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Hopx and Hdac2 interact to modulate Gata4 acetylation and embryonic cardiac myocyte proliferation

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Summary

Regulation of chromatin structure via histone modification has received intense recent attention. Here, we demonstrate that the chromatin modifying enzyme histone deacetylase 2 (Hdac2) functions with a small homeodomain factor, Hopx, to mediate deacetylation of Gata4, which is expressed by cardiac progenitor cells and plays critical roles in the regulation of cardiogenesis. In the absence of Hopx and Hdac2 in mouse embryos, Gata4 hyperacetylation is associated with a marked increase in cardiac myocyte proliferation, up-regulation of Gata4 target genes, and peri-natal lethality. Hdac2 physically interacts with Gata4, and this interaction is stabilized by Hopx. The ability of Gata4 to transactivate cell cycle genes is impaired by Hopx/Hdac2-mediated deacetylation and this effect is abrogated by loss of Hdac2-Gata4 interaction. These results suggest that Gata4 is a non-histone target of Hdac2-mediated deacetylation and that Hdac2, Hopx and Gata4 coordinately regulate cardiac myocyte proliferation during embryonic development.

Introduction

In the heart, cardiac myocyte proliferation is tightly controlled during embryonic development as the heart grows and simultaneously functions to circulate blood and nutrients. Cardiac myocyte progenitors rapidly proliferate during early stages of development and contribute to the trabecular and compact myocardium (Ieda et al., 2009; Pasumarthi and Field, 2002). During late gestation, myocytes progressively lose the ability to proliferate and shortly after birth the vast majority of cardiac myocyte proliferation ceases, as the heart transitions from hyperplastic to hypertrophic growth.

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The Gata family of transcription factors, Gata1–6, are known to be critical for embryonic development, cell growth, and differentiation. Gata transcription factors are defined by an evolutionarily conserved DNA binding domain consisting of two zinc finger motifs that recognize the consensus-binding site WGATAR (Ko and Engel, 1993; Merika and Orkin, 1993). Gata1–3 are predominantly expressed in hematopoietic cells, while Gata4–6 are expressed in several mesoderm and endoderm derived tissues (Charron and Nemer, 1999). Gata4 is one of the earliest genes expressed by specified cardiac precursors at the cardiac crescent stage of mouse development (Arceci et al., 1993; Kelley et al., 1993). Global loss of Gata4 in mice causes embryonic lethality at E9.5 as a result of severe defects in the extra-embryonic endoderm and aberrant heart and foregut morphogenesis (Kuo et al., 1997; Molkentin et al., 1997). Studies involving tissue-specific loss of Gata4 in murine cardiac myocytes have demonstrated a critical role for Gata4 in embryonic myocyte proliferation (Zeisberg et al., 2005). Mice lacking Gata4 in the anterior heart field (AHF), for example, die by E13.5 due to significant right ventricular and interventricular septal myocyte proliferation defects (Rojas et al., 2008). Gata4-null cardiomyocytes show down-regulation of a wide array of cell cycle associated genes, some of which are direct transcriptional targets of Gata4, including *cyclinD2* and *cdk4* (Rojas et al., 2008).

Gata4 activity is modulated in response to a number of intracellular signaling pathways, and Gata4 protein is subject to post-translational modifications including phosphorylation and acetylation (Kawamura et al., 2005; Liang et al., 2001). The histone acetyl transferase (HAT) p300 is able to acetylate Lys311, 318, 320, and 322 of Gata4, resulting in enhanced DNA binding and transcriptional activity (Takaya et al., 2008). In transgenic mice, p300 over-expression in the heart induces Gata4 acetylation and cardiac hypertrophy (Miyamoto et al., 2006; Yanazume et al., 2003). Interestingly, p300 null mice die at E9.5 exhibiting defects in proliferation and cardiac development (Yao et al., 1998). Mechanisms for Gata4 deacetylation have, to our knowledge, not been previously described.

Protein deacetylation can be mediated by members of the histone deacetylase (Hdac) family (Glozak et al., 2005). Based on phylogenetic analysis and sequence homology, the mammalian Hdacs are classified into five sub-families, class I (Hdac1, 2, 3, and 8), class IIa (Hdac4, 5, 7, and 9), class IIb Hdacs (Hdac6 and Hdac10), class III (sirtuins) and class IV (Hdac11) (de Ruijter et al., 2003; Gregoretti et al., 2004). Hdacs lacks intrinsic DNA binding ability and are recruited to target genes via their incorporation into large multiprotein transcriptional complexes as well as direct association with transcriptional activators or repressors (Grunstein, 1997). Eukaryotic Hdacs can deacetylate both histone and non-histone substrates (Ekwall, 2005; Glozak et al., 2005). Gene inactivation studies in mice have demonstrated potent functions for several class I and II Hdacs in cardiac development and in the regulation of cardiac hypertrophy and metabolism (Haberland et al., 2009). Recently, we have shown that global loss of Hdac2 results in partial perinatal lethality due to cardiac developmental defects that include enhanced cardiac myocyte proliferation (Trivedi et al., 2007a). Combined loss of Hdac1 and Hdac2 in cardiac myocytes results in complete perinatal lethality and cardiac defects that are more severe than those seen in either individual knock-out (Montgomery et al., 2007).

We have previously shown that Hdac2 is able to physically interact with an atypical homeodomain protein called Hopx, which is expressed in the developing heart (Kook et al., 2003). Hopx is a 73–amino acid protein that includes a 60–amino acid motif homologous to the homeodomain of Hox transcription factors (Kook and Epstein, 2003). Unlike most other homeodomain proteins, Hopx is unable to bind DNA as it lacks certain conserved amino acid residues that are required for protein-DNA interactions (Kook et al., 2006). However, Hopx is a nuclear protein that can function to modulate cardiac gene transcription by recruiting Hdacs (Kook et al., 2003). Inactivation of Hopx results in partial embryonic

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lethality and an increase in myocyte proliferation in late gestation suggesting that Hopx, perhaps in association with Hdac2, modulates the balance between growth and differentiation pathways in the heart (Chen et al., 2002; Shin et al., 2002).

Here we show that global loss of Hdac2 and Hopx in mice leads to complete perinatal lethality, severe cardiac developmental defects, and excessive myocyte proliferation. Hdac2 and Hopx regulate Gata4 acetylation and activation of Gata4 dependent cell cycle genes. Our results suggest an unexpected function for the homeodomain protein Hopx as an adapter that facilitates the ability of Gata4 to serve as a direct catalytic target of Hdac2 in the embryonic heart.

