p300 enhancement of myocardin-mediated gene expression as follows: (i) p300 functions as a general transcriptional coactivator or adaptor, (ii) p300 functions as a HAT to acetylate histones in nucleosomes at the regulatory regions of *ANF* and *SM22* genes, and (iii) p300 directly acetylates myocardin thereby enhancing its transactivity (19).

In this study, we tested the hypothesis that p300 functions as an acetyltransferase to directly acetylate myocardin. We report here that indeed myocardin is a substrate for p300-dependent acetylation modification. We also mapped acetylates sites to the N-terminal regions of the myocardin protein. We found that acetylation of myocardin enhances myocardin and SRF interaction as well as the formation of the myocardin-SRF-CArG box ternary complex. Our results indicate that acetylation of myocardin is critical for myocardin to activate smooth muscle target gene expression.

EXPERIMENTAL PROCEDURES

Plasmids and Reporter Genes—Myocardin and HDAC expression vectors have been described previously (2, 19, 27). The p300 expression vectors were as described previously (19). Myocardin mutants were generated through PCR-based mutagenesis using the QuikChange kit from Stratagene. All mutations were confirmed by DNA sequencing. The *SM22*-luciferase reporter contained the 1434-bp promoter (2), and the *ANF*-luciferase reporter contained the 638-bp promoter (2). *CMV*-lacZ was included as an internal control for variations in transfection efficiency.

Cell Culture and Transfection Assays—Transfection of COS7 and 10T1/2 cells and luciferase assays were performed as described previously (2, 19, 27). Unless otherwise indicated, 100 ng of reporter plasmid and 100 ng of each activator plasmid were used. The total amount of DNA per well was kept constant by adding the corresponding amount of expression vector without a cDNA insert. All the transfection experiments were repeated at least three times in duplicate.

GST-Protein Binding Assays—Plasmids encoding GST fusion proteins were transformed into BL21-codon plus cells (Stratagene). The cells were grown at 37 °C in 2× YT medium to an optical density of 1.0. Isopropyl 1-thio-β-D-galactopyranoside (50 μM) was then added to the culture to induce protein expression. After shaking at room temperature for 4–6 h, the cells were harvested, and the GST proteins were purified with glutathione beads according to the procedure from GE Healthcare.

Proteins translated *in vitro* were labeled with [^{35}S]methionine using a TnT T7-reticulocyte lysate system (Promega). Glutathione beads conjugated with 1 μg of protein were incubated with 10 μl of TnT product, at 4 °C for 2 h in 500 μl of GST binding buffer (20 mM Tris, pH 7.3, 150 mM NaCl, 0.5% Nonidet P-40, protease inhibitor mixture from Roche Applied Science, and 1 mM PMSF). The beads were washed three times with GST binding buffer. Fifty microliters of SDS-loading buffer was then added to the beads. After boiling, 20 μl were loaded onto a SDS-polyacrylamide gel.

Immunostaining and TUNEL Assays—Immunostaining was performed as described previously (19). To determine the cellular localization of myocardin and its mutants, COS7 cells were transfected with FLAG-tagged myocardin constructs and stained with anti-FLAG antibody (mouse monoclonal, M2, Sigma). Myogenic conversion assays in 10T1/2 cells were performed as described previously (19, 28), except that Lipofectamine reagent (Invitrogen) was used for transfection. Mouse anti-SM-α-actin monoclonal antibody (1A4, Sigma) was used to monitor smooth muscle gene induction.

10T1/2 cells cultured in DMEM containing 10% FBS were transfected with expression vectors encoding CMV-lacZ (control), myocardin, myocardin acetylation-deficient K4R mutant (myocardin-K4R), and MyoD. 48 h later, cells were switched to differentiation medium (DMEM containing 2% horse serum). After an additional 48 h, the cells were collected and fixed and then proceeded for TUNEL assay using the ApopTag®Plus fluorescein *in situ* apoptosis detection kit (S7111, Chemicon) according to the manufacturer's manual.

Coimmunoprecipitation Assays—COS7 cells were transiently transfected with plasmids encoding the epitope-tagged myocardin, SRF, HDAC5, and p300 proteins as indicated in the figure legends with FuGENE 6 reagent (Roche Applied Science). 48 h after transfection, cells were harvested in lysis buffer composed of phosphate-buffered saline (PBS) containing 0.5% Triton X-100, 1 mM EDTA, 1 mM PMSF, and complete protease inhibitors (Roche Applied Science). Following a brief sonication and removal of cellular debris by centrifugation, epitope-tagged proteins were precipitated with antibodies as indicated and protein A/G beads (Santa Cruz Biotechnology). The bound proteins were washed five times with lysis buffer or washing buffer with increasing salt concentrations (from 150, 350, and 550–750 mM NaCl), then resolved by SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Bio-Rad). Membranes were immunoblotted with antibodies as indicated, and proteins were visualized with a chemiluminescence detection system (Santa Cruz Biotechnology).

In Vitro Acetylation Assay—*In vitro* acetylation assays were performed as described previously (29). FLAG-tagged recombinant p300 protein (amino acids 1195–1810), which contains the HAT domain and possesses HAT activity, and GST myocardin or GST-myocardin mutant fusion proteins were expressed in bacteria and affinity-purified to homogeneity. *In vitro* acetylation assays were performed in 30 μl of solution containing 50 mM HEPES, pH 8.0, 10% glycerol, 1 mM DTT, 1 μl of [^{3}H]acetyl-CoA (Amersham Biosciences), 1 mM PMSF, 10 mM sodium butyrate, 1 μg of highly purified substrate proteins or 2.5 μg of GST fusion protein, and 100 ng of p300 and were incubated at 30 °C for 1 h. The reaction was resolved by SDS-

PAGE and dried and subjected to autoradiography at −70 °C for 1–3 days.

RT-PCR—Total RNA was isolated with TRIzol reagent (Invitrogen). After extraction and purification, 1 μg of RNA was used as template for reverse transcription with random hexamer primers. PCR conditions and primer sequences are the same as described previously (4, 19, 28). All PCR products span the intron region of the genes.

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was carried out essentially as described previously (2, 19) with minor modifications. The DNA-binding reactions (20 μl) contained 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM EDTA, 10% glycerol, 10 mM sodium butyrate, 0.5 mg/ml BSA, 100 ng poly(dI-dC), and proteins as indicated. Reaction mixtures were preincubated at room temperature for 20 min before a ^{32}P-labeled probe DNA (0.2 ng) was added and further incubated at room temperature for 20 min. Each reaction mixture was then loaded onto a native 4% polyacrylamide gel (acrylamide:bisacrylamide, 50:1) containing 0.5× TBE and electrophoresed in 0.25× TBE at 180–220 V for 3 h. In the case of supershift assays, the indicated monoclonal antibodies (200 ng) were added to reaction mixtures during preincubation.

