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 Hong Nguyen, Jung-Joon Min, Jong-Keun Kim, In-Kyu Lee, Rhonda Bassel-Duby, Eric N. Olson, Woo Jin Park, Hyun Kook

**Rationale:** Histone deacetylases (HDACs) are closely involved in cardiac reprogramming. Although the functional roles of 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 alternative post-translational activation mechanism of HDAC2 that involves acetylation of HDAC2 mediated by p300/CBP-associated factor/HDAC5.

**Methods and Results:** Hdac2 was acetylated in response to hypertrophic stresses in both cardiomyocytes and a mouse model. 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. p300/CBP-associated factor 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 antihypertrophic 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 p300/CBP-associated factor and HDAC5 in the development of cardiac hypertrophy. (Circ Res. 2014;114:1133-1143.)

**Key Words:** acetylation ■ cardiomyopathy, hypertrophic ■ histone deacetylase 2 ■ histone deacetylase 5, mouse ■ p300/CBP-associated factor ■ protein processing, post-translational

Proteins are frequently modified by diverse enzymes such as kinases, methyltransferases, ubiquitinylases, and acetyltransferases. Such modifications are called post-translational modifications, and the relevance of post-translational 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. Although biological roles of other modifications, especially acetylation, have not been intensively studied yet, the 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. Acetylation of lysine causes a loosening of the nucleosome by neutralizing the positive charge, p53, one of the most recognized oncoproteins, requires acetylation for stabilization, which thereby results in the subsequent activation for its own...
functions. Interestingly, a single molecule may be subject to multiple post-translational modifications; in addition, one modification often affects other types of modifications.

Mammalian histone deacetylases (HDACs) are divided into 4 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 histone 3 or 4. HDACs are so named because of this HDAC activity; however, many groups have reported that nonhistone proteins can also be deacetylated by HDACs. Such nonhistone proteins include p53 by HDAC1 in cancer physiology, glucocorticoid receptor by HDAC2 in chronic obstructive pulmonary diseases, v-rel reticuloeendotheliosis viral oncogene homolog A by HDAC3 in silencing mediator of retinoic acid and thyroid hormone receptor/nuclear receptor corepressor 1 complex formation, GATA1 by HDAC3/HDAC4/HDAC5 in murine erythroleukemia cell differentiation, α-tubulin by HDAC6 in control of cell motility, and pleiomorphic adenoma gene 1/2 by HDAC7 in tumorigenesis. Thus, to date, HDACs are also called lysine deacetylases 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 antihypertrophic genes. By contrast, Hdac5 and Hdac9, which are class IIa HDACs, 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 cytoplasmic redistribution of class IIa HDACs, whereas cardiac hypertrophy is prevented when class IIa HDACs are tethered in the nucleus. Although those 2 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 IIa 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 p300/CREB-associated factor (pCAF) acetylases HDAC2, whereas HDAC5, a class IIa HDAC, directly removes the acetyl moiety from HDAC2. We show that HDAC2 is a prohypertrophic mediator suppressed by class IIa 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 report 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) and 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) were inserted as described previously.

#### HDAC Activity

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

#### Trichostatin A Pulse

To minimize the unwanted effect by remnant inhibitors, the 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 reported. ARVCs were infected with adenovirus overnight and 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 previously.

#### In Vivo Delivery of Adenovirus

Adult male C57BL/6 mice (Daehan Biolink) 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, 1×10¹¹ 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 >3 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 performed with PASW Statistics 20 (SPSS, an IBM Company, Chicago, IL).

All reagents and detailed methods are described in the Online Data Supplement.

### Results

#### HDAC2 Acetylation in the Development of Cardiac Hypertrophy

Previously, we reported that the activation of HDAC2 induced by phosphorylation of S394 is critical in the development of cardiac hypertrophy. Here we further investigated whether other post-translational 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 antiacetyl lysine antibody and probed with anti-Hdac2 antisera. Hdad2 acetylation was significantly increased by phenylephrine (Figure 1A) and endothelin-1 (Figure 1B). The results of densitometer analysis were shown in the Online Table. We also examined the acetylation status in vivo in an animal model. An increase in the acetylation of Hdad2 in response to either isoproterenol (Figure 1C) or pressure overload (Figure 1D) was also observed in the mouse heart. Next, we examined the effects of modulators of acetylation. Interestingly, acetylation of Hdad2 was dramatically decreased by garcinol, a histone acetyltransferase inhibitor (Figure 1E, second lane). We also examined whether the inhibition of HDAC could induce hyperacetylation of the Hdad2 molecule itself. We treated NRVCs with trichostatin A (TSA) for 8 hours and then washed out TSA. This TSA pulse significantly increased Hdad2 acetylation (Figure 1E, right lane), which further suggested that Hdad2 is a target of acetylation by histone acetyltransferase or other HDACs.