Results

Genetic interaction between Hopx and Hdac2

Genetic inactivation of *Hopx* and *Hdac2* separately results in similar cardiac phenotypes characterized by partial embryonic lethality and cardiac myocyte hyperplasia, and we have previously demonstrated that Hopx and Hdac2 physically interact (Chen et al., 2002; Kook et al., 2003; Trivedi et al., 2007a). Hence, we sought to determine if *Hopx* and *Hdac2* genetically interact. Hdac2 is expressed by many, but not all, cardiac myocytes at P0 (Figure 1A), where Hopx is also expressed (Figure 1A). We crossed $Hopx^{+/-}$ mice with $Hdac2^{+/-}$ mice to generate compound heterozygotes, which were intercrossed to produce double homozygous knockout (DKO) offspring. DKO pups were identified at P0 when they appeared runted and milk was not evident in the stomachs (Figure 1B). DKO mice were not identified after P0 indicating complete peri-natal lethality (Table 1, see also Table S1). Compound heterozygous mice were found alive postnatally and at weaning, but several compound heterozygous animals appeared cyanotic at birth (not shown) and were euthanized. H&E stained heart sections of these compound heterozygous animals (Figure 1C), and of those lacking additional alleles of *Hopx* or *Hdac2* (not shown), revealed severe muscular ventricular septal defects (VSD), while these defects were never seen in single heterozygous animals or wild type controls. These data suggest a genetic interaction between *Hdac2* and *Hopx*. Examination of the hearts of DKO P0 pups revealed significant abnormalities in all cases (n=6) with thickening of the ventricular walls (Figure 1D). Cardiac myocyte proliferation, as assessed by phospho-histone H3 and myosin (MF-20) co-immunostaining, was increased in both *Hopx* and *Hdac2* single knockout hearts as compared to wild type litter-mates, and proliferation was even more dramatically increased in DKO hearts, likely explaining the thickened ventricular walls (Figure 1D, E).

Gata4 target genes are up-regulated in DKO hearts

We performed gene expression profiling of wild type, $Hdac2^{-/-}$, $Hopx^{-/-}$ and DKO hearts at E16.5 (Table S2). Gene ontogeny analysis of significantly altered transcripts indicated that cell cycle genes were markedly affected in both single and, to an even greater extent, double knockout hearts (Figure S1 A). Analysis of transcription factor binding sites (using the TRASFAC database) in the conserved non-coding regions within 5 Kb upstream of dysregulated genes in DKO hearts revealed frequent GATA binding sites, suggesting potential direct Gata4 transcriptional targets (Figure S1 B). Amongst these, we confirmed that several cell cycle genes were up-regulated in DKO hearts by RT-PCR (Table S3) including transcripts of known Gata4 targets, *cyclinD2* and *cdk4* (Figure 2A). Interestingly, *Gata4* mRNA and protein levels were unaltered in *Hopx* and *Hdac2* single knockout hearts, and in DKO hearts (Figure 2B–D).

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Acetylation of Gata4 is modulated by Hopx and Hdac2

Gata4 transcriptional activity is known to be regulated by post-translational modifications (Kawamura et al., 2005; Liang et al., 2001). Gata4 is acetylated by p300, which results in enhanced transcriptional activity (Kawamura et al., 2005; Takaya et al., 2008). Immunoprecipitation of endogenous Gata4 protein from wild type and DKO hearts followed by Western blotting using an anti-acetylated lysine specific antibody indicated that acetylated Gata4 was significantly upregulated in DKO hearts compared to controls and single knockouts (Figure 3A,B). Transfection experiments in 293T cells followed by anti-HA Gata4 immunoprecipitation and anti-acetyl lysine immunoblotting confirmed that acetylated Gata4 levels are diminished by co-transfection of Hdac2 (Figure 3C,D). Transfection of Hopx alone results in mild diminution of the acetylated Gata4 signal (Figure 3C,D). However, co-transfection of both Hdac2 and Hopx markedly reduced acetylated Gata4 levels (Figure 3C,D). Importantly, transfection of a mutant form of Hopx that does not interact with Hdac2, termed HopxH2 (Kook et al., 2003), does not reduce acetylated Gata4 levels, nor does it augment deacetylation induced by Hdac2 (Figure 3C,D).

Gata4 is able to activate expression of *cyclinD2* and *cdk4* by binding to promoter/enhancer regions resulting in direct transactivation (Rojas et al., 2008). Hence, we examined the ability of Hopx and Hdac2 to modify Gata4 activation of appropriate *cyclinD2* and *cdk4* luciferase reporter constructs. Transfection of Gata4 resulted in an approximate 10-fold activation of the *cyclinD2* reporter and this activation was moderately impaired by co-transfection of either Hdac2 or Hopx alone (Figure 3E). Co-transfection of Hopx and Hdac2 together markedly inhibited Gata4-mediated transactivation (Figure 3E). Mutant HopxH2 did not inhibit Gata4 dependent transactivation, nor did it enhance suppression of Gata4 dependent transactivation when co-transfected with Hdac2 (Figure 3E).

Gata4 contains 4 lysine residues near the second zinc finger, at positions 311, 318, 320 and 322, that are potentially capable of acetylation (Takaya et al., 2008). Replacement of lysines with alanine to mimic deacetylation resulted in a mutant form of Gata4 that could only weakly transactivate the *cyclinD2* luciferase reporter (Figure 3E). Replacement of lysine residues with glutamine to mimic constitutive acetylation allowed for approximately 7-fold activation of the *cyclinD2*-luciferase reporter. Importantly, co-transfection of Hdac2, Hopx, or both together failed to inhibit activation induced by this K → Q mutant (Figure 3E). Similar results were obtained using *cdk4* and *ANF*-luciferase reporter constructs (data not shown). These results suggest that Hdac2 and Hopx function, at least in part, by mediating deacetylation of lysine residues within Gata4 to modulate transcriptional activity.

Hdac2 and Gata4 physically interact

Immunoprecipitation of Gata4 protein from heart lysates, followed by immunoblotting for Hdac2, indicates that Gata4 and Hdac2 proteins can interact *in vivo* (Figure 4A). This interaction was markedly less robust when tested in heart lysates taken from $Hopx^{-/-}$ embryos, consistent with a model in which a complex containing Hdac2 and Gata4 is stabilized by Hopx (Figure 4A,B). In transfected 293T cells, Gata4 and Hdac2 can also interact (Figure 4C) and this interaction is stabilized by adding increasing amounts of Hopx (Figure 4C,D). Deletion analysis indicates that the interaction between Hdac2 and Hopx requires the lysine rich domain of Gata4 and is specific (Figure 4E,F). Loss of this interaction between Gata4 and Hdac2 abolishes Hdac2-Hopx mediated repression of Gata4 dependent *cyclinD2* reporter activity (Figure 4G).

