Regulation of Acetylation of Histone Deacetylase 2 by p300/CBP-Associated Factor/Histone Deacetylase 5 in the Development of Cardiac Hypertrophy

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ABSTRACT

**Rationale:** Histone deacetylases (HDACs) are closely involved in cardiac reprogramming. Although the functional roles of the class I and class IIa HDACs are well established, the significance of interclass crosstalk in the development of cardiac hypertrophy remains unclear.

**Objective:** Recently, we suggested that casein kinase-2α1-dependent phosphorylation of HDAC2 leads to enzymatic activation, which in turn induces cardiac hypertrophy. Here we report an alternate posttranslational activation mechanism of HDAC2 that involves acetylation of HDAC2 mediated by p300/CBP-associated factor (pCAF)/HDAC5.

**Methods and Results:** Hdac2 was acetylated in response to hypertrophic stresses in both cardiomyocytes and a mouse model. The acetylation was reduced by a histone acetyltransferase inhibitor but was increased by a nonspecific HDAC inhibitor. The enzymatic activity of Hdac2 was positively correlated with its acetylation status. pCAF bound to Hdac2 and induced acetylation. The HDAC2 K75 residue was responsible for hypertrophic stress-induced acetylation. The acetylation-resistant Hdac2 K75R showed a significant decrease in phosphorylation on S394, which led to the loss of intrinsic activity. Hdac5, one of class IIa HDACs, directly deacetylated Hdac2. Acetylation of Hdac2 was increased in Hdac5 null mice. When an acetylation-mimicking mutant of Hdac2 was infected into cardiomyocytes, the anti-hypertrophic effect of either nuclear tethering of Hdac5 with leptomycin B or Hdac5 overexpression was reduced.

**Conclusions:** Taken together, our results suggest a novel mechanism by which the balance of HDAC2 acetylation is regulated by pCAF and HDAC5 in the development of cardiac hypertrophy.

**Keywords:** Cardiac hypertrophy, HDAC2, posttranslational modification, acetylation, pCAF, HDAC5, gene regulation

**Nonstandard Abbreviations and Acronyms:**

CK2α1  Casein kinase 2α1
HDAC    Histone deacetylase
pCAF    p300/CBP-associated factor
RELA    V-rel reticuloendotheliosis viral oncogene homolog A
SMRT    Silencing mediator of retinoic acid and thyroid hormone receptor
N-CoR   Nuclear receptor corepressor 1
GATA    GATA binding protein
PLAG    Pleiomorphic adenoma gene
KDAC    Lysine (K) deacetylase
ARVCs   Adult rat ventricular cardiomyocytes
NRVCs   Neonatal rat ventricular cardiomyocytes
PBS     Phosphate buffered saline
ANOVA   Analysis of variance
TSA     Trichostatin A
Ad      Adenovirus
siRNA   Small interfering RNA
PKC/PKD Protein kinase C/protein kinase D
INTRODUCTION

Proteins are frequently modified by diverse enzymes such as kinases, methyltransferases, ubiquitinylases, and acetyltransferases. Such modifications are called posttranslational modifications, and the relevance of posttranslational modifications to both cellular function and human diseases has been highlighted. Among those modifications, phosphorylation is well-known as a critical process for enzymatic activity, signal transduction by recruiting complexes, DNA binding, and cell division. Though biological roles of other modifications, especially acetylation, have not been intensively studied yet, function of acetylation is as important as that of phosphorylation. For example, it is well-established that lysine-acetylation of histone 3 or 4 at the promoter region of certain genes is associated with transcriptional activation of downstream genes. The acetylation of lysine causes a “loosening” of the nucleosome by neutralizing the positive charge. p53, one of the best recognized oncoproteins, requires acetylation for stabilization, which thereby results in subsequent activation for its own functions. Interestingly, a single molecule may be subject to multiple posttranslational modifications; in addition, one modification often affects other types of modifications.

Mammalian HDACs are divided into four classes. Class I HDACs are ubiquitously expressed, whereas class II HDACs show tissue-specific expression. Class III HDACs require NAD+ for their activity and are associated with longevity. HDACs remove acetyl groups from an ε amino-terminal lysine on a histone 3 or 4. The HDACs are so named because of this histone deacetylase activity; however, many groups have reported that non-histone proteins can also be deacetylated by HDACs. Such non-histone proteins include p53 by HDAC1 in cancer physiology, glucocorticoid receptor by HDAC2 in chronic obstructive pulmonary diseases, RELA by HDAC3 in SMRT/N-CoR complex formation, GATA1 by HDAC3/HDAC4/HDAC5 in murine erythroleukemia cell differentiation, α-tubulin by HDAC6 in control of cell motility, and PLAG1/PLAGL2 by HDAC7 in tumorigenesis. Thus, to date, HDACs are also called lysine deacetylases (KDACs) to describe their function rather than their targets.

We and others have studied the functional importance of HDACs in cardiac hypertrophy. We have demonstrated that HDAC2 provokes cardiac hypertrophy and that phosphorylation-dependent activation of HDAC2 plays a critical role in the regulation of anti-hypertrophic genes. Hdac5 and Hdac9, which are class Ila HDACs, by contrast, are reported to inhibit cardiac hypertrophy as demonstrated by their knockout in mice in which hypertrophic phenotypes were exaggerated in response to hypertrophic stresses. Moreover, a variety of prohypertrophic agonists including inotropic agents induce the cytoplasmic redistribution of class Ila HDACs, whereas cardiac hypertrophy is prevented when the class Ila HDACs are tethered in the nucleus. Although those two classes of HDACs have opposite roles, both classes are still involved in the single disease of cardiac hypertrophy, which strongly suggests interconnections between them. In this study, we investigated the crosstalk between HDAC2 and class Ila HDACs in the development of cardiac hypertrophy. Here we suggest a novel mechanism of regulation of HDAC2 activity in the development of cardiac hypertrophy. We propose that pCAF acetylases HDAC2, whereas HDAC5, a class Ila HDAC, directly removes the acetyl moiety from HDAC2. We show that HDAC2 is a prohypertrophic mediator suppressed by class Ila HDACs through acetylation dynamics.

METHODS

Neonatal rat ventricular cardiomyocyte culture.
All animal experiments were performed after approval by the Chonnam National University Medical School Research Institutional Animal Care and Use Committee. Neonatal rat ventricular cardiomyocytes (NRVCs) were prepared as described previously.
Primary culture of adult rat ventricular cardiomyocytes.
Adult rat ventricular cardiomyocytes (ARVCs) were cultured from adult Sprague-Dawley rat hearts according to a previous report24 with slight modifications. Twelve-week-old male rats were used (Daehan Biolink, Daejeon, Korea).

Cardiac hypertrophy model.
Eight-week-old adult male CD1 mice were purchased (Daehan Biolink, Daejeon, Korea) and were housed in individual plastic cages. Mice were anesthetized with 2,2,2-tribromoethanol (300 mg/kg, intraperitoneally), and osmotic pumps (Alzet®, Durect Corp., Cupertino, CA, USA) were inserted as described previously19.