To elucidate whether acetylation of Hdad2 is related to its enzymatic activity, we tested the intrinsic activity of Hdad2 after treatment with garcinol or TSA. We first treated Hdad2 immunoprecipitates with TSA and measured the activity without washing out the TSA. This resulted in the complete inhibition of Hdad2 enzymatic activity (data not shown), which resulted from a direct inhibition of Hdad2 by the remaining TSA in the precipitates. Thus, we altered the experimental procedures to examine the activity after induction of acetylation of Hdad2, that is, TSA pulse. Hyperacetylation of Hdad2 by TSA was not decreased after washing out (Online Figure I). According to this preliminary experiment, we measured intrinsic Hdad2 activity after cleaning up TSA. Some chemical inhibitors that have recently been developed show relative selectivity on 1 class of HDACs; for example, apicidin25 and SK 704117 preferentially inhibit class I HDACs. Thus, we examined which class of HDAC mediates deacetylation of Hdad2 by using specific class I HDAC inhibitors or nonselective HDAC inhibitor. TSA pulse dramatically induced Hdad2 activation, whereas apicidin pulse failed to do so (Figure 1F). These results implied that deacetylation is mediated by class IIa HDACs.

Hdad2 K75 Residue as an Acetylation Target
We next examined which residue is responsible for acetylation of Hdad2 by using 2 truncated mutants of Hdad2: the N-terminal part (Hdad2-N; amino acids 1–260) and the C-terminal part (Hdad2-C; amino acids 250–488). The acetylation band was detected only in Hdad2-N (Online Figure II).

Hdad2-N contains 18 lysine residues, and the bioinformatics database indicated that both K75 and K90 could be a target for 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 Hdad2 was not detected when lysine 75 was substituted by arginine (Hdad2 K75R; third lane in the second panel in Figure 2A); however, we did not see any changes with K90R (fourth lane in Figure 2A). Furthermore, TSA-dependent hyperacetylation of Hdad2 was not observed in Hdad2 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 (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. Wild-type Hdac2 increased Nppa promoter activity (first and third columns in Figure 2E) as we reported previously, and the increase in activity was further potentiated by phenylephrine (third and fourth columns in Figure 2E). The activation induced by Hdac2 K75R was very weak compared with that by wild type in the basal condition (third and fifth column in Figure 2E). Interestingly, forced expression of Hdac2 K75R significantly blunted the phenylephrine-derived hypertrophy response (fourth and sixth columns in Figure 2E), which suggests that HDAC2 K75R works in a dominant-negative fashion in phenylephrine-treated NRVCs. A similar result was obtained when myosin heavy chain-7 promoter (Myh7, encoding β-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). The measurement of protein synthesis, a hallmark of cardiac hypertrophic phenotypes, 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 synergistically increased by both infection of wt Hdac2 and administration of PE; however, HDAC2 K75R mutant completely blocked PE-induced hypertrophic response. Results are shown as mean±SE. *P<0.05; @@P<0.01. IB indicates immunoblot; and IP, immunoprecipitation.

**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 (wt) control. B, Neither basal acetylation nor trichostatin A (TSA)-induced hyperacetylation was observed in Hdac2 K75R mutant. C, The intrinsic activity of Hdac2 K75R was ≈60% that of wt. HDAC indicates histone deacetylase. D, Hdac2 K75R could not induce Nppa promoter activation, whereas wt Hdac2 could. E, wt Hdac2–induced Nppa promoter activation was dramatically potentiated by simultaneous treatment with phenylephrine (PE); however, this potentiation was not observed when the acetylation-resistant mutant was overexpressed. F, PE further increased [3H]leucine incorporation in wt Hdac2–overexpressed adult rat ventricular cardiomyocytes (ARVCs), whereas it failed to do so in Hdac2 K75R–overexpressed ARVCs. G, Cell size of ARVCs was synergistically increased by both infection of wt Hdac2 and administration of PE; however, HDAC2 K75R mutant completely blocked PE-induced hypertrophic response. Results are shown as mean±SE. *P<0.05; @@P<0.01. IB indicates immunoblot; and IP, immunoprecipitation.
in cell size was potentiated by wild-type Hdac2 (second and fourth columns in Figure 2G). As in Figure 2E and 2F, the infection of Hdac2 K75R mutant successfully blocked the pro-hypertrophic feature of α-adrenergic agonist. However, like wild-type Hdac2, 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 post-translational modification in Hdac2 have been reported to modulate the intrinsic deacetylation activity of HDAC2; S394, S422, and S424 are phosphorylation sites responsible for activation,28 whereas H141 forms a critical component for a deacetylase pocket.29 In addition, S-nitrosylation of C262 and C274 also affects the enzymatic activity by an unknown mechanism.30 We previously reported that casein kinase 2α1 (CK2α1)-mediated phosphorylation of Hdac2 S394 is mandatory for the activation and plays an important role in the development of cardiac hypertrophy.19