Phosphorylated Hdac2 interacts with and deacetylates Gata4

Prior experimental data have suggested that a phosphorylated form of Hdac2 preferentially occupies promoter regions of regulated genes, while an un-phosphorylated form is more

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commonly found at coding regions of transcribed genes (Sun et al., 2007). Hence, we sought to determine if a phosphorylated form of Hdac2 preferentially interacts with Gata4 to mediate deacetylation and regulation of downstream gene transcription. We generated a mutant form of Hdac2 in which serine residues 394, 411, 422 and 424 are mutated to alanine to prevent serine phophorylation. This mutant form of Hdac2 failed to robustly interact with Gata4 (Figure 5A,B) and failed to repress Gata4-mediated activation of luciferase reporter constructs (Figure 5C). The Hdac2 phospho-mutant was expressed at equivalent levels when compared to wild type Hdac2 (Figure 5A) and was equally able to mediate deacetylation of histoneH3 and a histone pseudo-substrate in an in vitro Hdac activity assay (Figure 5D–F) suggesting that catalytic activity was intact. Taken together, these results suggest that Hdac2 functions coordinately with Hopx to deacetylate Gata4 and thus regulate transcriptional activity and myocyte proliferation in the developing heart (Figure 5G).

Discussion

In this report, we demonstrate a genetic and functional interaction between Hdac2 and Hopx. Heterozygous inactivation of either gene is well tolerated without any noted phenotype, while compound heterozygous mice exhibit muscular VSDs. This type of VSD is unusual in mouse models, while membranous VSDs located at the superior margin of the interventricular septum are more common, and suggests a defect of myocyte development. Mice homozygous deficient for both *Hdac2* and *Hopx* exhibit peri-natal lethality and cardiac myocyte proliferation is markedly up-regulated. The resulting ventricular walls are thickened and hyperplastic. Genes involved in embryonic cardiac myocyte proliferation are up-regulated. Our data strongly suggest that this is due, at least in part, to enhanced Gata4 transcriptional activity, which in turn is due to Gata4 hyper-acetylation. Furthermore, our results suggest that a phosphorylated form of Hdac2 interacts with Gata4 in a complex that is stabilized by Hopx (Figure 5G).

Hdacs are commonly thought to act as transcriptional co-repressors by deacetylating histone tails with resulting compaction of chromatin structure. Non-histone targets of Hdacs have been described, raising the possibility that Hdac-mediated effects on transcription can be caused by catalytic activity or other mechanisms unassociated with chromatin remodeling. Our results demonstrating that a Gata4 mutant in which lysine residues are mutated to mimic constitutive acetylation cannot be inhibited by Hdac2 allow us to conclude that direct deacetylation of Gata4, rather than deacetylation of histones, is responsible for Hdac2-mediated repression of Gata4 in this system.

Regulation of cardiac growth and proliferation requires a delicate balance of gene transcription and regulation of cell cycle machinery. Several transcription factors and cofactors maintain this balance during cardiac development (Pasumarthi and Field, 2002) and Hopx has previously been implicated in this process (Chen et al., 2002; Shin et al., 2002). Loss of either Hopx or Hdac2 individually leads to partially penetrant embryonic lethality with increased cardiomyocyte proliferation (Chen et al., 2002; Shin et al., 2002; Trivedi et al., 2007a). The results of the present study elucidate a mechanistic pathway by which an Hdac2-Hopx complex regulates embryonic myocardial proliferation. Overlapping features of the individual knockouts, the genetic interaction (particularly that seen in compound heterozygotes) and shared downstream target genes evident from microarray analysis are all consistent with this model. However, Hdac2 and Hopx individual knockout mice also have significant differences in their phenotypes and microarrays suggest distinct downstream genes in addition to those that are shared. Thus, each is likely to have functions that are independent of activities within a shared complex (Chen et al., 2002; Shin et al., 2002; Trivedi et al., 2007a).

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The fact that DKO mice have a worse phenotype than either Hopx or Hdac2 individual nulls indicates that neither protein is absolutely required for the activity of a shared complex. The data presented here suggest that Hopx stabilizes the interaction between Hdac2 and Gata4 and enhances Gata4 deacetylation, and we hypothesize that some degree of Hdac-mediated Gata4 deacetylation is preserved in the absence of Hopx. Likewise, when Hdac2 is absent, some degree of Gata4 deacetylation is still evident when compared to the absence of both Hopx and Hdac2 (both in vivo and in transfected cells, Figure 3B) and we hypothesize that this is due to partially redundant functions of other Hdacs. Indeed, we have previously shown that Hopx can interact with Hdac3 (Kook et al., 2003) and Hdac1 shares extensive homology with Hdac2 (de Ruijter et al., 2003).

Previous reports have indicated that Gata factors can recruit Hdacs to target genes, either by directly interacting or by associating with Fog (Friend of Gata) proteins that can recruit the NuRD complex containing Hdac1 and Hdac2 (Cantor and Orkin, 2005; Hong et al., 2005; Svensson et al., 1999; Tsang et al., 1997). For example, Hdac3 interacts with Gata2 to mediate transcriptional repression in hematopoietic progenitor cells (Ozawa et al., 2001). Similarly, Gata1 interaction with Hdac5 results in reduced Gata1 transcriptional activity (Watamoto et al., 2003). In each case, Hdac function suppresses Gata dependent transcriptional activity. Although it was generally assumed that Hdac-mediated transcriptional repression of Gata activity was mediated by histone deacetylation and chromatin remodeling, it will be interesting to re-address this question in light of our results to determine if other Gata proteins are regulated by Hdacs via direct deacetylation.