HDAC activity.
HDAC activity was assessed by use of a commercially available kit: HDAC Fluorometric Assay/Drug Discovery kit (Enzo Life Sciences, Inc., Plymouth Meeting, PA).

TSA-pulse.
To minimize an unwanted effect by remnant inhibitors, medium was replaced after incubation overnight. Then, NRVCs were incubated with inhibitor-free medium for 4 hours more after extensive washing with inhibitor-free PBS.

Cell size measurement.
Immunocytochemistry was performed as previously reported19. ARVCs were infected with adenovirus overnight and were exposed to reagent-containing media for 24 hours more. Both sarcomeric-actinin and GFP-positive cells were used for cell size measurement.

[^3H]Leucine incorporation assay.
After either transfection with plasmid for 6 hours (NRVCs) or infection with adenovirus overnight (ARVCs), the medium was changed to 1.0 μCi/ml[^3H]leucine-containing medium overnight. Tritiated leucine incorporation was measured as described previously19.

In vivo delivery of adenovirus.
Adult male C57BL/6 mice (Daehan Biolink, Daejeon, Korea) or Hdac5 null mice were prepared for in vivo delivery of adenovirus at 8 weeks of age. The day after insertion of the osmotic pump, 1x10^11 ifu Ad-GFP or Ad-Hdac2 K75R virus was injected via the tail vein.

Statistical analysis.
One-way ANOVA was used to test significances between more than three groups, which was followed by the Tukey multiple-comparison post hoc test. When the variances of each group were not equal by the Levene test, the Dunnett T3 test was used for a post hoc test. Statistical analysis was carried out with PASW Statistics 20 (SPSS, an IBM Company, Chicago, IL, USA).

All reagents and detailed methods are specifically described in the online supplement.

RESULTS

HDAC2 acetylation in the development of cardiac hypertrophy.

Previously, we reported that activation of HDAC2 induced by phosphorylation of the S394 is critical in the development of cardiac hypertrophy19. Here we further investigated whether other
posttranslational modifications of HDAC2 are involved in cardiac hypertrophy. We first examined the acetylation status of HDAC2 to hypertrophic stresses.

NRVCs were exposed to endothelin-1 or phenylephrine overnight. Cellular extracts were immunoprecipitated by anti-acetyl lysine antibody and probed with anti-Hdac2 antiserum. Hdac2 acetylation was significantly increased by phenylephrine (Figure 1A) and endothelin-1 (Figure 1B). The results of densitometer analysis were shown in Online Table. We also examined the acetylation status in vivo in an animal model. An increase in the acetylation of Hdac2 in response to either isoproterenol (Figure 1C) or pressure overload (Figure 1D) was also observed in mouse heart. Next, we examined the effects of modulators of acetylation. Interestingly, acetylation of Hdac2 was dramatically decreased by gavacinol, a histone acetyltransferase inhibitor (Figure 1E, second lane). We also examined whether inhibition of HDAC could induce hyper-acetylation of the Hdac2 molecule itself. We treated NRVCs with TSA for 8 hours and then washed out TSA. This “TSA-pulse” significantly increased Hdac2 acetylation (Figure 1E, right lane), which further suggested that Hdac2 is a target of acetylation by histone acetyltransferase or other HDACs.

To elucidate whether the acetylation of Hdac2 is related to its enzymatic activity, we tested the intrinsic activity of Hdac2 after treatment with gavacinol or TSA. We first treated Hdac2-immunoprecipitates with TSA and measured the activity without washing out the TSA. This resulted in complete inhibition of Hdac2 enzymatic activity (data not shown), which resulted from the direct inhibition of Hdac2 by the remaining TSA in the precipitates. Thus, we altered the experimental procedures to examine the activity after induction of acetylation of Hdac2, i.e., “TSA-pulse”. Hyper-acetylation of Hdac2 by TSA was not decreased after washing out (Online Figure I). According to this preliminary experiment, we measured intrinsic HDAC2 activity after cleaning up TSA. Some chemical inhibitors that have recently been developed showed relative selectivity on one class of HDACs; for example, apicidin25 and SK 704117 preferentially inhibit class I HDACs. Thus, we examined which class of HDAC mediates deacetylation of Hdac2 by using specific class I HDAC inhibitors or nonselective HDAC inhibitor. TSA-pulse dramatically induced Hdac2 activation, whereas apicidin-pulse failed to do so (Figure 1F). These results implied that the deacetylation is mediated by class IIa HDACs.

HDAC2 K75 residue as an acetylation target.

We next examined which residue is responsible for the acetylation of HDAC2 by using two truncated mutants of Hdac2: the N-terminal part (Hdac2-N, amino acids 1–260) and the C-terminal part (Hdac2-C, amino acids 250–488). The acetylation band was detected only in Hdac2-N (Online Figure II).

Hdac2-N contains 18 lysine residues and the bioinformatics database indicated that both K75 and K90 could be target for the acetylation (http://www.uniprot.org/, http://www.phosphosite.org/). Thus, we made acetylation-resistant mutants by use of a single point mutagenesis technique in which K75 or K90 was substituted with arginine. After transfection of those mammalian expression vectors, an immunoprecipitation-based acetylation assay was performed. Acetylation of Hdac2 was not detected when lysine 75 was substituted by arginine (Hdac2 K75R; 3rd lane in the second panel in Figure 2A); however, we did not see any changes with K90R (4th lane in Figure 2A). Furthermore, TSA-dependent hyper-acetylation of Hdac2 was not observed in Hdac2 K75R (Figure 2B), which strongly suggested that K75 was specific for acetylation.

The relationship between K75 acetylation and HDAC2 enzymatic activity was further examined by comparing the intrinsic activities of Hdac2 wild-type and K75R. The deacetylation activity was halved in K75R mutant (Figure 2C). We next examined whether acetylation of Hdac2 K75 is required for the development of cardiac hypertrophic phenotypes. We transfected mammalian expression vectors of either wild-type or K75R Hdac2 and performed a promoter assay with hypertrophic marker genes. Hdac2 K75R
failed to induce promoter activation of natriuretic peptide precursor A (Nppa), a gene encoding atrial natriuretic factor, ANF (Figure 2D). To confirm this further, NRVCs transfected with either wild-type or K75R Hdac2 were stimulated by phenylephrine and the promoter assay was performed. Hdac2 wild-type increased Nppa-promoter activity (1st and 3rd columns in Figure 2E) as we reported previously19, 26 and the increase in activity was further potentiated by phenylephrine (3rd and 4th columns in Figure 2E). Activation induced by Hdac2 K75R was very weak when compared with that by wild-type in the basal condition (3rd and 5th column in Figure 2E). Interestingly, forced expression of Hdac2 K75R significantly blunted the phenylephrine-derived hypertrophy response (4th and 6th columns in Figure 2E), which suggests that HDAC2 K75R works in a dominant-negative fashion in the phenylephrine-treated NRVCs. A similar result was obtained when myosin heavy chain 7-promoter (Myh7, encoding beta myosin heavy chain) was used for the promoter assay (Online Figure III). The protein amounts were the same in our experimental conditions (Online Figure IV). Measurement of protein synthesis, a hallmark of cardiac hypertrophic phenotypes27, by [3H]leucine incorporation using ARVCs (Figure 2F) or NRVCs (Online Figure V) revealed that adenoviral infection of Hdac2 K75R successfully blunted the increase in protein synthesis induced by phenylephrine. The cell size of ARVCs was significantly increased by overexpression of Hdac2 wild-type itself (1st and 3rd columns in Figure 2G), and the phenylephrine-induced increase in cell size was potentiated by Hdac2 wild-type (2nd and 4th columns in Figure 2G). As in Figure 2E and Figure 2F, infection of Hdac2 K75R mutant successfully blocked the prohypertrophic feature of alpha-adrenergic agonist. However, like Hdac2 wild type, both K75R and K75Q were still localized in the nucleus (Online Figure VI). Thus, we concluded that Hdac2 K75 is an acetylation target that mediates hypertrophic phenotypes in cardiomyocytes.

