Histone deacetylase 3 modulates Tbx5 activity to regulate early cardiogenesis

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Congenital heart defects often result from improper differentiation of cardiac progenitor cells. Although transcription factors involved in cardiac progenitor cell differentiation have been described, the associated chromatin modifiers in this process remain largely unknown. Here we show that mouse embryos lacking the chromatin-modifying enzyme histone deacetylase 3 (Hdac3) in cardiac progenitor cells exhibit precocious cardiomyocyte differentiation, severe cardiac developmental defects, upregulation of Tbx5 target genes and embryonic lethality. Hdac3 physically interacts with Tbx5 and modulates its acetylation to repress Tbx5-dependent activation of cardiomyocyte lineage-specific genes. These findings reveal that Hdac3 plays a critical role in cardiac progenitor cells to regulate early cardiogenesis.

INTRODUCTION

Multipotent cardiac progenitor cells are specified during the early stages of gastrulation from lateral plate mesoderm in the murine embryo (1). Around embryonic day 7.0 (E7.0), these cells migrate to form the cardiac crescent, which contains two populations of cardiac progenitors, the first and second heart fields (2). The cells of the cardiac crescent migrate medially to form a single linear heart tube, which subsequently gives rise to a four-chambered heart (3). During this process, multipotent cardiac progenitor cells differentiate into various endpoint lineages including cardiomyocytes, smooth muscle cells, endothelial cells and specialized conduction cells (4–6). For instance, Nkx2-5-positive bipotent cardiac progenitor cells give rise to cardiomyocytes and smooth muscle cells (7).

Despite recent progress in identifying cardiac progenitor cells, the epigenetic and chromatin modifiers regulating progenitor cell fate specification are poorly defined (8). Site-specific histone modifications, like acetylation and methylation, regulate chromatin structure and provide a signal to recruit lineage-defining transcription factors (9). For instance, histone acetyl-transferase (HAT)-mediated acetylation of core histones leads to relaxation of the chromatin structure and subsequent recruitment of transcription factors for gene activation. Conversely, histone deacetylase (Hdac)-dependent deacetylation leads to chromatin condensation and gene repression (10).

The mammalian Hdacs are classified into four sub-families based on their conserved sequences and structure (11). Class I Hdacs (Hdac1, 2, 3 and 8) are ubiquitously expressed and play critical roles during development (10). For example, we demonstrated that global loss of Hdac2 in mice causes severe cardiac developmental defects including cardiomyocyte hyperplasia (12). Global deletion of histone deacetylase 3 (Hdac3) results in embryonic lethality around E9.5 (13,14). In a tissue-specific context, Hdac3 regulates lipid metabolism and mitochondrial functions in the adult heart (14,15).

Hdacs lack 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 (11). For instance, our recent findings show that Hdac2 interacts with Gata4 and inhibits its acetylation and transcriptional activity to regulate embryonic cardiomyocyte proliferation (16). Several evolutionarily

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conserved transcription factors from the T-box, GATA, b-HLH, MADS box and homeodomain families are expressed in cardiac progenitor cells and regulate various stages of cardiogenesis (17). With regards to the T-box family, Tbx5 gain or loss-of-function mutations can result in Holt–Oram syndrome, characterized by the presence of atrial and ventricular septal defects (18,19). Recent reports demonstrate that gain-of-Tbx5 function in progenitor cells induces precocious differentiation into spontaneously beating cardiomyocytes, suggesting a lineage-defining role for Tbx5 during early cardiogenesis (20,21). However, how Tbx5 activity is regulated during cardiomyocyte lineage specification remains largely unknown.

Here we show that cardiac progenitor cell-specific loss of Hdac3 in mice leads to complete embryonic lethality, precocious cardiomyocyte differentiation and severe cardiac developmental defects. Hdac3 regulates Tbx5 acetylation and activation of Tbx5-dependent cardiomyocyte lineage-specific genes. Our results suggest a novel cardiac progenitor cell-specific function of ubiquitously expressed Hdac3 during early developmental stages of cardiogenesis.