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 an interplay may exist between the 2 post-translational modifications of acetylation and phosphorylation. Thus, we checked the phosphorylation status of Hdac2 K75R. In the basal condition, S394 phosphorylation was significantly decreased in K75R (Figure 3A). By contrast, no difference in acetylation status was seen between wild-type Hdac2 and S394A mutant (Figure 3B). In addition, hyperacetylation 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, the deacetylase activity in wild-type Hdac2 was further increased by TSA pulse (second and third columns in Figure 3E). However, the potentiation effect was completely absent when K75R was used (fourth and fifth columns in Figure 3E). Surprisingly, although acetylation was increased by TSA among the mutants tested, except for K75R, TSA-mediated Hdac2 activation was detected only in wild-type Hdac2 (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 prohypertrophic features both in HDAC activity (Online Figure VIII) and in [3H]leucine incorporation assay (Figure 3G); however, acetylation-associated hypertrophy was not observed in the Hdac2 K75Q/S394A double mutant.

Figure 3. Acetylation affects the 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 the substitution of serine 394 with alanine (B). C, Phosphorylation of S394 was significantly increased by trichostatin A (TSA) but was almost completely absent in Hdac2 K75R. D, Histone deacetylase (HDAC) activity of wild-type Hdac2 was dramatically induced by TSA pulse (see Methods section), 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: Hdac2 K75Q (acetylation mimicking).
To check the alteration of phosphorylation of different sites by acetylation, we performed an immunoprecipitation-based phosphorylation assay with Hdac2 S394A and Hdac2 K75R/S394A. Phosphorylation at the residues other than S394 was not changed by acetylation (Online Figure IXA). Likewise, the 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 CK2α that phosphorylates S394. The acetylation-resistant mutant failed to interact with CK2α, which implicates that K75 acetylation is dispensable for the interaction with CK2α for phosphorylation (Online Figure X). These results emphasized that acetylation precedes phosphorylation, but 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 acetylation of Hdac2 (Figure 1E) and that garcinol is an inhibitor of both p300 and pCAF,31 we checked their interaction with HDAC2 and the functional relevance of acetylation. Among the histone acetyltransferases tested, pCAF physically interacted with Hdac2 (Figure 4A–4C). 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). In addition, p300 was not involved in Hdac2 acetylation (Online Figure XIB), which agrees with the result of a previous report.32

**Hdac2 Deacetylation by Hdac5**

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

Both HDAC5 and HDAC9 are well-known antihypertrophic mediators.31 Thus, we examined whether either HDAC5 or HDAC9 could deacetylate HDAC2 and checked whether this deacetylation of HDAC2 worked as an antihypertrophic mechanism. Hdac2 acetylation was significantly decreased by the forced overexpression of Hdad5 (Figure 4G). However, we did not observe the deacetylation effect of Hdad9 in our experimental models (Online Figure XII C and XII D).

Hdac2 acetylation was significantly increased in hearts from Hdad5-null mice (Figure 5A). Hdad5-null mice showed mild cardiac hypertrophy when compared with their wild-type littermates (data not shown), as described previously.31 Like our previous observations in Figure 3A and 3C, Hdad2 phosphorylation was increased in Hdad5-knockout heart (Figure 5A). Hdad5 siRNA induced Hdad2 hyperacetylation (Figure 5B). Hdad2 activity was upregulated by siRNA against Hdad5 (Online Figure XIII). Hdad2 acetylation was reduced when Hdad5 expression was elevated, which was completely reversed by simultaneous treatment with TSA (Figure 5C). Phosphorylation of Hdad2 S394 showed the same trend as the alteration of Hdad2 acetylation by Hdad5. As in Figure 2C, the enzyme activity of Hdad2 was significantly decreased by the forced expression of Hdad5, whereas the effect of Hdad5 on Hdad2 activity was successfully recovered by the inhibition of Hdad5 (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 the recruitment of class I HDACs is required for the full activity of class IIa HDACs.31 To elucidate whether HDAC5 is dependent on class I HDACs to deacetylate Hdad2, we checked deacetylation of Hdad2 in the presence of apicidin. Hdad2 acetylation was significantly decreased whether the cells were simultaneously treated with apicidin or not (Figure 5D). To support this result, we generated 2 enzyme-dead mutants of HDAC5 according to a previous report34 and the bioinformatics site (http://www.uniprot.org/). One was a 767aa mutant lacking the HDAC domain and