RESULTS

Myocardin Is Acetylated by p300—To directly test whether myocardin is a substrate for p300-mediated acetylation, we performed *in vitro* acetylation assays, using GST-myocardin fusion proteins as a substrate. Indeed, the N-terminal region of myocardin (amino acids 1–274) was efficiently acetylated by p300 (Fig. 1*A*). In contrast, no acetylation was detected in the middle and the C-terminal portions (amino acids 278–670 and amino acids 670–935, respectively) of myocardin (Fig. 1*A*). As a negative control, GST protein was not acetylated. Notably, p300 also acetylates itself in these assays (Fig. 1*A*, *upper bands*), consistent with previous reports (24).

Acetylation by acetyltransferase(s) occurs at lysine residues (17, 24). The N terminus of myocardin (amino acids 1–274) contains 26 lysine residues, which are potential acetylation sites. A large proportion of those lysine residues are located within the basic domain of myocardin protein, which also contains its nuclear localization signal (Fig. 1*B*). To determine which lysine residue(s) are accessible to acetylation, we systematically mutated these lysine residues into arginines and assayed their ability to be acetylated by p300 *in vitro* (Fig. 1*C*). Because both arginine and lysine share similar chemical structure and charge, the lysine to arginine mutation will minimize the effect on other properties of the myocardin protein. Interestingly, replacing individual lysines to arginines did not significantly affect the acetylation state of myocardin (data not shown).

Although a definitive molecular signature has yet to be established for acetylation substrates, acetylation often occurs within clusters of lysine residues (24). Two closely spaced clusters of three lysines (amino acids 247–250 and 257–260) located within the nuclear localization signal region of myocardin resemble the acetylation sites identified in other transcription factors (24, 25, 30). To test their potential involvement, we mutated these lysine clusters into arginine clusters. Surprisingly, acetylation was not reduced but was further enhanced in the mutant myocardin protein, indicating that those lysine residues are unlikely the primary acetylation sites (Fig. 1*C*). Further introducing mutation into additional lysines at position 253 and 255 reduced myocardin acetylation moderately (*m2* and *m3* in Fig. 1*C*). We continued this process and discovered a dramatic reduction in acetylation when additional lysine residues (positions of 235 and 237) were also mutated (*m4, m5,* and *m6* in Fig. 1*C*). Together, those analyses suggest that lysine residues between amino acid 235 and 274 are critical for the acetylation of myocardin protein.

The above observations suggest that amino acids 235/237 and 253/255 could play a critical role in the acetylation of myocardin. However, such a conclusion could be confounded by the fact that simultaneous mutations were also introduced into other lysine residues in those experiments. We therefore decided to generate myocardin mutants in which only two (Lys-235/237 or Lys-253/255) or four lysines (Lys-235/237/253/255) were mutated into arginines. As shown in Fig. 1*D*, myocardin with two lysines being mutated (K2R) partially decreased its acetylation (*m7* and *m8* in Fig. 1*D*). However, the myocardin mutant with all four lysine residues mutated (thereafter called K4R mutant) was incapable of being acetylated (*m9* in Fig. 1*D*). These results indicate that multiple lysines in combination, rather than any single lysine residue, are responsible for the majority of myocardin acetylation by p300. These results are consistent with prior observations reported in the acetylation status of transcription factor YY1 and others (26, 31).

To determine whether myocardin is also acetylated *in vivo*, we overexpressed FLAG-tagged myocardin or the myocardin K4R mutant in COS7 cells together with a wild-type p300 or a HAT-deficient p300 mutant (p300-DY) (19). Myocardin proteins were immunoprecipitated with anti-FLAG antibodies, and their acetylation status was determined by anti-acetyl-lysine antibodies in Western blots. Whereas myocardin is clearly acetylated in the presence of wild-type p300 *in vivo*, the HAT-deficient p300 mutant failed to acetylate myocardin (Fig. 1*E*). Consistent with the *in vitro* result that the four lysine residues are critical for myocardin acetylation, the myocardin K4R mutant was not acetylated *in vivo* (data not shown). We have previously reported that myocardin and p300 directly interact *in vitro* and *in vivo,* and we have defined their interaction domains at the C terminus of the myocardin protein (19). Interestingly, myocardin(1–274), although sufficient to be acetylated *in vitro* (Fig. 1*A*), was incapable of being acetylated *in vivo* (data not shown). These observations suggest that a physical interaction between p300 and myocardin, which is mediated by myocardin C-terminal domains, is required for p300-mediated myocardin acetylation *in vivo*.

Acetylation Is Required for the Transcriptional Synergy between Myocardin and p300 to Activate Cardiac and Smooth Muscle Reporter Genes—Having established that myocardin is acetylated *in vitro* and *in vivo* by p300, we next investigated the functional significance of this modification. We first examined whether the HAT activity of p300 is required for the synergistic transactivation of cardiac and smooth muscle gene expression by myocardin. Myocardin was cotransfected with the wild-type or HAT-deficient p300 mutant into COS7 cells together with *SM22* or *ANF* luciferase reporter genes. As shown in Fig. 2*A*,