RESULTS

Loss of Hdac3 in cardiac progenitor cells results in embryonic lethality and severe cardiac developmental defects

Hdac3 is ubiquitously expressed in the developing heart (Fig. 1A). Germline deletion of Hdac3 results in embryonic lethality at E9.5 (13,14). To determine the function of Hdac3 during early cardiogenesis, we used Nkx2.5-driven Cre recombinase to delete Hdac3 in cardiac progenitor cells ($Hdac3^{Nkx2-5KO}$). $Hdac3^{Nkx2-5KO}$ mice were not identified at birth (P0), indicating complete embryonic lethality (Table 1). $Hdac3^{Nkx2-5KO}$ embryos displayed significant lethality as early as E11.5 (Supplementary Material, Tables S1 and S2). However, some $Hdac3^{Nkx2-5KO}$ embryos were identified until mid-gestation (Fig. 1B, Supplementary Material, Table S2). $Hdac3^{Nkx2-5KO}$ embryos were characterized by cardiac defects such as hypoplastic ventricular walls and membranous ventricular septal defects (Fig. 1B and C). Interestingly, genetic deletion of $Hdac3$ using αMHC-Cre, which is expressed in differentiated cardiomyocytes at E9.5 (22), did not reveal any embryonic lethality or
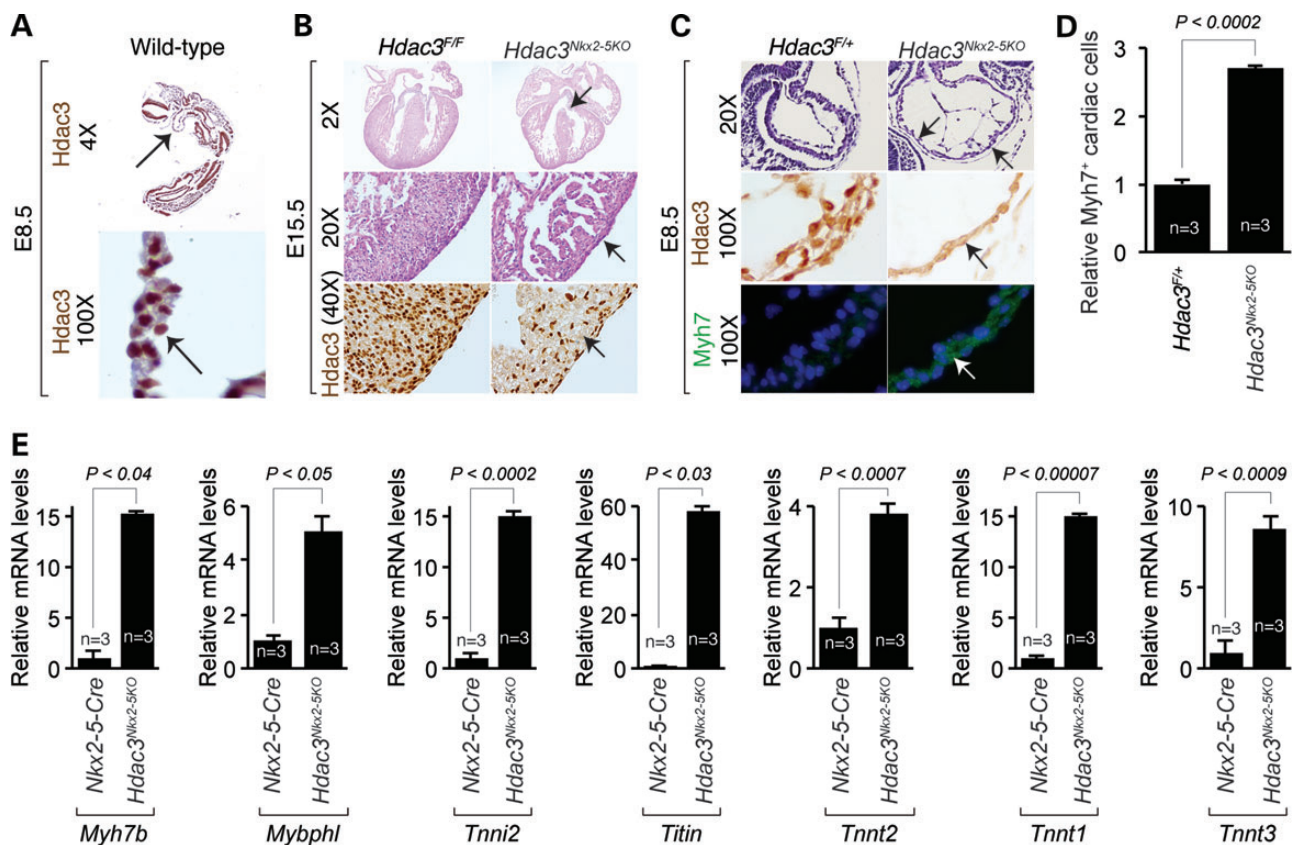