**Effect of acetylation on HDAC2 phosphorylation.**

Some residues that are susceptible to the posttranslational modification in Hdac2 have been reported to modulate the intrinsic deacetylation activity of HDAC2; S394, S422, and S424 are phosphorylation sites responsible for activation28, whereas H141 forms a critical component for a deacetylase pocket29. In addition, S-nitrosylation of C262 and C274 also affects enzymatic activity by an unknown mechanism30. We previously reported that casein kinase 2α1 (CK2α1)-mediated phosphorylation of Hdac2 S394 is mandatory for activation and plays an important role in the development of cardiac hypertrophy19.

In this report, we found that acetylation is also important for activity. Thus, we investigated the mechanism by which acetylation affects the intrinsic activity of Hdac2. Because of their functional similarities, we first assumed that interplay may exist between the two posttranslational modifications of acetylation and phosphorylation. Thus, we checked the phosphorylation status of Hdac2 K75R. In the basal condition, S394 phosphorylation was significantly decreased in the K75R (Figure 3A). By contrast, no difference in acetylation status was seen between Hdac2 wild-type and S394A mutant (Figure 3B). In addition, hyper-acetylation in either basal or TSA-provoked condition was also observed in diverse Hdac2 mutants such as phosphorylation-dead mutants of Hdac2 S394A, S394/422/424A, or even an enzyme-dead mutant of H141A (Online Figure VII), which further suggests that those modifications of phosphorylation do not affect acetylation, whereas acetylation influences phosphorylation. Next, S394 phosphorylation was measured after TSA-pulse. Hdac2 K75R was not phosphorylated by TSA, whereas wild-type was significantly increased (Figure 3D). As observed above, deacetylase activity in Hdac2 wild-type was further increased by TSA-pulse (2nd and 3rd columns in Figure 3E). However, the potentiation effect was completely absent when K75R was used (4th and 5th columns in Figure 3E). Surprisingly, even though acetylation was increased by TSA among the mutants tested except for K75R, TSA-mediated Hdac2 activation was detected only in Hdac2 wild-type (Figure 3F).

We further investigated the net effect of acetylation and phosphorylation by use of a double-mutant that contains both acetyl-mimicking (K75Q) and phosphor-dead (S394A) mutations. The
acetylation-mimic mutant of Hdac2, Hdac2 K75Q, showed pro-hypertrophic features both in HDAC activity (Online Figure VIII) and in the [³H]leucine incorporation assay (Figure 3G); however, acetylation-associated hypertrophy was not observed in the Hdac2 K75Q/S394A double mutant. To check the alteration of phosphorylation of different sites by acetylation, we performed an immunoprecipitation-based phosphorylation assay with the Hdac2 S394A and Hdac2 K75R/S394A. Phosphorylation at the residues other than S394 was not changed by acetylation (Online Figure IXA). Likewise, intrinsic activity of Hdac2 K75R/S394A was not further decreased when compared with that of Hdac2 S394A (Online Figure IXB), which suggests that basal phosphorylation was not affected by acetylation.

We further questioned how K75 acetylation regulates S394 phosphorylation; we tested whether acetylation is required for the interaction with CK2α1 that phosphorylates S394. Acetylation-resistant mutant failed to interact with CK2α1, which implicates that K75 acetylation is indispensable for the interaction with CK2α1 for phosphorylation (Online Figure X). These results emphasized that acetylation precedes phosphorylation but the phosphorylation is required for the acetylation-mediated development of hypertrophic phenotypes.

**Hdac2 acetylation by pCAF.**

Next, we questioned which histone acetyltransferase can induce acetylation of HDAC2. Because garcinol is effective for inhibiting the acetylation of Hdac2 (Figure 1E) and garcinol is an inhibitor of both p300 and p300/CBP-associated factor (pCAF), we checked their interaction with HDAC2 and functional relevance of acetylation. Among the histone acetyltransferases tested, pCAF physically interacted with Hdac2 (Figure 4A, B and C). Moreover, Hdac2 acetylation was increased by transfection of pCAF in a dose-dependent manner (Figure 4D). Interestingly, we could find no significant interaction between Hdac2 and p300 (Online Figure XIA). Besides, p300 was not involved in Hdac2 acetylation (Online Figure XIB), which agrees with the result of a previous report.

**Hdac2 deacetylation by Hdac5.**

As suggested by the results shown in Figure 1E, we assumed that one of the class II HDACs may work to remove acetylation of the Hdac2. First, we checked physical interaction between Hdac2 and class II HDACs. In our experimental model, among those tested, Hdac5 (Figure 4E and F) and Hdac9 (Online Figure XIIA and B) were successfully co-immunoprecipitated by Hdac2.

Both HDAC5 and HDAC9 are well-known anti-hypertrophic mediators. Thus, we examined whether either HDAC5 or HDAC9 could deacetylase HDAC2 and checked whether this deacetylation of HDAC2 worked as an anti-hypertrophic mechanism. Hdac2 acetylation was significantly decreased by forced overexpression of Hdac5 (Figure 4G). However, we did not observe a deacetylase effect of Hdac9 in our experimental models (Online Figure XIIIC and D).