The present study focused on the role of Hopx and Hdac2 during embryonic cardiac development. However, our prior studies have implicated important roles for both Hopx and Hdac2 in the adult heart (Chen et al., 2002; Kook and Epstein, 2003; Kook et al., 2003; Kook et al., 2006; Trivedi et al., 2007a). Gata4 also functions in homeostasis of the adult myocardium, and alterations in transcriptional activity, including those regulated by p300-mediated acetylation of Gata4, affect adult cardiac hypertrophy (Takaya et al., 2008). However, the model presented here to explain the roles of Hopx, Hdac2 and Gata4 in regulation of embryonic myocardial proliferation cannot easily explain the roles for these proteins during adult cardiac hypertrophic processes. Hdac2 deficiency results in resistance to cardiac hypertrophy, and Hopx over-expression induces hypertrophy. Cardiac hypertrophy is generally associated with enhanced Gata4 activity (which drives cardiac structural gene expression). A simple extension of the model presented here would suggest that loss of Hdac2 would result in enhanced Gata4 activity, and gain of Hopx might produce an inhibition of Gata4 activity. Neither of these predictions would be consistent with the adult phenotypes. However, we have noted a dramatic loss of Gata4 protein in newborn mice lacking Hopx and Hdac2, suggesting that hyper-acetylation results in protein degradation, with resulting loss of Gata4 activity. Consistent with this hyothesis, acetylation of Gata1 enhances activity and simultaneously accelerates degradation (Hernandez-Hernandez et al., 2006). Future studies will attempt to elucidate the mechanisms by which acetylation of Gata4 leads to degradation and the degree to which this pathway can account for the adult cardiac hypertrophy phenotypes in Hdac2 and Hopx mice.

Gata4 contains two distinct zinc finger domains and a C-terminal nuclear localization sequence that together constitute the DNA binding and protein-protein interaction domain (Molkentin, 2000). Gata4 also contains two transcriptional activation domains in the N-terminus (Pikkarainen et al., 2004). Some of these Gata4 domains are known to interact with other transcription factors or cofactors. For example, Gata4 interacts with p300 at the second zinc finger, with Fog2 at the first zinc finger, and with Gata6 at the region including both zinc fingers and their C-terminal tails (Charron et al., 1999; Dai and Markham, 2001; Lu et

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al., 1999; Svensson et al., 1999). Our studies identify the lysine rich domain located between the two zinc fingers as the region necessary for interaction with Hdac2.

Several HDACs are known to be regulated by phosphorylation (Haberland et al., 2009). Casein kinase II (CKII) is a putative Hdac2 kinase that can phosphorylate Hdac2 at Ser^{394}, Ser^{422}, and Ser^{424} (Adenuga et al., 2009; Tsai and Seto, 2002). Although the majority of Hdac2 in the cell is unphosphorylated, a recent study suggests that the phosphorylated form preferentially associates with mSin3A, NuRD, and CoREST complexes that are recruited to promoter regions by transcription factors such as p53, pRb, YY1 and NF-kappaB (Sun et al., 2007). Our data suggest that phosphorylated Hdac2 interacts with Gata4, resulting in Gata4 deacetylation and suppression of transcriptional activity. Thus, phosphorylation of Hdac2, by CKII or by another Hdac kinase in the heart, may serve as a mode of regulation of Gata4 activity and myocyte proliferation.

Experimental Procedures

Generation of Hdac2-Hopx DKO mice

Hdac2-null and *Hopx*-null mice have been previously described (Chen et al., 2002; Trivedi et al., 2007a). We interbred $Hdac2^{+/-}$ mice with $Hopx^{+/-}$ mice to generate $Hdac2^{+/-}$; $Hopx^{+/-}$. These compound heterozygous mice were crossed to generate *Hdac2-Hopx* DKO mice.

Histology, immunohistochemistry and in-situ hybridization

Tissues were fixed in 2% paraformaldehyde, ethanol dehydrated, embedded in paraffin, and sectioned as described previously (Trivedi et al., 2007a). Protocols for hematoxylin and eosin staining and immunohistochemistry have been previously described (Trivedi et al., 2007a). E16.5 and P0 wild-type, compound heterozygous, *Hdac2*-null, *Hopx*-null, and *Hdac2-Hopx* DKO heart sections were deparaffinized and pretreated using heat antigen retrieval with Bull'Eye Decloaker (BioCare Medical). After blocking endogenous peroxidase with 3% hydrogen peroxide, washing with 0.1% PBST and blocking with 2.5% horse serum, sections were incubated with anti-phospho-histone H3 (1:50; Cell Signaling) antibody overnight at 4°C. After washing with 0.1% PBST, sections were incubated with biotinyl tyramide amplification reagent (TSA Biotin System, PerkinElmer) at 25°C for four minutes, washed with 0.1% PBST and incubated with steptavidin-Texas red® (1:500; PerkinElmer) for one hour at 25°C. For MF-20 (myosin) co-staining, these sections were incubated with anti-MF-20 (1:20, Developmental Studies Hybridoma Bank) overnight at 4°C after washing with 0.1% PBST and blocking with 10% goat serum. Alexa Fluor 488 goat anti-mouse IgG (1:250; Invitrogen) was used as a secondary antibody. Slides were washed with 0.1% PBST and mounted with Vectashield mounting medium (Vector Laboratories). Using ImageJ software, phospho-histone H3-positive myocyte nuclei that were also MF20 positive were counted in five different sections of three independent heart samples. In-situ hybridization was performed on paraffin sections using S^{35} labeled *Hopx* RNA probe as described previously (Chen et al., 2002).

Microarray, gene ontology analysis and real time PCR

Microarray analysis was performed using 3 independent samples of wild type, *Hdac2*-null, *Hopx*-null, or *Hdac2-Hopx* DKO ventricular cardiac tissue from E16.5 embryos. RNA was extracted and reverse transcribed without amplification. Microarray analysis was performed by the University of Pennsylvania Microarray Core Facility using Affymetrix mouse cDNA arrays (MOE430 v2.0). Full data sets are available at Gene Expression Omnibus (GEO, accession number GSE23700) (Barrett et al., 2009). Gene ontology analysis was performed with Database for Annotation, Visualization and Integrated Discovery 6.7 (DAVID 6.7,

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http://david.abcc.ncifcrf.gov/) using PANTHER annotation (Panther_BP_All) with medium stringency (Dennis et al., 2003).

Quantitative real time PCR (qRT-PCR) was performed as described previously (Trivedi et al., 2008a). Briefly, total RNA was isolated from dissected mouse heart ventricles using Trizol (Invitrogen). RNA was reverse-transcribed using random hexamers and the Superscript First Strand Synthesis Kit (Invitrogen). Gene expression was then evaluated by qRT-PCR (ABI PRISM 7900) using the SYBR Green (Applied Biosystems). Signals were normalized to their corresponding GAPDH controls and the ratios expressed as fold changes compared to wild-type controls. PCR conditions and primer set sequences are available upon request.