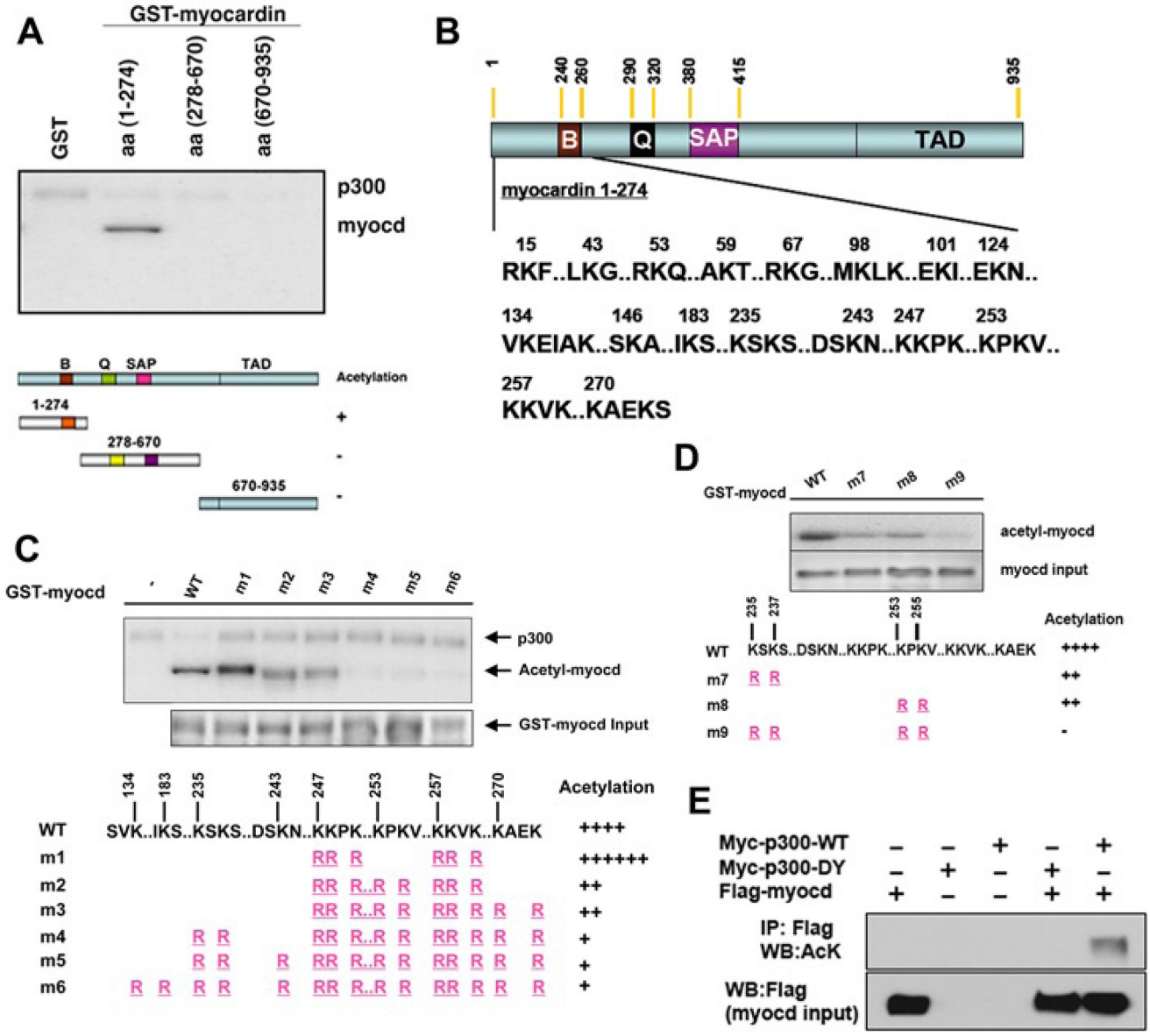

FIGURE 1. **Myocardin is acetylated by p300 *in vitro* and *in vivo*.** *A, in vitro* acetylation assays. GST-myocardin fusion proteins or GST alone was incubated with p300 in the presence of [^{3}H]acetyl-CoA and resolved on SDS-PAGE. Only myocardin (*myocd*) (amino acids 1–274) was acetylated. p300 auto-acetylates itself (*upper bands*). *B,* schematic diagram of myocardin protein with its signature domains marked. Partial amino acid sequences from the myocardin N-terminal region with all 26 lysine residues were displayed. *B*, basic domain; *Q*, glutamine-rich domain; *SAP*, SAP domain; *TAD*, transactivation domain. *C* and *D, in vitro* acetylation assays with GST-myocardin fusion protein (amino acids 1–274) and its derived Lys-to-Arg mutants or GST control. Acetylation results are summarized in the *lower panels*. *E,* myocardin is acetylated by p300 *in vivo*. FLAG-tagged myocardin was transfected with either a wild-type (*p300-WT*) or a HAT-dead mutant (*p300-DY*) of p300 into COS7 cells. Myocardin proteins were immunoprecipitated (*IP*) using an anti-FLAG antibody, and acetylated myocardin was detected by acetyl-lysine-specific antibody (*AcK*). *WB*, Western blot.

increasing concentrations of wild-type p300 in the presence of myocardin resulted in a synergistic and dose-dependent activation of *ANF* promoter luciferase reporter gene, consistent with our prior report (19). However, cotransfection of a HAT-deficient p300 mutant (p300-DY) failed to generate any synergy in these assays (Fig. 2*A*), suggesting that p300 HAT activity is required for the synergy between p300 and myocardin. Similar observations were obtained on the *SM22* luciferase reporter gene (data not shown). Accordingly, the myocardin K2R and K4R mutants significantly decreased the ability of myocardin in activating the *ANF* luciferase reporter gene. Most importantly, such mutations abolished the synergy between myocardin and p300 (Fig. 2*B*). Together, these results demonstrate that acetylation of myocardin is required for its synergistic cooperation with p300 to activate the expression of cardiac and smooth muscle genes.

Because these Lys-to-Arg mutations occur within or near the basic domain of myocardin, which also contains the nuclear localization signal, we examined if those mutations affect the nuclear localization of myocardin proteins. Transfection of the wild-type or the Lys-to-Arg myocardin mutant constructs into COS7 cells showed that the Lys-to-Arg mutations did not change the nuclear location pattern of myocardin proteins (Fig. 2*C*). These observations suggest that the myocardin acetylation did not affect the nuclear location of this protein, which further implies that the loss of synergy between myocardin acetylation-

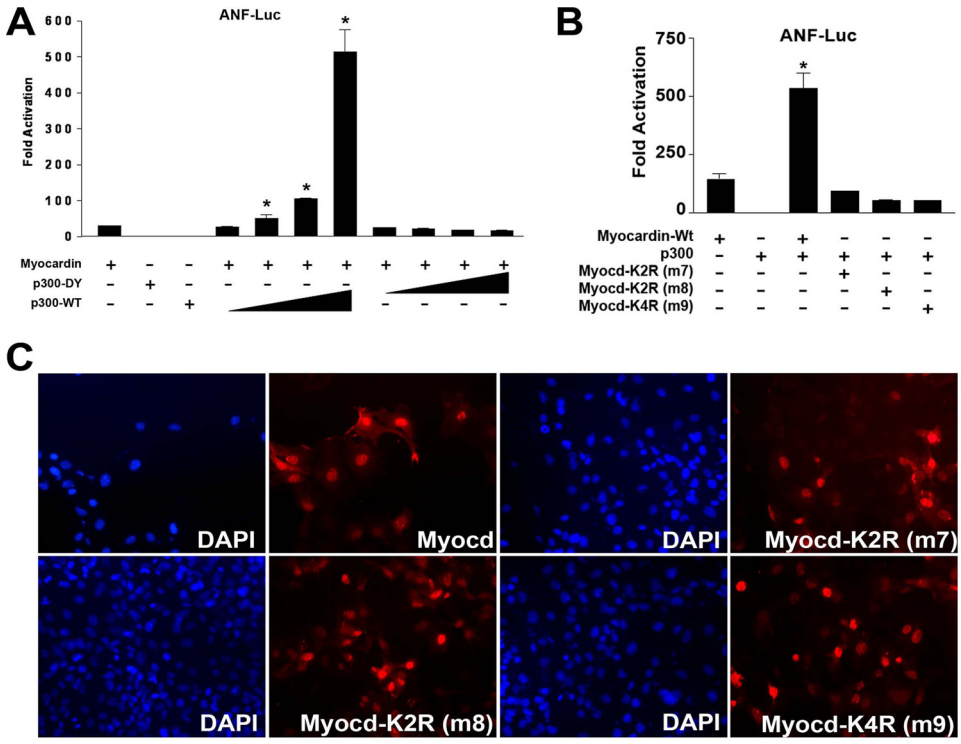

FIGURE 2. **Acetylation of myocardin is required for myocardin and p300 to synergistically activate cardiac reporter genes.** *A,* COS7 cells were transiently transfected with ANF-luciferase reporter and expression vectors encoding myocardin and increasing amounts of either wild-type (*p300-WT*) or mutant (*p300-DY*) p300, and luciferase activity was measured. *B,* COS7 cells were transiently transfected with ANF-luciferase reporter and expression vectors encoding p300 and either wild-type myocardin (*myocardin-WT*) or indicated myocardin mutants (*myocd-m7*, *m8*, and *m9*) and luciferase activity measured. The luciferase activity was determined 48 h after transfection and was presented as fold of activation in which the control was assigned a value of 1. Data represent the mean ± S.D. from at least three independent experiments in duplicate. *, $p < 0.05$. *C,* immunostaining of myocardin and its mutants. COS7 cells were transiently transfected with expression vectors for FLAG-tagged myocardin, and its mutant constructs and the subcellular distribution of the proteins were determined by immunostaining. DAPI staining marks the nuclei.

deficient mutants and p300 did not result from the disruption of myocardin nuclear localization in those mutants.