Figure 1. Developmental myocardial defects owing to loss of Hdac3 in Nkx2-5[+] cardiac progenitor cells. (**A**) Hdac3 expression was determined in E8.5 murine embryo (top) and heart (bottom) by immunohistochemistry. Arrows indicate expression of ubiquitous Hdac3. (**B**) Hematoxylin- and eosin-stained sections demonstrate membranous ventricular septal defect (top, arrow) and hypoplastic ventricular myocardium (middle, arrow) in $Hdac3^{Nkx2-5KO}$ E15.5 hearts. Immunohistochemistry staining of Hdac3 shows loss of Hdac3 in cardiac cells derived from Nkx2-5^{+} progenitor cells (bottom, arrow). (**C**) Hematoxylin- and eosin-stained sections demonstrate abnormally hypoplastic ventricular myocardium in $Hdac3^{Nkx2-5KO}$ E8.5 hearts (top, arrow). Hdac3 immunohistochemistry shows loss of expression in $Hdac3^{Nkx2-5KO}$ E8.5 hearts (middle, arrow) compared with control. Precocious cardiomyocyte differentiation was assessed by Myh7 immunofluorescent staining (green, bottom, arrow) in $Hdac3^{F/+}$ and $Hdac3^{Nkx2-5KO}$ E8.5 hearts. (**D**) Quantification of Myh7^{+} cells in $Hdac3^{F/+}$ and $Hdac3^{Nkx2-5KO}$ E8.5 hearts (mean ± SEM, $n = 3$). (**E**) Transcripts for $Myh7$, $Mybphl$, $Tnni2$, $Titin$, $Tnnt2$, $Tnnt1$ and $Tnnt3$ were detected by real-time qPCR in $Nkx2$-5-Cre and $Hdac3^{Nkx2-5KO}$ hearts derived from E8.5 embryos (mean ± SEM, $n = 3$).

Table 1. Genotypes of 72 mice aged P0

Genotype	Observed	Expected
Hdac3$^{+/+}$	9	9
Hdac3$^{F/+}$	24	18
Hdac3$^{F/F}$	10	9
Nkx2-5-Cre; Hdac3$^{+/+}$	11	9
Nkx2-5-Cre; Hdac3$^{F/+}$	18	18
Nkx2-5-Cre; Hdac3$^{F/F}$	0	9

Nkx2-5-Cre; Hdac3$^{F/+}$ mice were crossed with Hdac3$^{F/+}$ mice ($P < 0.04$).

developmental cardiac defects (Supplementary Material, Fig. S1, and Table S3) (14). Together, these data suggest a primary role of Hdac3 in cardiac progenitor cells during early cardiogenesis.

Cardiomyocyte marker immunostaining revealed a significant increase in precociously differentiated cardiomyocytes (Fig. 1C and D). Consistent with these findings, we observed robust precocious expression of cardiomyocyte lineage-specific genes, including *Myh7*, *Tnni2*, *Tnnt1* and *Tnnt2*, in E8.5 *Hdac3$^{Nkx2-5KO}$* hearts compared with control (Fig. 1E). We did not observe proliferation or apoptosis defects in differentiated *Hdac3$^{Nkx2-5KO}$* cardiomyocytes at E8.5 (Supplementary Material, Figs. S2 and S3). These results suggest that Hdac3 represses differentiation of cardiac progenitor cells and expression of cardiomyocyte lineage-specific genes during early cardiogenesis.

Hdac3 represses Tbx5-dependent transactivation during early cardiogenesis

We next explored the mechanism of cardiomyocyte lineage-specific gene regulation by Hdac3 during early cardiogenesis. We identified chromatin occupancy of Hdac3 in the conserved noncoding regions within 10 kb upstream of *Myh7*, *Tnni2*, *Tnnt1* and *Tnnt2*, using an Hdac3 ChIP-seq dataset (Supplementary Material, Fig. S4, unpublished). ChIP–qPCR analysis confirmed 13 sites occupied by Hdac3 in E8.5 wild-type hearts (Supplementary Material, Fig. S4). Comparison with a recent ChIP-seq dataset of core cardiac transcription factors revealed significant overlap ($>61\%$) between Tbx5-enriched regions and Hdac3 occupied sites (23). ChIP–qPCR analysis showed that 11 sites are occupied by both Hdac3 and Tbx5 (Fig. 2A, Supplementary Material, Fig. S4). To determine whether Tbx5 recruits Hdac3 to chromatin in the developing heart, we expressed Tbx5-shRNA in E8.5 cultured cardiac cells. Hdac3 ChIP–qPCR analysis revealed a significant decrease in Hdac3 enrichment in Tbx5-shRNA expressing compared with control cardiac cells at all overlapping regions (Fig. 2B). Tbx5 is known to activate the expression of cardiomyocyte-specific genes (20,21,24). Hence, we examined the requirement of Tbx5 for aberrant expression of cardiomyocyte-specific genes in *Hdac3$^{Nkx2-5KO}$* hearts. Loss of Hdac3 resulted in significant activation of *Myh7*, *Tnni2* and *Tnnt2*, and this activation was largely abolished by Tbx5 knockdown (Fig. 2C–E).