Immunoprecipitation

Embryonic heart samples or 293T cells were homogenized in immunoprecipitation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM DTT) containing 1 mM PMSF, phosphatase inhibitors (Sigma), and protease inhibitors mixture (Sigma). The samples were sonicated five times for 3 s. The lysate was collected by centrifugation at 16,000 × g for 10 min at 4°C. Pre-cleared lysates with beads were incubated with primary antibodies for 16 h at 4°C. After incubation for 1 h at 4°C with beads, immunocomplexes were collected, washed four times with immunoprecipitation buffer, and applied to 4–12% SDS-polyacrylamide gels for Western blot analysis.

Western blotting

Heart tissue lysates and cell lysates were prepared in lysis buffer consisting of 20 mmol/L Tris HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L Na_2EDTA, 1 mmol/L EGTA, 1% Triton X-100, 1 μg/mL leupeptin, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L Na_3VO_4, 1 mmol/L β-glycerophosphate. 1 mmol/L phenylmethylsulfonyl fluoride was added before use. Equal amounts of protein were resolved by 4%–12% SDS-PAGE acrylamide gel and transferred to poly(vinylidene difluoride) membranes. We used primary antibodies to Gata4 (1:1000 dilution, SantaCruz), Hdac2 (1:1000 dilution, Invitrogen), acetyl lysine (1:1000 dilution, Upstate), FLAG (1:1000 dilution, Sigma), Myc (1:1000 dilution, Sigma), HA (1:1000 dilution, SantaCruz), histone H3 (1:1000 dilution, Upstate) and acetylated histone H3 (1:1000 dilution, Upstate). Primary antibody binding was visualized by HRP-conjugated secondary antibody and detected by enhanced chemiluminescence (LumiGlo, Cell Signaling). Membranes were reprobed with primary antibody against β-actin (1:2500 dilution, Sigma) for loading control.

Transient transfections and luciferase assays

H9c2 myoblasts and 293T cells were transfected with PolyFect transfection reagent (Qiagen) as per manufacturer's protocol. In brief, for H9c2 myoblasts, each 100 mm plate containing a sub-confluent culture was transfected with total of 4 μg of DNA and 25 μl of PolyFect transfection reagent in 8 ml of 10% FBS medium. Total DNA was maintained constant by adding empty pcDNA3.1$^+$ vector when appropriate. 24 h after transfection, cells were lysed with passive lysis buffer (Promega) and lysates were analyzed for both firefly and renilla luciferase activity using a dual luciferase reporter assay kit (Promega) as described previously (Trivedi et al., 2008b). PerkinElmer multi-label plate reader was used to determine luciferase activity according to the manufacturer's instructions. For 293T cells, each 100 mm plate containing a sub-confluent culture was transfected with total of 8 μg of DNA and 25 μl of PolyFect transfection reagent in 8 ml of 10% FBS medium.

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Plasmids and site directed mutagenesis

Plasmids for expression of Hdac2, Hopx, and Gata4 are previously described (Durocher et al., 1997; Kook et al., 2003; Trivedi et al., 2007a). Various deletion mutants of HA tagged Gata4 cDNA were created by PCR amplification, sequence verified, and sub-cloned into the pGC eukaryotic expression vector (Durocher et al., 1997). The Gata4 N-terminal deletion mutants, pG4Δ80N, pG4Δ129N, and pG4Δ240N were named according to the last amino acid of the full-length protein that was deleted. Similarly, the C-terminal deletion mutants, pG4Δ390C, pG4Δ336C, pG4Δ310C, and pG4Δ274C were named to indicate the most C-terminal amino acid present in the recombinant protein. Finally, the in-frame GATA-4 deletion mutant, pG4Δ241-274N, was named to indicate the N- and C-terminal boundaries of the specified deletion. Plasmids HA-Gata4-KA (K311A/K318A/K320A/K322A), HA-Gata4-KQ (K318Q/K320Q/K322Q), and Hdac2SA (S394A/S411A/S422A/S424A) were used to generate Gata4KA, Gata4KQ and Hdac2SA, respectively, and were constructed by using a site-directed mutagenesis kit as per manufacturer's protocol (Stratagene). HopxH2 (HopH2) has been previously described (Kook et al., 2003). All the constructs were sequenced and tested for expression by Western blot analysis following transient transfection in 293T cells.

HDAC activity assay

HDAC activity was measured by using an HDAC activity assay kit—fluorometric detection (Upstate) according to the manufacturer's protocol as previously described (Trivedi et al., 2007b). In brief, H9c2 or 293T cells were transfected with plasmid containing either Hdac2 or mutant Hdac2SA. Thirty micrograms of total extract was incubated with HDAC assay substrate and incubated at 30 °C for 60 min. After addition of diluted activator reagent, samples were incubated at room temperature for 15 mins and examined by fluorescent microplate reader at excitation of 360 nm and emission 450 nm. A standard curve was performed according to the manufacturer's protocol.

Statistics

Data are expressed as mean ± SEM unless otherwise stated. The statistical significance of differences between groups was analyzed by Student's 2 tailed *t*-test. Differences were considered significant at a P-value < 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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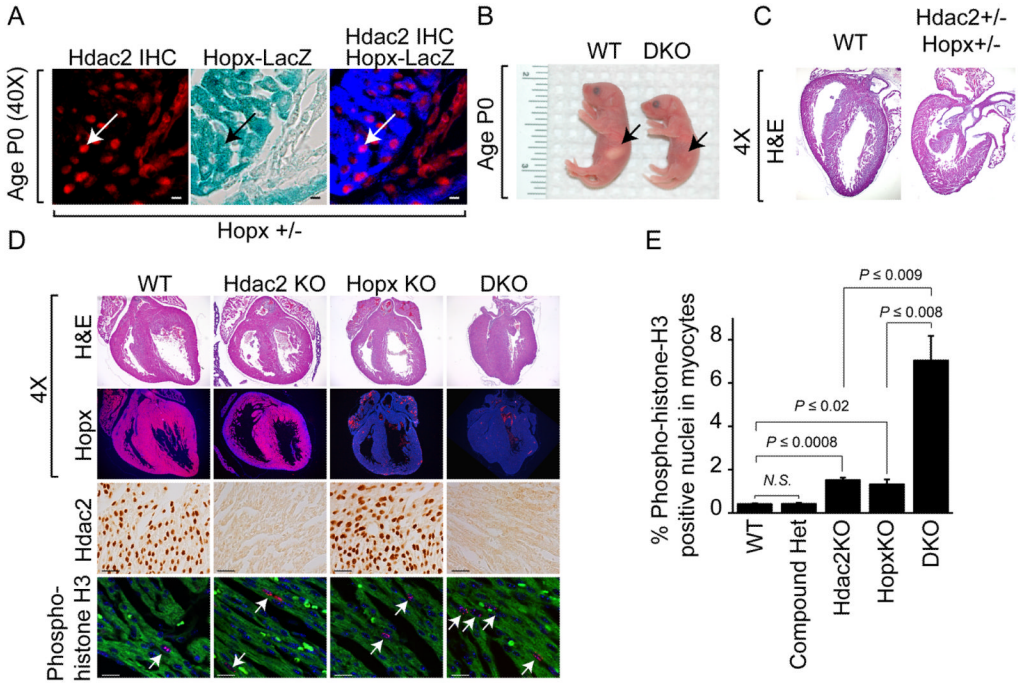