Acetylation of Myocardin Increases Its Affinity for SRF—To determine whether acetylation of myocardin could modulate its affinity for SRF, coimmunoprecipitation (Co-IP) assays were performed with cell lysates containing equal amounts of HA-tagged SRF, FLAG-tagged myocardin, and Myc-tagged p300 or the p300-DY mutant. After precipitation by anti-FLAG antibodies, followed by washes with increasing salt concentrations in washing buffer, the associated SRF was then detected by an anti-HA antibody in Western blot analyses. At low salt concentrations, the affinity of myocardin and SRF is 5–6-fold higher when WT p300 was included (and therefore, myocardin was predicted to be acetylated) than when p300-DY was included (and therefore, myocardin acetylation was predicted to be absent) (Fig. 3*A*). As salt concentration increases in the washing buffer (which correlates with increasing stringency for protein-protein interaction), the association of SRF and nonacetylated myocardin significantly diminished, whereas the association of SRF and acetylated myocardin decreased much slowly (Fig. 3*A*). These data demonstrate that acetylated myocardin displays higher affinity for SRF.

To further demonstrate that the observed difference in SRF-myocardin association is due to myocardin acetylation, we tested the ability of myocardin acetylation-deficient K4R mutant to associate with SRF in similar Co-IP assays. After precipitation by anti-FLAG antibodies, immunoprecipitates were subjected to washing with increasing salt concentrations in wash buffer. As expected, wild-type myocardin showed much stronger binding affinity for SRF than the acetylation-deficient myocardin K4R mutant (Fig. 3*B*). Together, these results demonstrate that acetylation of myocardin enhances its association with SRF.

Acetylation of Myocardin Enhances the Formation of the SRF-Myocardin-CArG Ternary Complex—Myocardin is a transcriptional cofactor of SRF that does not bind to DNA by itself. Instead, myocardin is recruited by SRF to the SRF-binding site, the CArG box (1, 2, 7). To determine whether acetylation of myocardin could modulate SRF binding to DNA as well as the formation of the SRF-myocardin-CArG complex, EMSAs were performed with nuclear extracts containing equal amounts of SRF and p300 (or the p300-DY mutant) and an increasing amount of myocardin. As shown in Fig. 4*A*, increasing myocardin protein amounts result in the formation of a more stable SRF-myocardin-CArG ternary complex in the presence of wild-type (WT) p300 (Fig. 4*A*, *lanes 2–5*). In contrast, the SRF-myocardin-CArG ternary complex formation was significantly deceased in the presence of p300-DY (Fig. 4*A*, *lanes 7–10*). The specificity of the ternary complex was demonstrated by antibody supershift (Fig. 4*A*, *lanes 11* and *12*). Together, these results suggest that acetylation of the myocardin enhances the formation of the SRF-myocardin-CArG ternary complex. Noticeably, p300 appears to enhance the binding of SRF to the

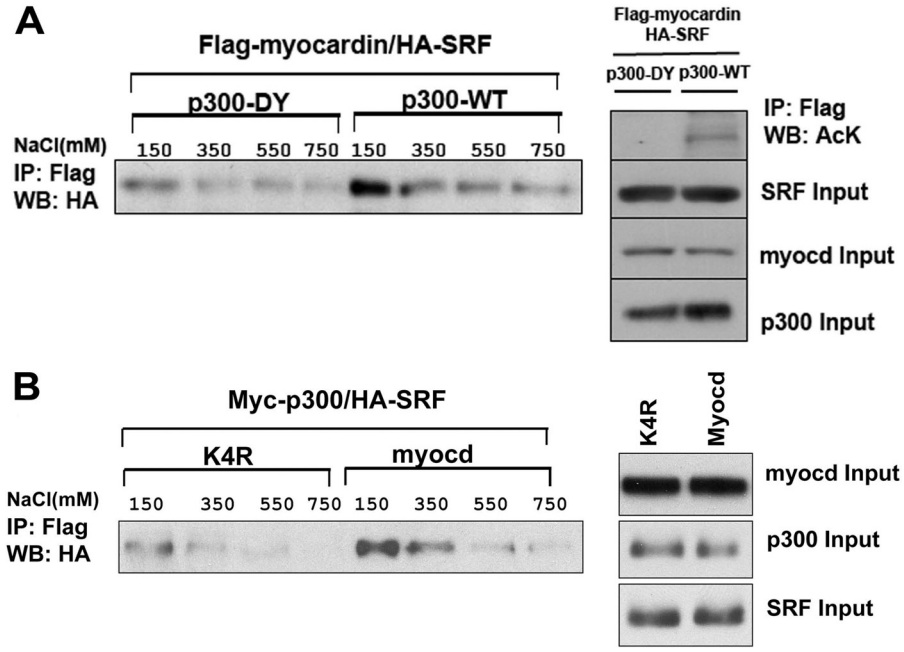

FIGURE 3. **Acetylation of myocardin enhances its association with SRF.** *A,* COS7 cells were transiently transfected with FLAG-tagged myocardin, HA-tagged SRF, and Myc-tagged p300 (*p300-WT*) or p300 mutants (*p300-DY*). FLAG-tagged myocardin (*myocd*) proteins were immunoprecipitated (*IP*) from cell lysates using an anti-FLAG antibody. The precipitates were washed in washing buffers containing the indicated NaCl concentration. Associated HA-tagged SRF proteins were then detected by an anti-HA antibody (*left panels*). Myocardin proteins were acetylated when cotransfected with wild-type p300 but not the p300-DY mutant. Comparable amount of each proteins were used in the Co-IP experiments (*right panels*). *WB*, Western blot. *B,* co-IP experiments same as described in *A* with the exception that both wild-type myocardin (*myocd*) and the myocardin K4R mutant were used.