Hdac2 acetylation was significantly increased in hearts from Hdac5 null mice (Figure 5A). Hdac5 null mice showed mild cardiac hypertrophy when compared with their wild-type littermates (data not shown), as described previously. Like our previous observations in Figure 3A and 3C, Hdac2 phosphorylation was increased in Hdac5 knockout heart (Figure 5A). Hdac5 siRNA induced Hdac2 hyper-acetylation (Figure 5B). Hdac2 activity was up-regulated by siRNA against Hdac5 (Online Figure XIII). Hdac2 acetylation was reduced when Hdac5 expression was elevated, which was completely reversed by simultaneous treatment with TSA (Figure 5C). Phosphorylation of Hdac2 S394 showed the same trend as the alteration of Hdac2 acetylation by Hdac5. As in Figure 2C, the enzyme activity of Hdac2 was significantly decreased by forced expression of Hdac5, whereas the effect of Hdac5 on Hdac2 activity was successfully recovered by inhibition of Hdac5 (Online Figure XIV).
It is well known that the activity of class IIa HDACs is extremely lower than that of class I HDACs and that recruitment of class I HDACs is required for the full activity of class IIa HDACs. To elucidate whether HDAC5 is dependent on class I HDACs to deacetylate Hdac2, we checked the deacetylation of Hdac2 in the presence of apicidin. Hdac2 acetylation was significantly decreased whether the cells were simultaneously treated with apicidin or not (Figure 5D). To support this result, we generated two enzyme-dead mutants of HDAC5 according to a previous report and the bioinformatics site (http://www.uniprot.org/). One was a 767aa mutant (lacking the HDAC domain) and the other was H833A (destruction of the HDAC pocket). We confirmed the intrinsic activity of HDAC5 mutants (Online Figure XV). Hdac2 acetylation was decreased when HDAC5 wild-type was co-transfected (2nd and 3rd lanes in Figure 5E); however, the enzyme-dead mutant of HDAC5 failed to show this effect (4th and 5th lanes in Figure 5E). These data strongly indicated that HDAC5 directly regulates HDAC2 acetylation.

Functional relevance of Hdad2 acetylation in cardiac hypertrophy.

HDAC5 is phosphorylated by PKC/PKD in response to various stimuli, including hypertrophy signals, and phosphor-HDAC5 undergoes nuclear export after recognition by 14-3-3 molecule. Leptomycin B (LMB), an anti-fungal antibiotic, inhibits the cytoplasmic shuttling of HDAC5 through direct binding to chromosomal region maintenance 1 (CRM1). Thus, we used LMB to tether Hdad5 in the nucleus. When Hdad2 K75Q was infected to ARVCs, the LMB-mediated repressive effect of Hdad5 was not seen either in cell size measurement (Figure 6A) or in the [3H]leucine incorporation assay (Figure 6B). In addition, as in the ARVCs, the LMB-mediated anti-hypertrophic effect was completely abolished by simultaneous overexpression of Hdad2 K75Q in NRVCs (Figure 6C and Online Figure XVI). Likewise, overexpression of Hdad5 significantly reduced activation of the Nppa-promoter by Hdad2 wild-type; however, the anti-hypertrophic effect of forced expression of Hdad5 was not observed when Hdad2 K75Q was transfected (Figure 6D). Likewise, the exaggeration effect by Hdad5 siRNA was not observed in Hdad2 K75R-transfected NRVCs (Figure 6E).

pCAF is also known as a putative prohypertrophic molecule. We tested whether pCAF could induce cardiac hypertrophy, which is dependent on Hdad2 acetylation. Forced expression of pCAF in NRVCs induced cardiac hypertrophy and the hypertrophic effect by pCAF was completely blocked when Hdad2 K75R was co-transfected (Figure 6F and Online Figure XVII). Furthermore, we challenged whether Ad-Hdad2 K75R would function in a dominant-negative fashion in the development of cardiac hypertrophy in vivo. Isoproterenol-induced cardiac hypertrophy was significantly attenuated both in wild-type mice and Hdad5 null mice by injection of Ad-Hdad2 K75R (Figure 6G). These results suggested that HDAC5 directly regulates HDAC2 activity by modulating the acetylation of HDAC2, which further implicates pCAF-dependent HDAC2 acetylation as a crucial step in the development of cardiac hypertrophy.

DISCUSSION

Though initial hypertrophy is physiologic; sustained stresses lead to a pathologic phase of hypertrophy. When the heart undergoes transition into pathologic hypertrophy, the myocardium becomes stiffened by interstitial fibrosis, and thereby diastolic dysfunction induces global remodeling of the heart, dilated cardiomyopathy, and heart failure. We previously demonstrated that blocking class I HDACs effectively prevents the development of cardiac hypertrophy and that activation of HDAC2 is associated with CK2α1-dependent HDAC2 S394 phosphorylation and with binding of molecular chaperone, inducible heat shock protein.
Although both HDAC2 and class IIa HDACs are involved in cardiac hypertrophy, they play opposite roles. HDAC2 works as a prohypertrophic molecule\textsuperscript{18, 19, 26}, whereas class IIa HDACs are negative regulators of cardiac hypertrophy\textsuperscript{21, 22}. Considering previous reports showing that the development of cardiac hypertrophy is completely blocked not only by a nonselective HDAC inhibitor but also by a class I HDAC specific inhibitor\textsuperscript{17, 25}, it is plausible that interplay may exist between class I and class IIa HDACs in the regulation of cardiac hypertrophy and that class IIa HDACs directly regulate HDAC2 in an enzyme-substrate manner when cardiac hypertrophy develops.

In this study, we have elucidated a novel regulation mechanism of HDAC2 acetylation in cardiac hypertrophy; pCAF-dependent HDAC2 K75 acetylation induces cardiac hypertrophy, which is closely linked to increase in S394 phosphorylation induced by CK2\textsubscript{\alpha}1. In contrast, HDAC5-mediated deacetylation of HDAC2 reduces the phosphorylation status and decreases the intrinsic activity of HDAC2. These results suggest that the acetylation dynamics of HDAC2 are also notable modification in addition to phosphorylation. Taken together, we suggest the working hypothesis depicted in Figure 7; in the basal state, HDAC5 binds to and suppresses HDAC2 by deacetylation (Left). When hypertrophic insults stimulate the myocardium, phosphor-HDAC5 is redistributed to the cytoplasm as reported previously\textsuperscript{35}. In the HDAC5-free nucleus, HDAC2 is acetylated by pCAF and then phosphorylated by CK2\textsubscript{\alpha}1, which allows HDAC2 to be activated. As a result, quiescent fetal genes are reactivated and cardiac hypertrophy develops (Right).

The most important finding of this study is the delineation of the new crosstalk mechanism in the regulation of cardiac hypertrophy; that is, HDAC2 is a direct target of class IIa HDACs. It is known that class II HDACs recruit class I HDACs to form a large complex and thereby repress the transcription of downstream targets\textsuperscript{33}. However, in that case, it is not evident whether the acetylation status of HDACs in those complexes is altered by other histone acetyltransferases or HDACs. In contrast, in this study, we have clearly shown that HDAC5-mediated deacetylation of HDAC2 is critical in the regulation of the activity of HDAC2 in cardiomyocytes. HDAC9 also interacted with HDAC2, however, HDAC9 failed to deacetylate HDAC2. This finding also provides an alternate explanation as to how two opposite actions of prohypertrophic class I HDACs and anti-hypertrophic class IIa HDACs can work together in the regulation of cardiac hypertrophy.