Figure 1. Myocardial defects in Hdac2-Hopx-null mice

(**A**) Immunohistochemistry (Hdac2) and LacZ staining (Hopx) of P0 $Hopx^{lacZ/+}$ heart sections shows Hdac2 and Hopx co-expression (arrow) in developing heart. (**B**) Wild-type and Hdac2-Hopx DKO littermates are shown at P0. (**C**) H&E-stained sections of wild-type and compound heterozygous hearts show muscular type of ventricular septal defect (VSD) at P0. (**D**) Abnormally thickened compact zone of the ventricular myocardium and increased cardiac myocyte proliferation in DKO hearts. Immunohistochemistry staining of Hdac2 shows loss of Hdac2 in DKO hearts at P0. *Hopx* in situ shows loss of *Hopx* mRNA in DKO hearts at P0. Cardiac myocyte proliferation (green, stained by MF-20) was assessed by phosphohistone H3 co-staining (arrows) in wild-type, $Hdac2^{-/-}$, $Hopx^{-/-}$ and DKO P0 hearts. Scale bar, 20 μm. (**E**) Quantification of phospho-histone H3 positive cells in WT, compound heterozygous, $Hdac2^{-/-}$, $Hopx^{-/-}$, and DKO P0 cardiac myocytes (mean ± SEM, n=3). N.S., not significant.

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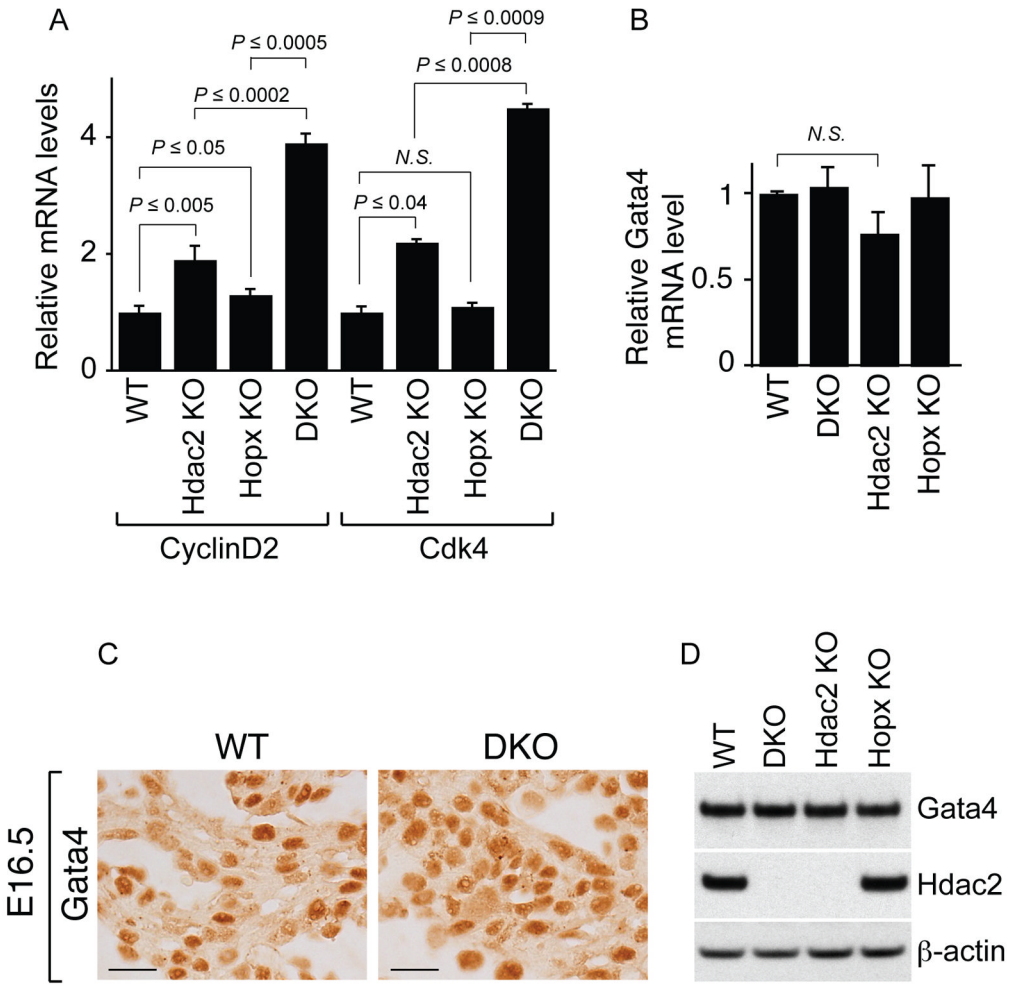

Figure 2. Aberrant expression of Gata4 target genes in DKO hearts

(**A**) Transcripts for Gata4 target genes, *cyclinD2* and *cdk4*, were detected by qRT-PCR from E16.5 wild-type, *Hdac2*$^{-/-}$, *Hopx*$^{-/-}$ and DKO myocardium (mean ± SEM, n=3) (**B**) *Gata4* transcripts were detected by qRT-PCR from E16.5 wild-type, *Hdac2*$^{-/-}$, *Hopx*$^{-/-}$ and DKO myocardium (mean ± SEM, n=3). (**C**) Gata4 protein levels are unchanged in DKO mice. Gata4 expression was assessed by immunohistochemistry in WT and DKO E16.5 hearts. Scale bar, 10 μm. (**D**) Western blot analysis was performed on total lysates from WT, DKO, *Hdac2*$^{-/-}$, and *Hopx*$^{-/-}$ E16.5 hearts. β-actin is shown as a loading control. N.S., not significant.