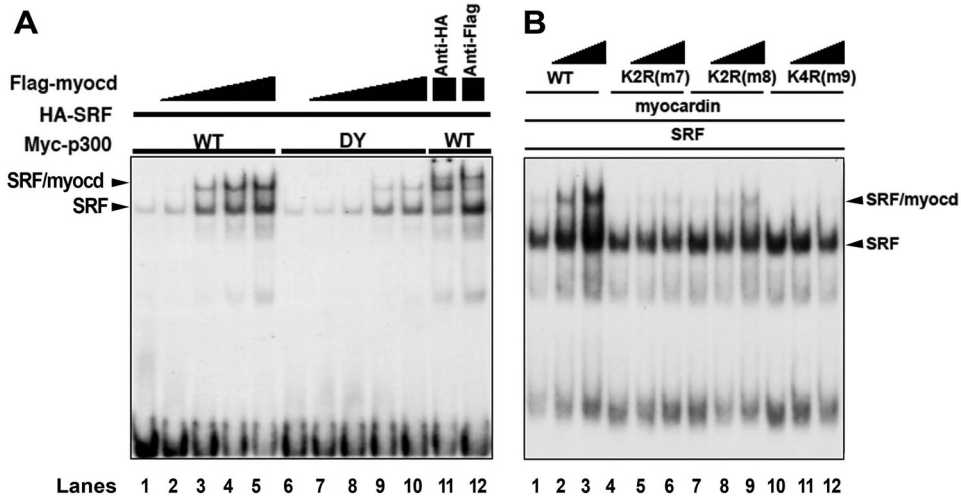

FIGURE 4. **Acetylation of myocardin is required for the formation of myocardin-SRF-CArG ternary complex.** EMSAs were performed with a ^{32}P-labeled oligonucleotide probe for c-*fos* CArG and nuclear extracts containing the indicated proteins. *A,* FLAG-tagged myocardin and HA-tagged SRF were included in the assay when either wild-type (*WT*) or mutant (*DY*) p300 proteins were also present. Note increased formation of myocardin-SRF-CArG ternary complex at the presence of p300-WT. Anti-HA and anti-FLAG antibodies were used for supershift (*lanes 11* and *12*). *B,* same EMSAs as described in *A* with the exception that both wild-type (*WT*) and acetylation-deficient K2R and K4R myocardin mutant proteins were used.

CArG boxes as well, presumably due to an acetylation modification of SRF (Fig. 4*A*).

To further confirm that the enhanced SRF-myocardin-CArG complex formation was indeed due to acetylation of myocardin, we performed EMSAs using the acetylation-deficient myocardin mutants (both the K2R and the K4R mutants). When comparable levels of wild-type and mutant myocardin proteins were used in these experiments, wild-type myocardin formed a very stable SRF-myocardin-CArG ternary complex (Fig. 4*B*, *lanes 1–3*), In contrast, the K2R mutants significantly decreased the formation of such ternary complexes (Fig. 4*B*, *lanes 4–9*). Most importantly, the K4R mutant completely abolished the formation of the SRF-myocardin-CArG ternary complexes. Notably, acetylation-deficient myocardin K2R and K4R mutants appear to have no (or very little) effect on the formation of the SRF-CArG complex (Fig. 4*B*, *lanes 10–12*). Together, these data demonstrate that acetylation modification of myocardin is essential for its association with DNA-bound SRF.

Acetylation of Myocardin Decreases the Association of HDAC and Myocardin—Myocardin transactivity is positively or negatively modulated by HAT and HDAC, respectively. This is con-

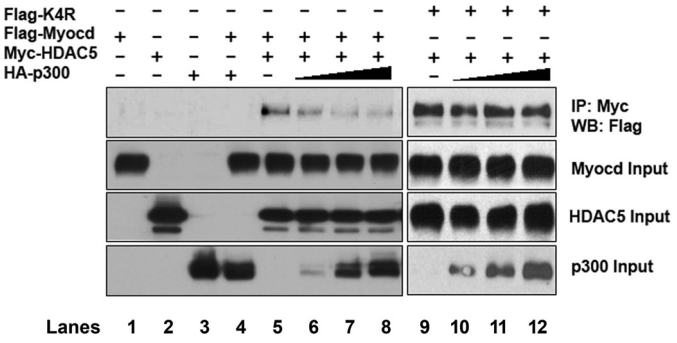

FIGURE 5. **Acetylation of myocardin decreases its HDAC5 association.** COS7 cells were transiently transfected with FLAG-tagged myocardin (*lanes 1* and *4–8*) or the myocardin K4R mutant (*lanes 9–12*), Myc-tagged HDAC5, and increasing amounts of HA-tagged p300 as indicated. Myc-tagged HDAC5 proteins were immunoprecipitated (*IP*) from cell lysates using an anti-Myc antibody. The precipitates were washed, and associated FLAG-tagged myocardin (*Myocd*) (or myocardin K4R mutant) proteins were then detected by an anti-FLAG antibody. Western blots (*WB*) demonstrate comparable amounts of each proteins were used in the Co-IP experiments.

sistent with the observation that myocardin can interact with p300 and HDAC proteins simultaneously (19). Previous studies mapped the HDAC interacting domain to the N-terminal region and p300 interacting domain to the C-terminal transactivation domain of myocardin (19). Interestingly, we found that myocardin acetylation by p300 occurs in its N-terminal region (Fig. 1). We asked whether binding of p300 to myocardin and subsequent acetylation modification could lead to de-association of HDAC from myocardin. Coimmunoprecipitation experiments were performed in which FLAG-tagged myocardin and Myc-tagged HDAC5 were coexpressed in COS7 cells at fixed levels, although HA-tagged p300 was expressed with increasing levels. As shown in Fig. 5, the affinity of HDAC5 for myocardin showed a decreasing trend when the level of p300 protein increased (Fig. 5, *lanes 5–8*). Consistent with the hypothesis that myocardin acetylation status influences its HDAC5 association, the acetylation-deficient myocardin K4R mutant remains highly associated with HDAC5, even at the presence of high p300 expression levels (Fig. 5, *lanes 9–12*). Together, these data support the view that the acetylation status of myocardin serves as a nodal point to determine whether myocardin is associated with positive or negative transcriptional regulators.

Acetylation of Myocardin Is Required to Activate Smooth Muscle Gene Expression—Previously, myocardin has been shown to sufficiently activate endogenous smooth muscle gene expression when ectopically overexpressed in 10T1/2 fibroblasts (5, 28). To investigate the functional significance of myocardin acetylation, we decided to test whether acetylation of myocardin is required for the activation of smooth muscle genes. As expected, *SM22*, *SM-α-actin*, and *SM-MHC*, markers of smooth muscle cell but not cardiac α-myosin heavy chain (*α-MHC*) or cardiac α-actin, were induced by ectopic overexpression of wild-type myocardin (Fig. 6*A*), consistent with previous reports (19, 28). However, the induction of smooth muscle markers was dramatically impaired in 10T1/2 cells transfected with the acetylation-deficient myocardin K4R mutant (Fig. 6*A*). As a control, MyoD potently induced the expression of skeletal muscle *α-actin* gene but not that of cardiac or smooth muscle genes (Fig. 6*A*).

To further confirm the above observations, we transfected 10T1/2 fibroblasts with expression plasmid for wild-type myocardin or the K4R mutant. The cells were then treated with curcumin to inhibit the activity of p300. Curcumin is a natural polyphenolic compound, and recent work has demonstrated that curcumin could inhibit the p300-histone acetyltransferases and cardiomyocyte hypertrophy (32, 33). As shown in Fig. 6*B*, myocardin, but not its K4R mutant, was able to potently activate the expression of smooth muscle genes *SM22* and *SM-MHC* (Fig. 6*B*, *lanes 4*). Curcumin treatment dramatically inhibited myocardin-mediated activation of smooth muscle genes but not that of controls (Fig. 6*B*, compare *lanes 4* and *6*). These data suggest that acetylation modification of myocardin is essential for the activation of its target smooth muscle genes.