The role of the class II HDACs as enzymes in human disease is not as well understood as their role as transcriptional regulators owing to their lack of enzyme activities\textsuperscript{33}. Indeed, their enzymatic activity does not seem to be directly associated with their anti-hypertrophic action. For example, MITR, an alternative splice form of HDAC9 that lacks a deacetylase domain, is still effective in preventing cardiac hypertrophy\textsuperscript{22}. In this study, however, we demonstrated that LMB-dependent inhibition of nuclear export of HDAC5 completely suppresses the prohypertrophic effect of HDAC2 wild-type, whereas an acetylation-mimicking mutant of HDAC2 is resistant to LMB. In addition to the previously known mechanism of transcriptional regulation by binding to other transcription factors\textsuperscript{32}, our finding suggests that HDAC5 plays a role as a deacetylase enzyme targeting HDAC2, a novel non-histone substrate. This eccentric relationship must be considered in the development of new HDAC inhibitors, because class IIa HDAC inhibitors may aggravate disease by the activation of prohypertrophic class I HDACs through their acetylation.

The functional importance of histone acetyltransferases in the development of cardiac hypertrophy is well-established. Both p300 and pCAF successfully induce cardiac hypertrophy either by transcriptional activation of heart-specific genes\textsuperscript{36} or by acetylation of non-histone substrates such as GATA\textsuperscript{48}. In this study, in addition to those mechanisms, we have demonstrated that pCAF, but not p300, induces acetylation and following activation of HDAC2.
Study of the posttranslational modifications of Hdac2 has been intensively focused on phosphorylation. According to a study by the Seto group, four serine residues, 394, 411, 422, and 424, are susceptible to phosphorylation. Among them, S394, S422, and S424 are targets of CK2, and S411 is a target of PKC. Moreover, CK2-dependent phosphorylation is essential for either intrinsic activity (422/424) or hypertrophy-associated transactivation (394), however, the ability of HDAC2 to repress transcription is not affected. These reports suggest that the HDAC2-mediated hypertrophy response takes place not by suppression of downstream genes but by modulation of HDAC2’s own non-histone substrates.

In addition to HDAC2 phosphorylation, SUMOylation, ubiquitylation, S-nitrosylation, and acetylation are also reported. SUMOylation and phosphorylation regulate complex formation. Both DNA binding affinity and the following transcriptional inhibitory activity are affected by S-nitrosylation. As shown in the chronic obstructive pulmonary diseases model utilizing cigarette smoke extract in lung epithelial cells and in macrophages, HDAC2 phosphorylation seems to be closely related to its acetylation. Indeed, in our experimental model, HDAC2 phosphorylation-resistant mutants, S394A and S394/422/424A, and an enzyme activity-inert mutant, H141A, were successfully hyper-acetylated. On the other hand, their deacetylation activities were not upregulated by hyper-acetylation in either the phosphorylation-resistant mutants or the enzyme-dead mutant we investigated. These findings suggest that the acetylation-associated increase in activity is not a direct effect but rather a secondary phenomenon by modulation of interaction with kinase and thereby phosphorylation. Although we demonstrated that Hdad2 K75R fails to interact with CK2α1, it is still not clear how acetylation affects binding of CK2 to HDAC2. Considering that acetylation causes conformational changes, certain HDAC2 domains which is responsible for the interaction with CK2 might be exposed by K75 acetylation. Studies for posttranslational modification and following alterations of HDAC2 structure will provide better understanding. To summary, our results suggest that crosstalk between CK2-mediated phosphorylation and pCAF/HDAC5-induced acetylation plays a critical role in the development of cardiac hypertrophy by regulation of HDAC2 activity. Such crosstalk requires further intensive investigations.

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DISCLOSURES
None.
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42. Sun JM, Chen HY, Davie JR. Differential distribution of unmodified and phosphorylated histone

FIGURE LEGENDS

**Figure 1.** HDAC2 is acetylated in the development of cardiac hypertrophy. To study endogenous Hdac2 acetylation in response to hypertrophic stresses, 2 mg of protein was immunoprecipitated with 2 μg anti-acetyl lysine antibody and acetylation of Hdac2 was visualized by antisera against Hdac2. 2% of input was utilized for showing loading controls. Hdac2 acetylation was significantly upregulated by either 100 μM of phenylephrine (PE) (A) or 10 nM of endothelin-1 (ET-1) (B) in the primary-cultured neonatal rat ventricular cardiomyocytes (NRVCs). Hdac2 hyper-acetylation was also detected in hearts obtained either from mice treated with continuous infusion of isoproterenol (ISP) (C) or mice undergone pressure overload (D). (E) Hdac2 acetylation was decreased by garcinol, a histone acetyltransferase inhibitor, but was increased by TSA, an HDAC inhibitor. (F) The deacetylase activity of Hdad2 was down-regulated by garcinol but induced by pan-HDAC inhibitor. Apicidin, a specific class I HDAC inhibitor, did not affect HDAC2 activity. Results are shown as mean±SE. ** indicates p<0.01. NS, not significant.

**Figure 2.** HDAC2 K75 is acetylation residue. (A) Acetylation of Hdac2 was not detected when lysine 75 was substituted by arginine (K75R); however, lysine 90 mutation (K90R) did not result in significant changes compared with the wild-type control. (B) Neither basal acetylation nor TSA-induced hyper-acetylation was observed in Hdac2 K75R mutant. (C) The intrinsic activity of Hdac2 K75R was about 60% that of wild-type. (D) Hdac2 K75R could not induce Nppa-promoter activation, whereas Hdac2 wild-type could. (E) Hdac2 wild-type-induced Nppa-promoter activation was dramatically potentiated by simultaneous treatment with phenylephrine; however, this potentiation was not observed when the acetylation-resistant mutant was overexpressed. (F) Phenylephrine further increased [3H]leucine incorporation in Hdac2 wild-type-overexpressed adult rat ventricular cardiomyocytes (ARVCs), whereas it failed to do so in the Hdac2 K75R-overexpressed ARVCs. (G) Cell size of ARVCs was synergistically increased by both infection of Hdac2 wild-type and administration of phenylephrine (PE); however, Hdac2 K75R mutant completely blocked PE-induced hypertrophic response. Results are shown as mean±SE. * indicates p<0.05; @@ p<0.01.

**Figure 3.** Acetylation affects phosphorylation status of Hdac2. (A and B) Phosphorylation of serine 394 was significantly decreased in an acetylation-resistant mutant of Hdac2 (A), whereas acetylation was not affected despite substitution of serine 394 with alanine (B). (C) Phosphorylation of S394 was significantly increased by TSA but was almost completely absent in Hdac2 K75R. (D) HDAC activity of Hdac2 wild-type was dramatically induced by TSA-pulse (see Methods), whereas Hdac2 K75R was not. (E) HDAC activity of various Hdac2 mutants: Hdac2 K75R (acetylation-resistant), Hdac2 S394A (hypertrophy-induced phosphorylation-resistant), Hdac2 S394/422/424A (all phosphorylation-inert), Hdac2 H141A (enzyme-dead). TSA-mediated induction of intrinsic activity of Hdac2 was dependent on phosphorylation status. (F) [3H]Leucine incorporation amount by each Hdac2 mutant in neonatal rat ventricular cardiomyocytes (NRVCs): Hdac2 K75Q (acetylation-mimicking).