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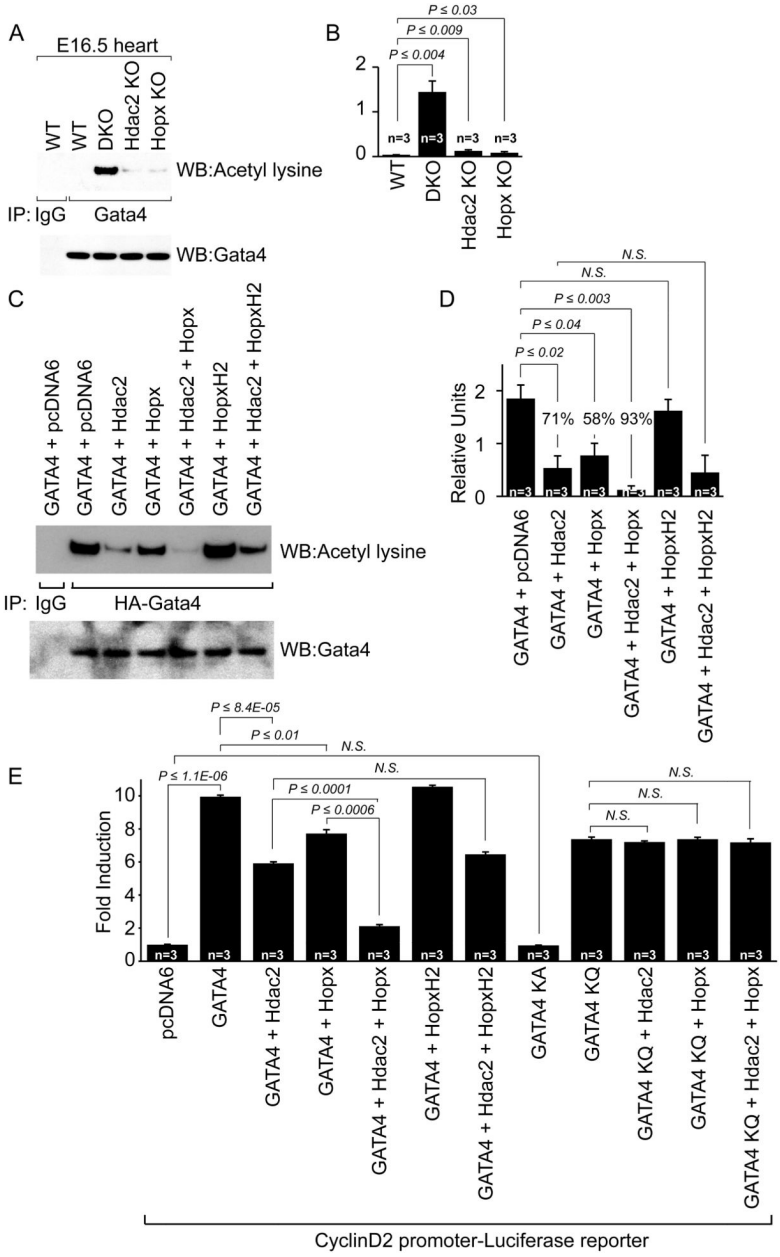

Figure 3. Hdac2-Hopx modulates Gata4 transcriptional activity and acetylation

(**A**) Increased Gata4 acetylation in DKO hearts. Total lysates from E16.5 WT, DKO, *Hdac2*$^{-/-}$, and *Hopx*$^{-/-}$ hearts were immunoprecipitated by anti-Gata4 antibody and Western blot was performed using anti-acetyl lysine antibody to detect acetylated Gata4. Western blot for total Gata4 is shown below. (**B**) Acetylated Gata4 was quantified and normalized to total Gata4 in WT, DKO, *Hdac2*$^{-/-}$, and *Hopx*$^{-/-}$ myocardium using ImageJ software (mean ± SEM, n=3). (**C**) Hdac2-Hopx over-expression inhibits Gata4 acetylation. Total lysates from transfected 293T cells were immunoprecipitated by anti-HA antibody to immunoprecipitate Gata4 and acetylated Gata4 was detected by Western blot analysis using anti-acetyl lysine antibody. (**D**) Acetylated Gata4 was quantified and normalized to total Gata4 using ImageJ software (mean ± SEM, n=3). (**E**) Hdac2-Hopx inhibits Gata4 dependent transactivation. CyclinD2-luciferase reporter construct was transfected in H9c2

myoblast cells with or without Gata4, Hdac2, Hopx, HopxH2, Gata4KA, and Gata4KQ expression constructs. Firefly luciferase activity was measured from total lysates 24 hrs after transfection and was normalized to renilla luciferase activity. The induction is represented as fold-induction over the normalized luciferase activity in the untreated cells (mean ± SEM, n=3). N.S., not significant.

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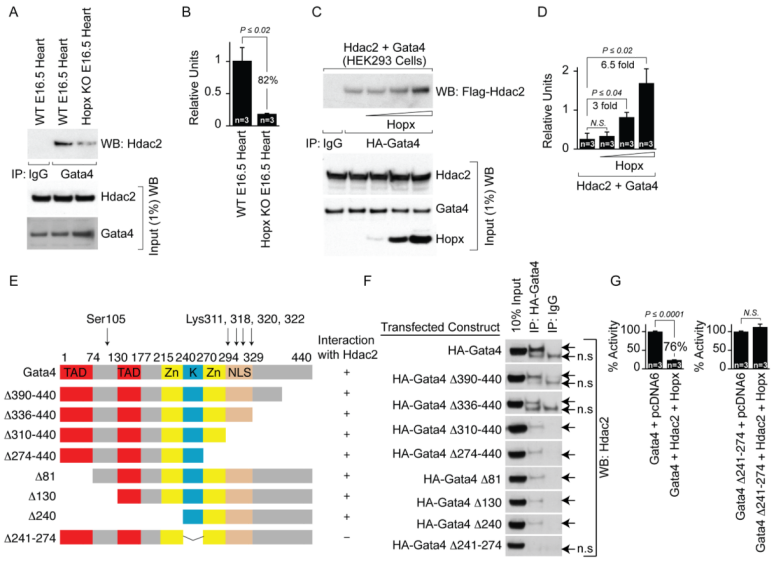