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Figure 4. p300/CBP-associated factor (pCAF)/HDAC5 regulates Hdac2 acetylation. A to C, pCAF physically interacted with Hdac2. D, Hdac2 was acetylated in a pCAF dose-dependent fashion. E and F, Hdad5 interacted with Hdac2. G, Hdad2 acetylation was significantly decreased by forced overexpression of Hdad5.
We confirmed the intrinsic activity of HDAC5 mutants (Online Figure XV). Hdac2 acetylation was decreased when wild-type HDAC5 was cotransfected (second and third lanes in Figure 5E); however, the enzyme-dead mutant of HDAC5 failed to show this effect (fourth and fifth lanes in Figure 5E). These data strongly indicated that HDAC5 directly regulates HDAC2 acetylation.

Functional Relevance of Hdac2 Acetylation in Cardiac Hypertrophy

HDAC5 is phosphorylated by protein kinase C/D in response to various stimuli, including hypertrophy signals, and phosphorylated HDAC5 undergoes nuclear export after recognition by the 14-3-3 molecule.35 Leptomycin B (LMB), an antifungal antibiotic, inhibits the cytoplasmic shuttling of HDAC5 through direct binding to chromosomal region maintenance 1.23 Thus, we used LMB to tether Hdac5 in the nucleus. When Hdac2 K75Q was infected to ARVCs, the LMB-mediated repressive effect of Hdac5 was not seen either in cell size measurement (Figure 6A) or in [3H]leucine incorporation assay (Figure 6B). In addition, as in ARVCs, the LMB-mediated antihypertrophic effect was completely abolished by the simultaneous overexpression of Hdac2 K75Q in NRVCs (Figure 6C; Online Figure XVI). Likewise, the overexpression of Hdac5 significantly reduced the activation of Nppa promoter by wild-type Hdac2; however, the antihypertrophic effect of forced expression of Hdac5 was not observed when Hdac2 K75Q was cotransfected (Figure 6D). Likewise, the exaggeration effect by Hdac5 siRNA was not observed in Hdac2 K75R–transfected NRVCs (Figure 6E).

pCAF is also known as a putative prohypertrophic molecule.36 We tested whether pCAF could induce cardiac hypertrophy, which is dependent on Hdac2 acetylation. The forced expression of pCAF in NRVCs induced cardiac hypertrophy, and the hypertrophic effect by pCAF was completely blocked when Hdac2 K75R was cotransfected (Figure 6F; Online Figure XVII). Furthermore, we challenged whether Ad-Hdac2 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 Hdac5-null mice by the injection of Ad-Hdac2 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

Although initial hypertrophy is physiological, sustained stresses lead to a pathological phase of hypertrophy. When the heart undergoes transition into pathological hypertrophy, the myocardium becomes stiffened by interstitial fibrosis, and thereby diastolic dysfunction induces global remodeling of the heart, dilated cardiomyopathy, and heart failure.17 We previously demonstrated that blocking class I HDACs effectively prevents the development of cardiac hypertrophy and that the activation of HDAC2 is associated with CK2α1-dependent HDAC2 S394 phosphorylation19 and with binding of molecular chaperone, inducible heat shock protein 70.20

Although both HDAC2 and class IIa HDACs are involved in cardiac hypertrophy, they play opposite roles. HDAC2 works as a prohypertrophic molecule,18,19,26 whereas class IIa HDACs are negative regulators of cardiac hypertrophy.21,22 Considering

Figure 5. Hdac5 regulates Hdac2 activity by deacetylation. A, Both Hdac2 acetylation and S394 phosphorylation were increased in Hdac5-knockout (KO) mice. B, Hdac5 knockdown dramatically induced Hdac2 acetylation. C, Both Hdac2 acetylation and phosphorylation were significantly decreased by transient overexpression of Hdac5, which was completely reversed by trichostatin A (TSA). D, Hdac2 acetylation was successfully reduced by the overexpression of Hdac5, even when apicidin was added. E, Two enzyme-dead mutants, 767aa and H833A, failed to deacetylate Hdac2, whereas wild-type Hdac5 did.
the 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, it is plausible that an 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 elucidated a novel regulation mechanism of HDAC2 acetylation in cardiac hypertrophy; pCAF-dependent HDAC2 K75 acetylation induces cardiac hypertrophy, which is closely linked to an increase in S394 phosphorylation induced by CK2α1. 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. In HDAC5-free nucleus, HDAC2 is acetylated by pCAF and then phosphorylated by CK2α1, 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. 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 clearly show 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 alternative explanation as to how 2 opposite actions of prohypertrophic class I HDACs and antihypertrophic class IIa HDACs can work together in the regulation of cardiac hypertrophy.

