Acetylation of myocardin is required for the activation of cardiac and smooth muscle genes

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Running title: acetylation of myocardin
Word and character counts: 5657 words; 39,337 characters (including space)

Capsule

**Background:** Myocardin is a cardiac- and smooth muscle specific transcription factor.
**Result:** Myocardin is directly acetylated by p300.
Conclusion: Acetylation of myocardin is critical for myocardin to activate cardiac and smooth muscle target gene expression.

Significance: 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.

Myocardin and myocardin-related transcription factors (MRTFs) belong to a family of 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 serum response factor (SRF) bound to the DNA consensus sequence CC(A/T)$_n$GG, known as a CArG box. Such recruitment of myocardin and MRTFs brings the powerful transcription activation domain (TAD) 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 (7) (1,8). 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 histone acetyltransferase (HAT), and HDAC4 and HDAC5, respectively. p300 interacts with myocardin at its C-terminal transactivation domain (TAD) to enhance the transactivity 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 change the DNA-binding affinity of transcription factors (25), or disrupt the ability of transcription factors to recruit additional co-factors (26). Previously we proposed three putative mechanisms for p300 enhancement of myocardin-mediated gene expression, i) p300 functions as a general transcriptional coactivator or adaptor, ii) p300 functions as a histone acetyltransferase (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 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 myocardin/RF ternary complex on CArG boxes. 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 (2,19,27). The p300 expression vectors were as previously described (19). Myocardin mutants were generated through PCR-based mutagenesis using the QuickChange 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 (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 duplication.

**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 2X YT medium to an optical density of 1.0. IPTG (50 µM) was then added to the culture to induce protein expression. After shaking at room temperature for 4-6 hrs, the cells were harvested and the GST proteins were purified with glutathione beads according to Amersham's procedure.

Proteins translated *in vitro* were labeled with 35S-methionine using a TNT T7-reticulocyte lysate system (Promega). Glutathione beads conjugated with 1 µg protein were incubated with 10 µl TNT product, at 4 °C for 2 hrs in 500 µl GST binding buffer (20 mM Tris, pH7.3, 150 mM NaCl, 0.5% NP40, protease inhibitor cocktail from Roche, 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-PAGE.

**Immunostaining, TUNEL assays**
Immunostaining was performed as described (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 (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 (Myocd), myocardin acetylation-deficient K4R mutant (Myocd-K4R) and MyoD. 48hr later, cells were switched to differentiation medium (DMEM containing 2% horse serum). After additional 48hr, the cells were collected, fixed and proceeded for TUNEL assay using the ApopTag®Plus Fluorescein In Situ Apoptosis Detection Kit (S7111, Chemicon) according to the manufacture’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 Molecular Biochemicals). 48 hrs 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 Molecular Biochemicals). 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). The bound proteins were washed five times with lysis buffer, or washing buffer with increasing salt concentration (from 150, 350, 550 to 750mM of NaCl), and 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).

**In vitro acetylation assay**
*In vitro* acetylation assays were performed as described (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 solution containing 50 mM HEPES (pH8.0), 10% glycerol, 1 mM DTT, 1 µl of [3H]-acetyl CoA (Amersham), 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 hr. The reaction was resolved by SDS-PAGE and dried and subject 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 same as previously described (4,19,28). All PCR products span intron region of the genes.

**Electrophoretic Mobility Shift Assay (EMSA)**

EMSA was carried out essentially as described (2,19) with minor modifications. The DNA binding reactions (20 µl) contained 20 mM Tris–HCl (pH 7.5), 50 mM KCl, 5 mM MgCl₂, 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 32P-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:Bis, 50:1) containing 0.5 X TBE and electrophoresed in 0.25 X TBE at 180–220 V for 3 hrs. In the case of supershift assays, the indicated monoclonal antibodies (200 ng) were added to reaction mixtures during pre-incubation.

**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. 1A). 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. 1A). As a negative control, GST protein was not acetylated. Notably, p300 also acetylates itself in these assays (Fig. 1A, upper bands), consistent with previous reports (24).