We tested whether the acetylation of myocardin is involved in apoptosis. We transfected 10T1/2 cells with myocardin wild-type or the K4R mutant. As shown in Fig. 6*C*, overexpression of myocardin, but not the myocardin-K4R mutant, induced the expression of caspase-3, indicating an increase in apoptosis. As a control, we showed that MyoD also induced the expression of caspase-3 (Fig. 6*C*). Such observation is further supported by the results of TUNEL assays. Overexpression of myocardin, but not the myocardin-K4R mutant, resulted in more TUNEL-positive cells when compared with controls (Fig. 6*D*). Together, these data suggest that the acylation of myocardin also plays a role in the regulation of apoptosis.

DISCUSSION

Regulation of gene expression plays a central role during the cellular proliferation and differentiation processes (34, 35). Dysregulation of gene expression is often associated with varieties of pathophysiological conditions (34). It is known that both sequence-specific DNA binding by transcription factors and chromatin modification play an important role in the regulation of gene transcription (36). Previously, we have shown that myocardin directly interacts with p300 and HDACs, which positively or negatively modulates myocardin transactivity (19). In this study, we further explored the consequence of the myocardin and p300 interaction. We found that myocardin is a target for acetylation modification by p300. Acetylation of myocardin plays a central role in modulating myocardin's affinity for SRF association as well as for the formation of the myocardin-SRF-CArG ternary complex. Most importantly, we showed that acetylation of myocardin is essential for myocardin to activate cardiac and smooth muscle gene expression. These studies provide a molecular mechanism to explain how chromatin-remodeling enzymes participate in the regulation of tissue-specific gene expression by directly modulating key transcription factors.

Post-transcriptional modifications, including acetylation, methylation, phosphorylation, and ubiquitination, are critical to the function of modified proteins (15, 35). In the nucleus, such modifications occur to both histone proteins and many other nuclear proteins, including transcription factors. It is well recognized that post-translational modification of histone proteins is critical to chromatin structure and to the transcriptional control of gene expression (15, 16). Recent studies have also

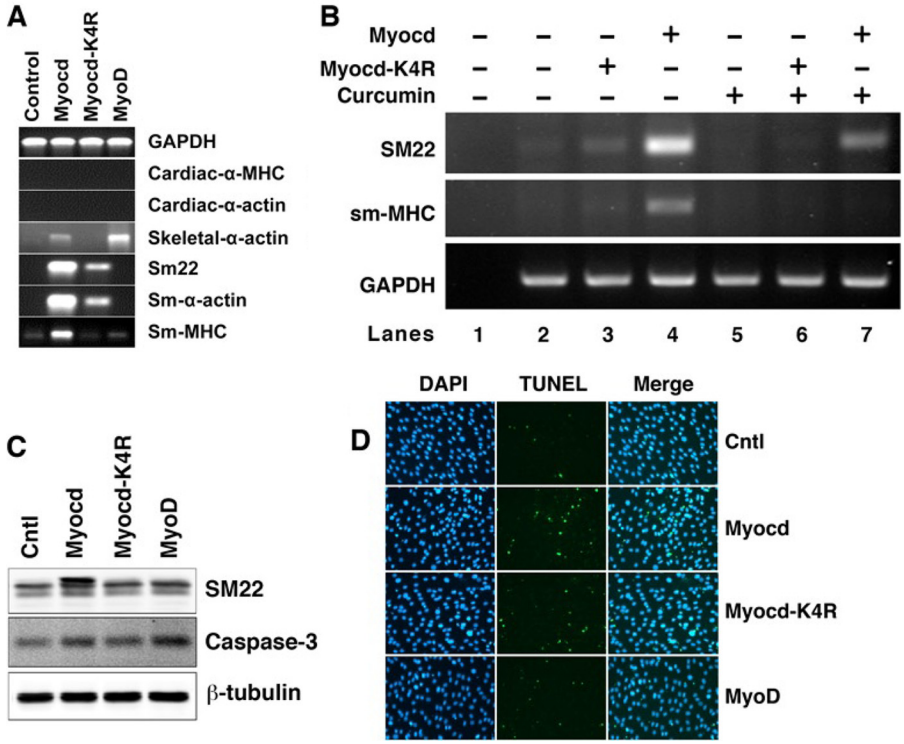

FIGURE 6. **Myocardin acetylation-deficient mutant failed to activate smooth muscle gene expression.** *A,* 10T1/2 cells were transfected with expression vectors encoding CMV-lacZ (*control*), myocardin (*Myocd*), myocardin acetylation-deficient K4R mutant (*Myocd-K4R*), and MyoD. Total RNAs were isolated, and muscle gene expression was assayed by RT-PCR. *GAPDH* was measured as a loading control. *B,* 10T1/2 cells were transfected with expression vectors encoding myocardin (*Myocd*) or myocardin acetylation-deficient K4R mutant (*Myocd-K4R*). Cells were treated with curcumin (or without treatment to serve as controls), and total RNAs were isolated, and the expression of smooth muscle genes was assayed by RT-PCR. GAPDH was measured as a loading control. *C,* 10T1/2 cells were transfected with the indicated expression vectors, and cell extracts were subjected to Western blots to document the expression of SM22 protein and cleaved caspase-3. β-Tubulin was used as a loading control (*Cntl*). *D,* 10T1/2 cells were transfected with indicated expression vectors, and apoptosis was detected by TUNEL assays. DAPI marks the nuclei.

documented that post-translational modification of transcription factors contribute significantly to the activation of gene expression (35). The first example of a non-histone protein target for HAT acetylation is p53, a well studied tumor suppressor and transcription factor (24). It was shown that p300 acetylates p53 at multiple lysine residues located at the C-terminal DNA binding domain of the protein. As a consequence of acetylation, p53 transactivity was enhanced. Conversely, HDAC1 deacetylates p53 *in vitro* and *in vivo*, thereby reversing the function of p53 (24, 25). Another example of a p300 non-histone target for acetylation is Yin Yang 1, or YY1, a transcription factor involved in many biological processes. Intriguingly, YYI has been shown to be either a transcriptional activator or repressor, depending on the context (31). It is now known that YY1 can be acetylated by p300 and deacetylated by HDACs (HDAC1, -2, and -3). These acetylation and deacetylation modifications, together with other post-translational modifications such as phosphorylation, determine whether YY1 acts as a transcriptional activator or transcriptional repressor (31). In skeletal muscle, MyoD, which belongs to the family of myogenic transcription factors that function as "master" regulators to activate myogenic gene expression, was also identified as a target of p300 acetylation. Again, acetylation of MyoD increased its transcriptional activity, at least in part, by increasing its affinity for DNA binding on the promoters and/or enhancers of genes induced during muscle differentiation (22, 23). Our study demonstrates myocardin as yet another key transcription factor target of acetylation modification. Similar to our analysis of the nonacetylated form, acetylated myocardin does not bind to DNA directly; instead, this post-translational modification promotes formation of a more stable complex with SRF to potentiate its binding to DNA and the formation of the myocardin-SRF-CArG ternary complex. Interestingly, myocardin was shown to repress cell proliferation and could function as a tumor suppressor (37, 38). It will be important to determine whether acetylation modification of myocardin contributes to its function in inhibiting cell proliferation.