**Figure 4.** pCAF/HDAC5 regulates Hdac2 acetylation. (A, B, and C) P300/CBP-associated factor (pCAF) physically interacted with Hdac2. (D) Hdac2 was acetylated in a pCAF dose-dependent fashion. (E and F) Hdad5 interacted with Hdac2. (G) Hdac2 acetylation was significantly decreased by forced overexpression of Hdad5.

**Figure 5.** Hdad5 regulates Hdac2 activity by deacetylation. (A) Both Hdac2 acetylation and S394 phosphorylation were increased in Hdad5 knockout (KO) mice. (B) Hdad5 knock-down dramatically induced Hdac2 acetylation. (C) Both Hdac2 acetylation and phosphorylation were significantly decreased by transient overexpression of Hdad5, which was completely reversed by TSA. (D) Hdac2 acetylation was successfully reduced by overexpression of Hdad5, even when apicidin was added. (E) Two enzyme-dead mutants, 767aa and H833A, failed to deacetylase Hdac2, whereas wild-type Hdad5 did.
Figure 6. HDAC2 acetylation is a critical event for cardiac hypertrophy. (A and B) PE induced hypertrophy in ARVCs, which was completely blocked by simultaneous treatment of leptomycin B (LMB). Hdac2 wild-type-derived increase in cell size (A) or in [3H]leucine incorporation (B) was significantly potentiated by PE. However, LMB blocked the Hdac2 wild-type-induced hypertrophy. In contrast, Hdac2 K75Q successfully escaped the effects of LMB. Dots indicate data on size from a single cell; horizontal bars depict the mean of group data. (C) Nppa-luciferase activity in NRVCs showed the same pattern as in (A&B). (D) Forced expression of Hdac5 suppressed Hdac2 wild-type-derived Nppa-promoter activation, whereas Hdac5 failed to inhibit the Hdac2 K75Q-induced luciferase activation. (E) Nppa-promoter activity was significantly up-regulated by down-regulation of Hdac5, which was not observed when acetylation-resistant mutant was expressed instead of wild-type. (F) pCAF-mediated prohypertrophic effect was blocked by simultaneous expression of Hdac2 K75R. (G) The infection of Ad-Hdac2 K75R significantly attenuated isoproterenol-induced cardiac hypertrophy both in wild-type and Hdac5 knock-out mice. Dots indicate data from individual mouse.

Figure 7. Working hypothesis. In the basal condition, HDAC5 inhibits HDAC2 by deacetylation (left). When hypertrophic stresses stimulate the myocardium, phosphor-HDAC5 is exported to the cytoplasm. CK2α1 is shuttled into the nucleus and phosphorylates HDAC2, and pCAF binds to HDAC2 and induces acetylation. Activated HDAC2 induces hypertrophy of the myocardium to satisfy the increased hemodynamic requirement (right).
Novelty and Significance

What Is Known?

- Class II histone deacetylases (HDACs) inhibit hypertrophy, whereas class I HDACs promote hypertrophy.

- Activation of HDAC2, a class I HDAC, and the ensuing transcriptional repression of anti-hypertrophic genes is required for the development of cardiac hypertrophy.

- In response to hypertrophic stimuli, casein kinase 2α-phosphorylates HDAC2 serine 394 to activate its enzymatic activity, which plays an essential role in cardiac hypertrophy.

What New Information Does This Article Contribute?

- HDAC2 lysine 75 is acetylated by hypertrophic stresses.

- The acetylation of lysine 75 precedes the phosphorylation of serine 394, which activates HDAC2 and thereby induces cardiac hypertrophy.

- HDAC2 acetylation is mediated by p300/CBP-associated factor (pCAF), whereas its deacetylation is mediated by HDAC5, one of the class II HDACs in the heart.

Acetylation of the lysine residue of histones is closely related to transcriptional activation of downstream target genes and is regulated by a fine balance between histone acetyltransferases (HATs) and HDACs. Recently, many proteins other than histones have been shown to undergo acetylation by HATs, which is reversed by HDACs. The pathological implications of protein acetylation are being highlighted in diverse cardiovascular diseases. We showed previously that activation of HDAC2, one of the class I HDACs, is indispensable for hypertrophy and that hypertrophic stresses phosphorylate HDAC2 S394 to induce its activation. Here, we have shown that acetylation of HDAC2 K75 is mandatory for S394 phosphorylation and thereby activation of HDAC2 in hypertrophy. The acetylation status of HDAC2 K75 is balanced by pCAF, one of the HATs, and HDAC5, one of the class II HDACs. The anti-hypertrophic effect of HDAC5 is mediated by its deacetylase activity on HDAC2. Thus, we have shown that HDAC2 itself is an acetylation target and that its acetylation and ensuing enzyme activation cause hypertrophy. Our work establishes the existence of a previously unknown crosstalk between class I and class II HDACs by acetylation dynamics in the regulation of cardiac hypertrophy.
Figure 1

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IP: IgG α-Hdac2
Figure 3

A

B

C

D

E

F

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Figure 4

A

IB: α-pCAF
IB: α-Hdac2

Input
Output

95kDa
70kDa
55kDa

B

IB: α-pCAF
IB: α-Hdac2

Input
Output

95kDa
70kDa
55kDa

C

Flag-pCAF

α-Flag
α-Hdac2

Input

IB: α-Hdac2

55kDa

D

Flag-pCAF

α-Flag
α-Hdac2

Input

AdK
IB: α-Hdac2

70kDa
55kDa

E

IB: α-Hdac5
IB: α-Hdac2

Input

Flag-pCAF

2% input

IB: α-Hdac5

130kDa
95kDa

F

IB: α-V5
IB: α-V5

Input

Hdac5-Flag
Hdac2-V5

2% input

IB: α-Flag
IB: α-V5

α-Flag
α-V5

70kDa
55kDa

G

IB: α-V5
IB: α-V5

Input

Hdac5-Flag
Hdac2-V5

2% input

IB: α-Flag

α-Flag
α-V5

70kDa
55kDa
Figure 5
Figure 6

A

B

C

D

E

F

G

Heart weight/tibia length ratio

Ad-GFP

Ad-HDAC2 K75R

Ad-Hdac2 K75Q

Wild type

Hdac5 KO

[Graphs and data plots showing cellular activity and promoter activity changes under different conditions]
Regulation of Acetylation of Histone Deacetylase 2 by p300/CBP-Associated Factor/Histone Deacetylase 5 in the Development of Cardiac Hypertrophy

Gwang Hyeon Eom, Yoon Seok Nam, Jae Gyun Oh, Nakwon Choe, Hyun-Ki Min, Eun-Kyung Yoo, Gaeun Kang, Vu H Nguyen, Jung-Joon Min, Jong-Keun Kim, In-Kyu Lee, Rhonda Bassel-Duby, Eric N Olson, Woo Jin Park and Hyun Kook

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SUPPLEMENTAL MATERIAL

Detailed Methods

Reagents
Antibodies against Hdac2 (1:5000), Hdac2 pS394 (1:1000), Hdac4 (1:1000), Hdac5 (1:1000), Hdac7 (1:1000), Hdac9 (1:1000), CK2α1 (1:5000), acetyl lysine (for immunoprecipitation), and α-actinin (for immunocytochemistry) were from Abcam (Abcam, Cambridge, UK); anti-Flag (1:1000) and anti-phosphoserine (1:1000) were from Sigma (Sigma-Aldrich Corp., St. Louis, MO, USA); anti-GAPDH (1:1000) and anti-pCAF (1:1000) were from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); and anti-V5 was from Invitrogen (Invitrogen Corporation, Camarillo, CA, USA).