Acetylation by acetyltransferase(s) occurs at lysine residues (17,24). The N-terminus of myocardin (aa 1-274) contains 26 lysine residues, of 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 (NLS) (Fig. 1B). To determine which lysine residue(s) are accessible to acetylation, we systematically mutated these lysine residues into arginines (K-to-R) and assayed their ability to be acetylated by p300 in vitro (Fig. 1C). Because both arginine and lysine share similar chemical structure and charge, the lysine to arginine mutation will minimize the effect on other properties of 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.
Two closely spaced clusters of three lysines (aa 247-250 and aa 257-260) located within the NLS 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 further enhanced in the mutant myocardin protein, indicating that those lysine residues are unlikely the primary acetylation sites (Fig. 1C). Further introducing mutation into additional lysines at position 253 and 255 reduced myocardin acetylation moderately (m2 and m3 in Fig. 1C). 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. 1C). 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 (K235/237 or K253/255) or four lysines (K235/237/253/255) were mutated into arginines. As shown in Fig. 1D, myocardin with two lysines being mutated (K2R) partially decreased its acetylation (m7 and m8 in Fig. 1D). However, the myocardin mutant with all four lysine residues mutated (thereafter called K4R mutant) was incapable of being acetylated (m9 in Fig. 1D). 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 over-expressed 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 determined by anti-acetyllysine 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. 1E). 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 myocardin protein (19). Interestingly, myocardin 1-274, though sufficient to be acetylated in vitro (Fig. 1A), 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 trans-activation of cardiac and smooth muscle gene
expression by myocardin. Myocardin was co-transfected with wild-type or HAT-deficient p300 mutant into COS7 cells together with SM22 or ANF luciferase reporter genes. As shown in Figure 2A, increasing concentration 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, co-transfection of a HAT-deficient p300 mutant (p300-DY) failed to generate any synergy in these assays (Fig. 2A), suggesting that p300 HAT activity is required for the synergy between p300 and myocardin. Similar observations were obtained on 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. 2B). 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.

Since these K-to-R mutations occur within or near the basic domain of myocardin which also contains the NLS, we examined if those mutations affect the nuclear localization of myocardin proteins. Transfection of the wild-type or the K-to-R myocardin mutant constructs into COS7 cells showed that the K-to-R mutations did not change the nuclear location pattern of myocardin proteins (Fig. 2C). 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-deficient mutants and p300 was not resulted 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, co-immunoprecipitation (Co-IP) assays were performed with cell lysates containing equal amount 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 concentration 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 to 6 fold higher when WT p300 was included (and therefore, myocardin predicted to be acetylated) than when p300-DY was included (and therefore, myocardin acetylation predicted to be absent) (Fig. 3A). As salt concentration increases in the washing buffer (which correlates with increasing stringency for protein-protein interaction), the association of SRF and non-acetylated myocardin significantly diminished, whereas the association of SRF and acetylated myocardin decreased much slowly (Fig. 3A). 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 precipitated by anti-Flag antibodies, immunoprecipitates were subjected to wash with increasing salt concentration in wash buffer. As expected, wild-type myocardin showed much stronger binding affinity for SRF than the acetylation-deficient myocardin K4R mutant (Fig. 3B). 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, electrophoretic mobility shift assays (EMSAs) were performed with nuclear extracts containing equal amount of SRF and p300 (or the p300-DY mutant) and an increasing amount of myocardin. As shown in Figure 4A, increasing myocardin protein amount results in the formation of more stable SRF/myocardin/CArG ternary complex in the presence of wild-type p300 (WT) (Fig. 4A, lanes 2-5). In contrast, the SRF/myocardin/CArG ternary complex formation was significantly deceased in the presence of p300-DY (Fig. 4A, lanes 7-10). The specificity of the ternary complex was demonstrated by anti-body supershift (Fig. 4A, lanes 11-12). Together, these results suggest that acetylation of myocardin enhances the formation of the SRF/myocardin/CArG ternary complex. Noticeably, p300 appears to enhance the binding of SRF to the CArG boxes as well, presumably due to an acetylation modification of SRF (Fig. 4A).

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 level of wild-type and mutant myocardin proteins were used in these experiments, wild-type myocardin (WT) formed a very stable SRF/myocardin/CArG ternary complex (Fig 4B, lanes 1-3). In contrast, the K2R mutants significantly decreased the formation of such ternary complexes (Fig. 4B, 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 SRF/CArG complex (Fig. 4B, 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 consistent 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 (TAD) 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 HADC from myocardin. Co-immunoprecipitation experiments were performed in which Flag-tagged myocardin and Myc-tagged HDAC5 were co-expressed in COS7 cells at fixed levels while 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 level (Fig. 5, lanes 9-12). Together, these data support the view that 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 alpha myosin heavy chain (α-MHC) or cardiac α-actin, were induced by ectopic over-expression of wild-type myocardin (Fig. 6A), 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. 6A). As a control, MyoD potently induced the expression of skeletal muscle α-actin gene, but not that of cardiac or smooth muscle genes (Fig. 6A).