Figure 4. Hdac2 interacts with Gata4

(**A**) Total lysates from E16.5 WT and *Hopx*$^{-/-}$ hearts were immunoprecipitated by anti-Gata4 antibody and Western blot was performed using anti-Hdac2 antibody. The interaction between Hdac2 and Gata4 is more robust in WT hearts (lane 2) than in Hopx-deficient hearts (lane 3). (**B**) Quantification of the interaction between Hdac2 and Gata4 in WT and *Hopx*$^{-/-}$ hearts using ImageJ software (mean ± SEM, n=3). (**C**) Hopx modulates Hdac2-Gata4 interaction. Total lysates from transfected 293T cells were immunoprecipitated by anti-HA antibody to immunoprecipitate Gata4 and Western blot was performed with anti-Flag antibody to detect Hdac2. Increasing amounts of Hopx resulted in an increasingly robust interaction between Hdac2 and Gata4. (**D**) Quantification of the interaction between Hdac2 and Gata4 in presence of increasing amount of Hopx (mean ± SEM, n=3). (**E**) Schematic representation of Gata4 showing post-translational modification sites and deletion constructs. Gata4 contains two distinct zinc (Zn) finger domains and two transcriptional activation domains (TAD). Gata4 also contains lysine rich domain (K) and nuclear localization sequence (NLS). p300 dependent acetylation of Gata4 at Lys 311, 318, 320, and 322 and MAPK dependent phosphorylation at Ser105 is important for Gata4 DNA binding and transcriptional activation abilities. (**F**) Total lysates from Gata4 or various Gata4 deletion constructs transfected 293T cells were immunoprecipitated with anti-HA antibody and Westernblot analysis was performed with anti-Hdac2 antibody to detect Hdac2. n.s. – non specific. (**G**) Loss of Hdac2 and Gata4 interaction prevents Hdac2-Hopx mediated repression of Gata4 dependent *cyclinD2* reporter activity. *CyclinD2*-luciferase reporter construct was transfected in H9c2 cells with or without Gata4, Gata4 Δ241-274, Hdac2, and Hopx expression constructs. Firefly luciferase activity was measured from total lysates 24 hrs after transfection and was normalized to renilla luciferase activity. The induction is represented as % activity of *cyclinD2* reporter over the normalized luciferase activity in the Gata4 or Gata4 Δ241-274 transfected cells (mean ± SEM, n=3).

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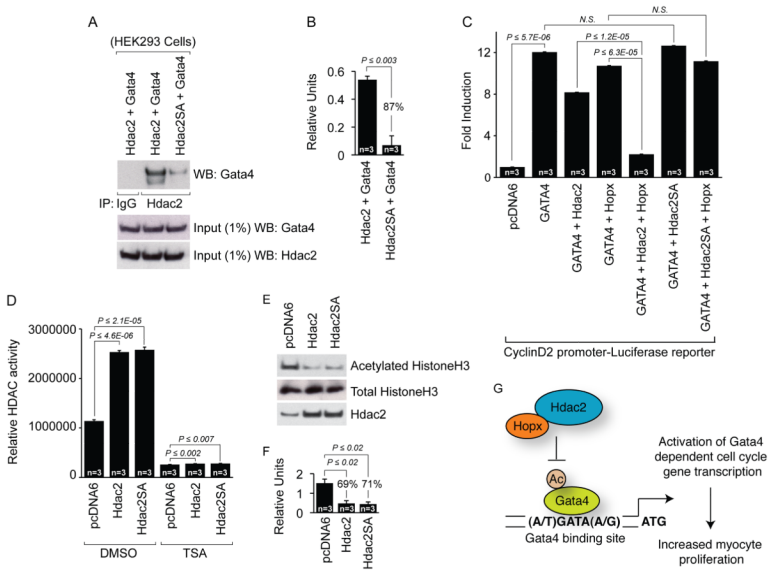

Figure 5. Phosphorylated Hdac2 preferentially interacts with Gata4 and modulates transcriptional activity

(**A**) Total lysates from transfected 293T cells were immunoprecipitated with anti-Hdac2 antibody to immunoprecipitate Hdac2 and Western blot analysis was performed with anti-Gata4 antibody. Mutation of serine 394, 411, 422 and 424 of Hdac2 to alanine (Hdac2SA) reduced interaction with Gata4. (**B**) Quantification of the interaction between phospho-mutant Hdac2 and Gata4 using ImageJ software (mean ± SEM, n=3). (**C**) CyclinD2-luciferase reporter construct was transfected in H9c2 myoblast cells with or without Gata4, Hdac2, Hopx, and Hdac2SA expression constructs. Normalized luciferase activity is reported as fold-induction over mock transfected cells (mean ± SEM, n=3). (**D**) Hdac2SA retains deacetylase activity. Total lysates from transfected 293T cells with or without Hdac2 and Hdac2SA were assayed for HDAC activity against a pseudo-substrate (mean ± SEM, n=3). (**E**) Western blot analysis was performed on total lysates from transfected 293T cells with or without Hdac2 or Hdac2SA using an anti-acetylated histoneH3 antibody. Hdac2 expression was determined by using an anti-Hdac2 antibody that recognized endogenous (lane 1) and exogenous Hdac2. Transfection of either Hdac2 or Hdac2SA resulted in diminished histoneH3 acetylation compared to mock transfected cells. (**F**) Acetylated histoneH3 was quantified and normalized to total histoneH3 in Hdac2 or Hdac2SA transfected cells using ImageJ software (mean ± SEM, n=3). (**G**) Model depicting Hdac2 interacting with Hopx to induce deacetylation of Gata4 and modulation of cell cycle genes.

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Table 1

Genotypes of 380 mice aged P2-P21. Parents of these mice were $Hdac2^{+/-}$ $Hopx^{+/-}$

Genotype	Actual #	Actual %	Expected #	Expected %
$Hdac2^{+/+}$ $Hopx^{+/+}$	22	5.8	20.25	6.3
$Hdac2^{-/-}Hopx^{-/-}$	0	0.0	20.25	6.3
$Hdac2^{+/+}Hopx^{-/-}$	33	8.7	20.25	6.3
$Hdac2^{-/-}Hopx^{+/+}$	8	2.1	20.25	6.3
$Hdac2^{+/+}Hopx^{+/-}$	71	18.7	40.5	12.5
$Hdac2^{-/-}Hopx^{+/-}$	10	2.6	40.5	12.5
$Hdac2^{+/-}Hopx^{+/+}$	49	12.9	40.5	12.5
$Hdac2^{+/-}Hopx^{-/-}$	73	19.2	40.5	12.5
$Hdac2^{+/-}Hopx^{+/-}$	114	30.0	81	25.0