In this study, we have defined four lysine residues in myocardin that are primarily responsive to p300 acetylation. Myocardin belongs to the family of SAP-containing myocardin and MRTFs (3, 39). We have examined the conservation of those lysine residues among myocardin and MRTF protein cross-species. We found that Lys-235, Lys-237, and Lys-253 are highly conserved among all myocardin family of transcription factors in species from *Xenopus* to human. This is particular intriguing given that myocardin was previously shown to activate cardiac gene expression ectopically in *Xenopus* embryos (40). We speculate that acetylation modification is required for myocardin to activate cardiac gene expression in this setting. However, Lys-255 is conserved between myocardin and MRTF-A but not in MRTF-B.[4] It will be important to investigate whether MRTFs

[4] D. Cao and D.-Z. Wang, unpublished data.

are also subjected to acetylation modification and, if so, how such modification alters their ability in activating target gene expression.

Both p300 (HAT) and class II HDACs directly interact with and regulate the transactivity of myocardin (19). Our data indicate that binding of p300 to myocardin and subsequent acetylation led to its de-association from HDAC5. It should be noted that increasing p300 did not cause complete de-association of HDAC5 from myocardin, suggesting that additional mechanisms regulating myocardin activity might exist. It will be interesting for future studies to investigate whether the acetylation and deacetylation of myocardin by p300 and HDACs is reversible, and if so, how such dynamic switching between acetylated and deacetylated states might affect the transcriptional property of myocardin; more importantly, it will be crucial to determine whether such regulation is associated with cardiac and smooth muscle gene expression during development and/or pathological conditions.

It has previously been shown that HDAC5 was also able to repress the transcriptional activity of MEF2C, a member of the myocyte enhancer factor-2 (MEF2) family (41–45). Interestingly, this repression can be released by calcium/calmodulin-dependent protein kinase signaling, which apparently functions to disrupt MEF2-HDAC complexes and stimulate HDAC nuclear export (46). This would suggest that HDAC-mediated transcription repression is signal-dependent. Interestingly, we have recently demonstrated that myocardin transactivity is enhanced by TGF-β and bone morphogenetic protein signaling pathways (27, 47), raising the possibility that those signaling pathways could be involved in the activation of p300. It will be intriguing to test whether calcium/calmodulin-dependent protein kinase signaling will release the repression of HDAC5 on myocardin. In particular, it is important to determine whether a similar nuclear export mechanism is involved.

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REFERENCES

1. Wang, D. Z., and Olson, E. N. (2004) Control of smooth muscle development by the myocardin family of transcriptional coactivators. *Curr. Opin. Genet. Dev.* **14,** 558–566
2. Wang, D., Chang, P. S., Wang, Z., Sutherland, L., Richardson, J. A., Small, E., Krieg, P. A., and Olson, E. N. (2001) Activation of cardiac gene expression by myocardin, a transcriptional cofactor for serum response factor. *Cell* **105,** 851–862
3. Wang, D. Z., Li, S., Hockemeyer, D., Sutherland, L., Wang, Z., Schratt, G., Richardson, J. A., Nordheim, A., and Olson, E. N. (2002) Potentiation of serum response factor activity by a family of myocardin-related transcription factors. *Proc. Natl. Acad. Sci. U.S.A.* **99,** 14855–14860
4. Wang, Z., Wang, D. Z., Hockemeyer, D., McAnally, J., Nordheim, A., and Olson, E. N. (2004) Myocardin and ternary complex factors compete for SRF to control smooth muscle gene expression. *Nature* **428,** 185–189
5. Chen, J., Kitchen, C. M., Streb, J. W., and Miano, J. M. (2002) Myocardin. A component of a molecular switch for smooth muscle differentiation. *J. Mol. Cell. Cardiol.* **34,** 1345–1356
6. Long, X., Creemers, E. E., Wang, D. Z., Olson, E. N., and Miano, J. M. (2007) Myocardin is a bifunctional switch for smooth *versus* skeletal muscle differentiation. *Proc. Natl. Acad. Sci. U.S.A.* **104,** 16570–16575
7. Pipes, G. C., Creemers, E. E., and Olson, E. N. (2006) The myocardin family of transcriptional coactivators. Versatile regulators of cell growth, migration, and myogenesis. *Genes Dev.* **20,** 1545–1556
8. Miano, J. M. (2004) Channeling to myocardin. *Circ. Res.* **95,** 340–342
9. Liu, Z. P., Wang, Z., Yanagisawa, H., and Olson, E. N. (2005) Phenotypic modulation of smooth muscle cells through interaction of Foxo4 and myocardin. *Dev. Cell* **9,** 261–270
10. Li, S., Wang, D. Z., Wang, Z., Richardson, J. A., and Olson, E. N. (2003) The serum response factor coactivator myocardin is required for vascular smooth muscle development. *Proc. Natl. Acad. Sci. U.S.A.* **100,** 9366–9370
11. Li, J., Zhu, X., Chen, M., Cheng, L., Zhou, D., Lu, M. M., Du, K., Epstein, J. A., and Parmacek, M. S. (2005) Myocardin-related transcription factor B is required in cardiac neural crest for smooth muscle differentiation and cardiovascular development. *Proc. Natl. Acad. Sci. U.S.A.* **102,** 8916–8921
12. Oh, J., Richardson, J. A., and Olson, E. N. (2005) Requirement of myocardin-related transcription factor-B for remodeling of branchial arch arteries and smooth muscle differentiation. *Proc. Natl. Acad. Sci. U.S.A.* **102,** 15122–15127
13. Sun, Y., Boyd, K., Xu, W., Ma, J., Jackson, C. W., Fu, A., Shillingford, J. M., Robinson, G. W., Hennighausen, L., Hitzler, J. K., Ma, Z., and Morris, S. W. (2006) Acute myeloid leukemia-associated Mkl1 (Mrtf-a) is a key regulator of mammary gland function. *Mol. Cell. Biol.* **26,** 5809–5826
14. Parmacek, M. S. (2007) Myocardin-related transcription factors. Critical coactivators regulating cardiovascular development and adaptation. *Circ. Res.* **100,** 633–644
15. Ruthenburg, A. J., Li, H., Patel, D. J., and Allis, C. D. (2007) Multivalent engagement of chromatin modifications by linked binding modules. *Nat. Rev. Mol. Cell Biol.* **8,** 983–994
16. Haberland, M., Montgomery, R. L., and Olson, E. N. (2009) The many roles of histone deacetylases in development and physiology. Implications for disease and therapy. *Nat. Rev. Genet.* **10,** 32–42
17. Shahbazian, M. D., and Grunstein, M. (2007) Functions of site-specific histone acetylation and deacetylation. *Annu. Rev. Biochem.* **76,** 75–100
18. Backs, J., and Olson, E. N. (2006) Control of cardiac growth by histone acetylation/deacetylation. *Circ. Res.* **98,** 15–24
19. Cao, D., Wang, Z., Zhang, C. L., Oh, J., Xing, W., Li, S., Richardson, J. A., Wang, D. Z., and Olson, E. N. (2005) Modulation of smooth muscle gene expression by association of histone acetyltransferases and deacetylases with myocardin. *Mol. Cell. Biol.* **25,** 364–376
20. Mal, A., Sturniolo, M., Schiltz, R. L., Ghosh, M. K., and Harter, M. L. (2001) A role for histone deacetylase HDAC1 in modulating the transcriptional activity of MyoD. Inhibition of the myogenic program. *EMBO J.* **20,** 1739–1753
21. Polesskaya, A., Duquet, A., Naguibneva, I., Weise, C., Vervisch, A., Bengal, E., Hucho, F., Robin, P., and Harel-Bellan, A. (2000) CREB-binding protein/p300 activates MyoD by acetylation. *J. Biol. Chem.* **275,** 34359–34364
22. Polesskaya, A., and Harel-Bellan, A. (2001) Acetylation of MyoD by p300 requires more than its histone acetyltransferase domain. *J. Biol. Chem.* **276,** 44502–44503
23. Sartorelli, V., Puri, P. L., Hamamori, Y., Ogryzko, V., Chung, G., Nakatani, Y., Wang, J. Y., and Kedes, L. (1999) Acetylation of MyoD directed by PCAF is necessary for the execution of the muscle program. *Mol. Cell* **4,** 725–734
24. Gu, W., and Roeder, R. G. (1997) Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell* **90,** 595–606
25. Luo, J., Li, M., Tang, Y., Laszkowska, M., Roeder, R. G., and Gu, W. (2004) Acetylation of p53 augments its site-specific DNA binding both *in vitro* and *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* **101,** 2259–2264
26. Bereshchenko, O. R., Gu, W., and Dalla-Favera, R. (2002) Acetylation inactivates the transcriptional repressor BCL6. *Nat. Genet.* **32,** 606–613
27. Callis, T. E., Cao, D., and Wang, D. Z. (2005) Bone morphogenetic protein signaling modulates myocardin transactivation of cardiac genes. *Circ. Res.* **97,** 992–1000
28. Wang, Z., Wang, D. Z., Pipes, G. C., and Olson, E. N. (2003) Myocardin is a master regulator of smooth muscle gene expression. *Proc. Natl. Acad. Sci. U.S.A.* **100,** 7129–7134

29. Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996) The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* **87,** 953–959
30. Tang, Y., Luo, J., Zhang, W., and Gu, W. (2006) Tip60-dependent acetylation of p53 modulates the decision between cell-cycle arrest and apoptosis. *Mol. Cell* **24,** 827–839
31. Yao, Y. L., Yang, W. M., and Seto, E. (2001) Regulation of transcription factor YY1 by acetylation and deacetylation. *Mol. Cell. Biol.* **21,** 5979–5991
32. Feng, B., Chen, S., Chiu, J., George, B., and Chakrabarti, S. (2008) Regulation of cardiomyocyte hypertrophy in diabetes at the transcriptional level. *Am. J. Physiol. Endocrinol. Metab.* **294,** E1119–E1126
33. Morimoto, T., Sunagawa, Y., Kawamura, T., Takaya, T., Wada, H., Nagasawa, A., Komeda, M., Fujita, M., Shimatsu, A., Kita, T., and Hasegawa, K. (2008) The dietary compound curcumin inhibits p300 histone acetyltransferase activity and prevents heart failure in rats. *J. Clin. Invest.* **118,** 868–878
34. Han, P., Hang, C. T., Yang, J., and Chang, C. P. (2011) Chromatin remodeling in cardiovascular development and physiology. *Circ. Res.* **108,** 378–396
35. Bonasio, R., Tu, S., and Reinberg, D. (2010) Molecular signals of epigenetic states. *Science* **330,** 612–616
36. Hasselgren, P. O. (2007) Ubiquitination, phosphorylation, and acetylation. Triple threat in muscle wasting. *J. Cell Physiol.* **213,** 679–689
37. Milyavsky, M., Shats, I., Cholostoy, A., Brosh, R., Buganim, Y., Weisz, L., Kogan, I., Cohen, M., Shatz, M., Madar, S., Kalo, E., Goldfinger, N., Yuan, J., Ron, S., MacKenzie, K., Eden, A., and Rotter, V. (2007) Inactivation of myocardin and p16 during malignant transformation contributes to a differentiation defect. *Cancer Cell* **11,** 133–146
38. Tang, R. H., Zheng, X. L., Callis, T. E., Stansfield, W. E., He, J., Baldwin, A. S., Wang, D. Z., and Selzman, C. H. (2008) Myocardin inhibits cellular proliferation by inhibiting NF-kappaB(p65)-dependent cell cycle progression. *Proc. Natl. Acad. Sci. U.S.A.* **105,** 3362–3367
39. Meadows, S. M., Warkman, A. S., Salanga, M. C., Small, E. M., and Krieg, P. A. (2008) The myocardin-related transcription factor, MASTR, cooperates with MyoD to activate skeletal muscle gene expression. *Proc. Natl. Acad. Sci. U.S.A.* **105,** 1545–1550
40. Small, E. M., Warkman, A. S., Wang, D. Z., Sutherland, L. B., Olson, E. N., and Krieg, P. A. (2005) Myocardin is sufficient and necessary for cardiac gene expression in *Xenopus*. *Development* **132,** 987–997
41. McKinsey, T. A., Zhang, C. L., and Olson, E. N. (2001) Control of muscle development by dueling HATs and HDACs. *Curr. Opin. Genet. Dev.* **11,** 497–504
42. Haberland, M., Arnold, M. A., McAnally, J., Phan, D., Kim, Y., and Olson, E. N. (2007) Regulation of HDAC9 gene expression by MEF2 establishes a negative-feedback loop in the transcriptional circuitry of muscle differentiation. *Mol. Cell. Biol.* **27,** 518–525
43. Grégoire, S., Tremblay, A. M., Xiao, L., Yang, Q., Ma, K., Nie, J., Mao, Z., Wu, Z., Giguère, V., and Yang, X. J. (2006) Control of MEF2 transcriptional activity by coordinated phosphorylation and sumoylation. *J. Biol. Chem.* **281,** 4423–4433
44. Zhang, C. L., McKinsey, T. A., Chang, S., Antos, C. L., Hill, J. A., and Olson, E. N. (2002) Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy. *Cell* **110,** 479–488
45. Olson, E. N., Backs, J., and McKinsey, T. A. (2006) Control of cardiac hypertrophy and heart failure by histone acetylation/deacetylation. *Novartis Found. Symp.* **274,** 3–12
46. McKinsey, T. A., Zhang, C. L., Lu, J., and Olson, E. N. (2000) Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. *Nature* **408,** 106–111
47. Qiu, P., Ritchie, R. P., Fu, Z., Cao, D., Cumming, J., Miano, J. M., Wang, D. Z., Li, H. J., and Li, L. (2005) Myocardin enhances Smad3-mediated transforming growth factor-β1 signaling in a CArG box-independent manner. Smad-binding element is an important cis element for SM22α transcription *in vivo*. *Circ. Res.* **97,** 983–991