Endothelin-1 (ET-1), phenylephrine (PE), isoproterenol (ISP), leptomycin B (LMB), trichostatin A (TSA), apicidin, 2,3-butanedione monoxime, and 2,2,2-tribromoethanol were purchased from Sigma (Sigma-Aldrich Corp., St. Louis, MO, USA); laminin and collagenase type B were from Roche (Roche Diagnostics GmbH, Mannheim, Germany); hyaluronidase was from Worthington (Worthington Biochemical Corporation, Lakewood, NJ, USA); and garcinol was from Enzo Life Sciences (Enzo Life Sciences, Inc., Plymouth Meeting, PA, USA).

Scramble, Hdac5 siRNA, and Hdac9 siRNA were products of Dharmacon (Dharmacon, Lafayette, CO, USA).

Neonatal rat ventricular cardiomyocyte culture
All animal experiments were performed after approval by the Chonnam National University Medical School Research Institutional Animal Care and Use Committee. Neonatal rat ventricular cardiomyocytes (NRVCs) were prepared as described previously1. Postnatal 1- or 2-day-old Sprague-Dawley rats were used (Daehan Biolink, Daejeon, Korea). After large vessels and atria were completely removed, ventricles were perfused with ADS buffer (20 mM HEPES pH 7.4, 120 mM NaCl, 5.5 mM glucose, 11 mM NaH2PO4, 5.4 mM KCl, and 0.44 mM MgSO4 in distilled water) to wash out the red blood cells. Ventricles were minced and continuously agitated with 0.1% type 2 collagenase/ADS buffer for 30 min at 37 °C. The collagenase reaction was terminated by the addition of an equal amount of 10% FBS containing DMEM. Fibroblasts were removed by the Percoll gradient method. The isolated cardiomyocytes were counted and seeded on 1% gelatin-coated culture plates and were maintained in 10% FBS in DMEM.

Primary culture of adult rat ventricular cardiomyocytes
Adult rat ventricular cardiomyocytes (ARVCs) were cultured from adult Sprague-Dawley rat hearts according to a previous report2 with slight modifications. Twelve-week-old male rats (250-320 g, (Daehan Biolink, Daejeon, Korea) were sacrificed by cervical dislocation after injection of heparin (50 U). The heart was removed and perfused with calcium-free Tyrode buffer (10 mM HEPES pH 7.4, 137 mM NaCl, 5.4 mM KCl, 1 mM MgCl2, 10 mM glucose, 5 mM taurine, and 10 mM 2,3-butanedione monoxime) gassed with 100% O2 through the aorta at 37 °C for 3 min. The dissociation into individual cells was performed by digestion buffer (adding hyaluronidase [0.1 mg/ml] and collagenase type B [0.35 U/ml] to the Ca2+-free Tyrode buffer). When the heart became pale and swollen after digestion for 10 min, the left ventricle was quickly cut into several pieces. Further dissociation was encouraged by gentle stirring (60 rpm) for 10 min at 37 °C in the same digestion buffer. The supernatants were filtered through a cell strainer (pore size: 100 μm). To reduce damage caused by the Ca2+ paradox, extracellular calcium was supplemented to 1.25 mM over a span of 30 min. Rod-shaped myocytes with clear sarcomere striations were utilized.
Cardiac hypertrophy model
Eight-week-old adult male CD1 mice were purchased from Daehan Biolink (Daehan Biolink, Daejeon, Korea) and were housed in individual plastic cages. Under anesthesia with 2,2,2-tribromoethanol (300 mg/kg, intraperitoneally), osmotic pumps (Alzet®, Durect Corp., Cupertino, CA, USA) were inserted as described previously1. Isoproterenol (30 mg/kg/day) was continuously infused by a micro-osmotic pump for the induction of cardiac hypertrophy. Hypertrophy was assessed by comparing heart weight to body weight. For the in vitro hypertrophy model, NRVCs were exposed to 100 μM phenylephrine overnight.

HDAC activity
HDAC activity was assessed by use of a commercially available kit: HDAC Fluorometric Assay/Drug Discovery kit (Enzo Life Sciences, Inc., Plymouth Meeting, PA) with slight modifications. Two milligrams of lysates were preincubated with 2 μg of specific antibodies or normal IgG. The absorbance obtained from IgG-precipitated controls was used for the basal level, and the values from vehicle or wild-type HDACs were regarded as 100% to calculate the relative change. Activity was measured from more than three independent sets of experiments.

TSA-pulse
To minimize an unwanted effect by remnant inhibitors, medium containing inhibitors was removed after incubation overnight. Then, NRVCs were incubated with inhibitor-free medium for 4 hours more after extensive washing with inhibitor-free PBS. In the case of TSA, we described this step as the ‘TSA-pulse.’

Cell size measurement
Immunocytochemistry was performed according to a previous report1 with minor modifications. ARVCs were infected with appropriate adenovirus overnight and were exposed to reagent-containing media for 24 hours more. After brief washing with room temperature PBS, ARVCs were fixed by ice-cold methanol for 10 min and washed twice by prewarmed PBS. Blocking was carried out with 10% BSA/PBS at 37 °C for 30 min and sarcomeric-actinin (1:500) was probed at 37 °C for 2 hours. Primary antibodies were labeled by Alexa-568-conjugated goat anti-mouse antibodies at 37 °C for 45 min. DAPI was used for nuclear staining. Both sarcomeric-actinin- (red) and GFP- (green) positive rod-shaped cells were included for study. Cell size measurement was performed in five independent sets. Outliers were determined by Grubb’s test and significant data (p<0.05) were excluded.

[^3H]Leucine incorporation assay
Tritium-leucine incorporation assay was measured as described previously1. After transfection for 6 hours (NRVCs) or overnight (ARVCs), the medium was changed to 1.0 μCi/ml [^3H]leucine-containing medium with or without 100 μM PE overnight. After sequential denaturation with 10% trichloroacetic acid (TCA) followed by 5% TSA, proteins were extracted into 0.25 N NaOH, and de novo synthesis was measured in a liquid scintillation counter (Wallac, Gaithersburg, MD, USA). Scintillation counts were normalized by the protein concentration of each well.