The role of 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. Indeed, their enzymatic activity does not seem to be directly associated with their antihypertrophic action. For example, MTR, an alternative splice form of HDAC9 that lacks a deacetylase domain, is still effective in preventing cardiac hypertrophy. In this study, however, we demonstrated that LMB-dependent inhibition of nuclear export of HDAC5 completely suppresses the prohypertrophic effect of wild-type HDAC2, 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, our finding suggests that HDAC5 plays a role as a deacetylase enzyme targeting HDAC2, a novel nonhistone 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 or by acetylation of nonhistone substrates such as GATA4. In this study, in addition to these mechanisms, we demonstrated that pCAF, but not p300, induces acetylation and subsequent activation of HDAC2.

Studies of post-translational modifications of HDAC2 have intensively focused on phosphorylation. According to a study by the Seto group, 4 serine residues, S394, S411, S422, and S424, are susceptible to phosphorylation. Among them, S394, S422, and S424 are targets of CK2, and S411 is a target of protein kinase C. Moreover, CK2-dependent phosphorylation is essential for either intrinsic activity (S422/S424) or hypertrophy-associated transactivation (S394); however, the ability of HDAC2 to repress transcription is not affected. These reports suggest that the HDAC2-mediated hypertrophy response takes place not by the suppression of downstream genes but by the modulation of HDAC2's own nonhistone 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 subsequent transcriptional inhibitory activity are affected by S-nitrosylation. As shown in a chronic obstructive pulmonary disease model using 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 hyperacetylated. Whereas, their deacetylation activities were not upregulated by hyperacetylation in either phosphorylation-resistant mutants or the enzyme-dead mutant we investigated. These findings suggest that acetylation-associated increase in activity is not a direct effect but rather a secondary phenomenon by the modulation of interaction with kinase and thereby phosphorylation. Although we demonstrated that HDAC2 K75R fails to interact with CK2x1, it is still not clear how acetylation affects the binding of CK2 to HDAC2. Considering that acetylation causes conformational changes, certain HDAC2 domains that are responsible for the interaction with CK2 might be exposed by K75 acetylation. Studies of post-translational modification and subsequent alterations of HDAC2 structure will provide a better understanding. In summary, our results suggest that the crosstalk between CK2-mediated phosphorylation and pCAF/HDAC5-induced acetylation plays a critical role in the development of cardiac hypertrophy by the regulation of HDAC2 activity. Such crosstalk requires further intensive investigations.

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Disclosures

None.

References


What Is Known?

• Class II histone deacetylases (HDACs) inhibit hypertrophy, whereas class I HDACs promote hypertrophy.
• The activation of HDAC2, a class I HDAC, and the ensuing transcriptional repression of antihypertrophic genes are required for the development of cardiac hypertrophy.
• In response to hypertrophic stimuli, casein kinase 2α1 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 the 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 previously showed that the activation of HDAC2, one of the class I HDACs, is indispensible for hypertrophy and that hypertrophic stresses phosphorylate HDAC2 S394 to induce its activation. Here, we showed that acetylation of HDAC2 K75 is mandatory for S394 phosphorylation and thereby activation of HDAC2. The acetylation status of HDAC2 K75 is balanced by pCAF, one of the HATs, and HDAC5, one of the class II HDACs. The antihypertrophic effect of HDAC5 is mediated by its deacetylase activity on HDAC2. Thus, we showed 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.
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 Hong 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), Hdad2 pS394 (1:1000), Hdad4 (1:1000), Hdad5 (1:1000), Hdad7 (1:1000), Hdad9 (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, Hdad5 siRNA, and Hdad9 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 report5 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 previously. 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 report 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.

**[^H]Leucine incorporation assay**

Tritium-leucine incorporation assay was measured as described previously. After transfection for 6 hours (NRVCs) or overnight (ARVCs), the medium was changed to 1.0 μCi/ml[^H]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. \[^{3}H\]Leucine incorporation in NRVCs. \[^{3}H\]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