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 works demonstrated that curcumin could inhibit the p300-histone acetyltransferases and cardiomyocyte hypertrophy (32,33). As shown in Fig. 6B, myocardin, but not its K4R mutant, was able to potently activate the expression of smooth muscle genes SM22 and SM-MHC (Fig. 6B, lanes 4). Curcumin treatment dramatically inhibited myocardin-mediated activation of smooth muscle genes, but not that of controls (Fig. 6B, 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. 6C, overexpression of myocardin, but not the myocardin-K4R mutant, induced the expression of caspase-3, indicating a increase in apoptosis. As a control, we showed that MyoD also induced the expression of caspase-3 (Fig. 6C). 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. 6D). Together, these data suggest that the acetylation 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 modulate 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 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 as well as the regulation of cardiomyocyte hypertrophy. These studies provide a molecular mechanism to explain how chromatin-remodeling enzymes participate the regulation of tissue-specific gene expression by directly modulating key transcription factors.

Post-transcriptional modification, 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 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 acylates 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, YY1 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 non-acetylated 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 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 which are primarily responsive to p300 acetylation. Myocardin belongs to the family of SAP-containing myocardin and myocardin-related transcription factors (MRTFs) (3,39). We have examined the conservation of those lysine residues among myocardin and MRTF proteins across species. We found that
K235, K237 and K253 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. On the other hand, K255 is conserved between myocardin and MRTF-A, but not in MRTF-B (Unpublished data). It will be important to investigate whether MRTFs 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 (CaMK) 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 BMP 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 CaMK 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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FOOTNOTES
We thank members of the Wang lab for their support and discussion. We thank Drs. Ronald Neppl and John Mably for reading the manuscript and comments. Research in the Wang lab was supported by the March of Dimes Foundation. The project was supported by HL085635 and HL075251 from the National Heart, Lung, and Blood Institute. M Tatsuguchi was a postdoctoral fellow and DZ Wang is an Established Investigator of the American Heart Association.

Author contributions:
DC, CW and DZW designed the experiment; DC, CW, RT, MT, HC and ZZ performed experiments; DC, CW, RT, MT and DZW analyzed the data; DC and DZW wrote the manuscript.

The authors have declared that no competing interests exist.

FIGURE LEGENDS
Figure 1 Myocardin is acetylated by p300 in vitro and in vivo. (A) In vitro acetylation assays. GST-myocardin fusion proteins or GST alone were incubated with p300 in the presence of [3H]-acetyl-CoA and resolved on SDS-PAGE. Only myocardin (aa 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 (K) residues were displayed. B, basic domain; Q, glutamine-rich domain; SAP, SAP domain; TAD, transactivation domain. (C-D) In vitro acetylation assays with GST-myocardin fusion protein (aa 1-274) and its derived K-to-R 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).

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 amount of either wild-type (p300-WT) or mutant (p300-DY) p300 and luciferase activity 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 (myocdm7, m8 and m9) and luciferase activity measured. The luciferase activity was determined 48 hr 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 was determined by immunostaining. DAPI staining marks nuclei.

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 mutant (p300-DY). Flag-tagged myocardin proteins were immunoprecipitated from cell lysates using an anti-Flag antibody. The precipitates were washed in washing buffers containing indicated NaCl concentration. Associated HA-tagged SRF proteins were then detected by an anti-HA antibody (left panels). Myocardin proteins were acetylated when co-transfected with wild type p300, but not the p300-DY mutant. Comparable amount of each proteins were used in the Co-IP experiments (right panels). (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. Electrophoretic mobility shift assays (EMSAs) were performed with a 32P-labeled oligonucleotide probe for c-fos CArG and nuclear extracts containing 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.

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

**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. DAPDH 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. DAPDH was measured as a loading control. (C) 10T1/2 cells were transfected with 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. (D) 10T1/2 cells were transfected with indicated expression vectors and apoptosis was detected by TUNEL assays. DAPI marks nuclei.
Cao et al., Figure 2

Panel A: Bar graph showing fold activation of ANF-Luc with combinations of Myocardin, p300, Myocardin-Wt, and p300-DY.

Panel B: Bar graph showing fold activation of ANF-Luc with combinations of Myocardin-Wt, p300, Myocardin-K2R (m7), Myocardin-K2R (m8), and Myocardin-K4R (m9).

Panel C: Images showing DAPI staining and Myocardin localization with different combinations as indicated.
Cao et al., Figure 5
Cao et al., Figure 6

A

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Lanes

1 | 2 | 3 | 4 | 5 | 6 | 7

C

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D

DAPI  | TUNEL  | Merge
Cntl  |         |      |
Myocd |         |      |
Myocd-K4R |       |      |
MyoD  |         |      |
Acetylation of myocardin is required for the activation of cardiac and smooth muscle genes
Dongsun Cao, Chunbo Wang, Ruhang Tang, Huaqun Chen, Zheng Zhang, Mariko Tatsuguchi and Da-Zhi Wang

J. Biol. Chem. published online September 23, 2012

Access the most updated version of this article at doi: 10.1074/jbc.M112.353649

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