In vivo delivery of adenovirus
Adult male C57BL/6 mice were purchased from Daehan Biolink (Daehan Biolink, Daejeon, Korea) or Hdac5 null mice were prepared for in vivo delivery of adenovirus at 8 weeks of age. The day after implantation of the osmotic pump containing isoproterenol, 1x10^11 ifu Ad-GFP or Ad-Hdac2 K75R virus was injected via the tail vein by use of 100 μl sterile saline as the vehicle. Mice were sacrificed four days after viral injection, and infection was confirmed by Western blot analysis with anti-V5 antibody.
**Densitometer analysis**
Alteration of acetylation or phosphorylation was quantified by use of ImageJ software. Results were normalized compared with their input densities, divided by control again, and presented as fold-change. The results were shown in Online Table.

**Statistical analysis**
One-way ANOVA was used to test significances between more than three groups, which was followed by the Tukey honestly significant difference (HSD) multiple-comparison post hoc test. When the variances of each group were not equal by the Levene test, the Dunnett T3 test was used for a post hoc test. Statistical analysis was carried out with PASW Statistics 20 (SPSS, an IBM Company, Chicago, IL, USA).
Online Figures

Online Figure I. **TSA-pulse**. To reduce unwanted effects by remnant TSA when performing the HDAC activity assay, the medium containing inhibitors was changed after incubation overnight. Then, NRVCs were incubated with TSA-free medium for 4 hours more after extensive washing with TSA-free PBS (TSA-pulse). The TSA-induced hyper-acetylation of Hdac2 was not decreased after washing out (2nd lane) compared with the inhibitor-containing condition until the NRVCs were lysed (TSA).
Online Figure II. HDAC2 acetylation in the N-part. Hdac2 was divided into two parts: the Hdac2-N (amino acids 1~260) and the Hdac2-C (amino acids 250~488). The acetylation signal was detected in Hdac2-N but not in Hdac2-C.
Online Figure III. **Dominant negative Hdac2 K75R mutant.** The prohypertrophic feature of Hdac2 wild type dramatically potentiated *myosin heavy chain 7* (Myh7, encoding beta myosin heavy chain)-promoter activity by simultaneous treatment with phenylephrine (PE); however, further induction by hypertrophic stimuli was not observed when the acetylation-resistant mutant was overexpressed. ** indicates p<0.01; NS, not significant.
Online Figure IV. **Expression level of HDAC2 mutants.** The expression amount of the K75R or of the K75Q was unchanged compared with the wild type.
Online Figure V. [3H]Leucine incorporation in NRVCs. [3H]Leucine incorporation was notably increased with phenylephrine (PE) treatment in the Hdac2 wild-type transfection group, whereas PE failed to induce the hypertrophic phenotype in the group in which Hdac2 K75R was overexpressed. ** indicates p<0.01; NS, not significant.
Online Figure VI. **Intracellular localization of HDAC2 mutants.** After ARVCs were infected with adenovirus containing HDAC2 wild type or mutants, the proteins were visualized by utilizing V5 antibodies. Either K75R or K75Q mutant was localized in the nucleus as the wild type.
Online Figure VII. **Hyper-acetylation of various Hdac2 mutants by TSA.** TSA-mediated hyper-acetylation was repeated in various Hdac2 mutants. Hdac2 S394A (hypertrophy-associated phosphorylation-resistant), Hdac2 S394/422/424A (all phosphorylation-inert), Hdac2 H141A (enzyme-dead by destruction of HDAC pocket). The perpendicular black bars indicate the separation of two independent blots.
Online Figure VIII. **HDAC activity of various Hdac2 mutants.** The acetylation mimicking mutant, Hdac2 K75Q, possessed similar deacetylase activity compared with Hdac2 wild type. Hdac2 K75Q/S394A, however, lost its activity as much as Hdac2 S394A, a hypertrophy-associated phosphorylation-resistant mutant. This result suggests that the HDAC activity of Hdac2 is mainly regulated not by K75 acetylation but by S394 phosphorylation.
Online Figure IX. **Basal phosphorylation of Hdac2.** (A) Phosphorylation at the residues other than S394 in Hdac2 was not changed by acetylation. (B) Compared with that of Hdac2 S394A, the enzyme activity of Hdac2 K75R/S394A was not altered.
Online Figure X. **HDAC2 K75 acetylation is required for the interaction with casein kinase 2α1.** Compared with that of Hdac2 wild type, interaction between CK2α1 and Hdac2 K75R was significantly decreased.
Online Figure XI. **Functional relevance of p300 in Hdac2 acetylation.** (A) p300 and Hdac2 did not bind to each other. (B) Hdac2 acetylation was not increased by overexpression of p300.
Online Figure XII. **Functional relevance of Hdac9 in Hdac2 deacetylation.** (A and B) Hdac2 and Hdac9 physically interacted with each other. (C and D) Acetylation status of Hdac2 was not changed by overexpression nor by down-regulation of Hdac9.
Online Figure XIII. **HDAC activity of Hdac2 by knock-down of Hdac5.** Intrinsic activity of Hdac2 was significantly induced by down-regulation of Hdac5 by small interfering RNA against Hdac5. ** indicates p<0.01.
Online Figure XIV. Hdac5 regulates Hdac2 activity. HDAC activity of Hdac2 was significantly decreased by forced expression of Hdac5, which was completely recovered by TSA. ** indicates p<0.01.
Online Figure XV. **Intrinsic activity of Hdac5 mutants.** According to a previous study or the bioinformatics site (http://www.uniprot.org/), enzyme-inert mutants were generated: 767aa, lacking HDAC domain; H833A, destroyed HDAC pocket by substitution of histidine 833 with alanine. HDAC activity of 767aa or H833A mutant was significantly decreased. * indicates p<0.05; ** indicates p<0.01; NS, not significant.
Online Figure XVI. **Nuclear tethering effect of Hdac5 by leptomycin B.** Wild-type (wt) Hdac2 activated Myh7-promoter activity, which was completely blocked by simultaneous treatment with leptomycin B (LMB). Hdac2 wt-derived Myh7-luciferase activation was dramatically potentiated by PE. However, LMB completely normalized the Hdac2 wt-induced promoter activation. Acetylation-mimicking mutant, Hdac2 K75Q, escaped the anti-hypertrophic effect of LMB. * indicates p<0.05; ** indicates p<0.01; NS, not significant.
Online Figure XVII. **Dominant-negative characteristics of Hdac2 K75R.** pCAF induced -3003 Nppa-luciferase activity in a dose-dependent manner, which was completely blocked by co-transfection of Hdac2 K75R. pCAF-associated hypertrophy is dependent on Hdac2 acetylation.
### Online Table. **Densitometer analysis.**

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* acetylation; # phosphorylation. SE; standard error
Supplemental References