To determine the effect of Hdac3 gain-of-function on Tbx5-dependent transactivation, we generated a *Tbx5*–luciferase reporter construct containing five consensus Tbx5-binding

sites. Transfection of Tbx5 resulted in an approximate 15-fold activation of the *Tbx5*–luciferase reporter, and this activation was significantly inhibited by co-transfection of Hdac3, but not by Hdac1 (Fig. 3A, Supplementary Material, Fig. S5A). Likewise, Hdac3 gain-of-function repressed Tbx5-mediated activation of *Myh7*, *Tnni2* and *Tnnt2* in developing cardiac cells (Fig. 3B–D). Importantly, Tbx5 mRNA and protein levels were unaltered in *Hdac3$^{Nkx2-5KO}$* hearts (Fig. 3E and F).

Hdac3 and Tbx5 physically interact

Immunoprecipitation of endogenous Hdac3 protein from E8.5 heart lysates, followed by immunoblotting for Tbx5, indicates that Hdac3 and Tbx5 proteins interact *in vivo* (Fig. 4A). Hdac3 and Tbx5 also interact in transfected HEK-293 T cells (Fig. 4B). Deletion analysis indicates that the interaction between Hdac3 and Tbx5 requires the partial T-box domain of Tbx5 and is specific (Supplementary Material, Fig. S6). Approximately 40 different genetic mutations of Tbx5 have been identified in human patients with Holt–Oram syndrome (25,26). Interestingly, the human TBX5^{G125R} gain-of-function mutation (27) coincides with the T-box domain required for interaction with Hdac3 (Supplementary Material, Fig. S6). We found that the TBX5^{G125R} mutation affects its interaction with Hdac3 (Fig. 4B, Supplementary Material, Fig. S7). This finding had functional implications, as Hdac3 failed to repress TBX5^{G125R}-mediated activation of the *Tbx5*–luciferase reporter construct and transcription of *Myh7*, *Tnni2* and *Tnnt2* in developing cardiac cells (Fig. 4C and D). These results suggest that Hdac3 interacts with Tbx5 to repress its transcriptional activity during early cardiogenesis.

Hdac3 and EP300 regulate Tbx5 acetylation

Post-translational modifications such as acetylation and deacetylation regulate the activity of cardiac transcription factors (16,28). We examined the ability of various Tbx5-associated HATs to modify Tbx5-dependent activation of the *Tbx5*–luciferase reporter construct (29,30). TBX5 activity was significantly augmented by co-transfection of EP300 but not by KAT5 or KAT2B (Fig. 5A, Supplementary Material, Fig. S8). Transfection experiments in 293 T cells followed by TBX5 immunoprecipitation and acetyl lysine immunoblotting revealed that acetylated TBX5 levels are markedly enhanced by co-transfection of EP300 (Fig. 5B, Supplementary Material, Fig. S9). Transfection of mutant TBX5^{G125R} alone results in a significant increase in the acetylated signal compared with wild-type TBX5 (Fig. 5B, Supplementary Material, Fig. S9). However, co-transfection of mutant TBX5^{G125R} with EP300 does not augment the acetylation signal (Fig. 5B, Supplementary Material, Fig. S9). An *in vitro* acetylation assay confirmed that EP300 acetylates TBX5 (Fig. 5C). Immunoprecipitation experiment revealed that Tbx5 is significantly acetylated in E9.5 wild-type hearts (Fig. 5D). Co-transfection with HDAC3 significantly diminished acetylated TBX5 but not acetylated TBX5^{G125R} (Fig. 5E, Supplementary Material, Fig. S10).

We identified Lys157 and Lys159 as conserved acetylation sites of TBX5 using three independent acetylation site prediction software programs (Supplementary Material, Fig. S11A and B). Replacement of lysines with alanine to mimic deacetylation
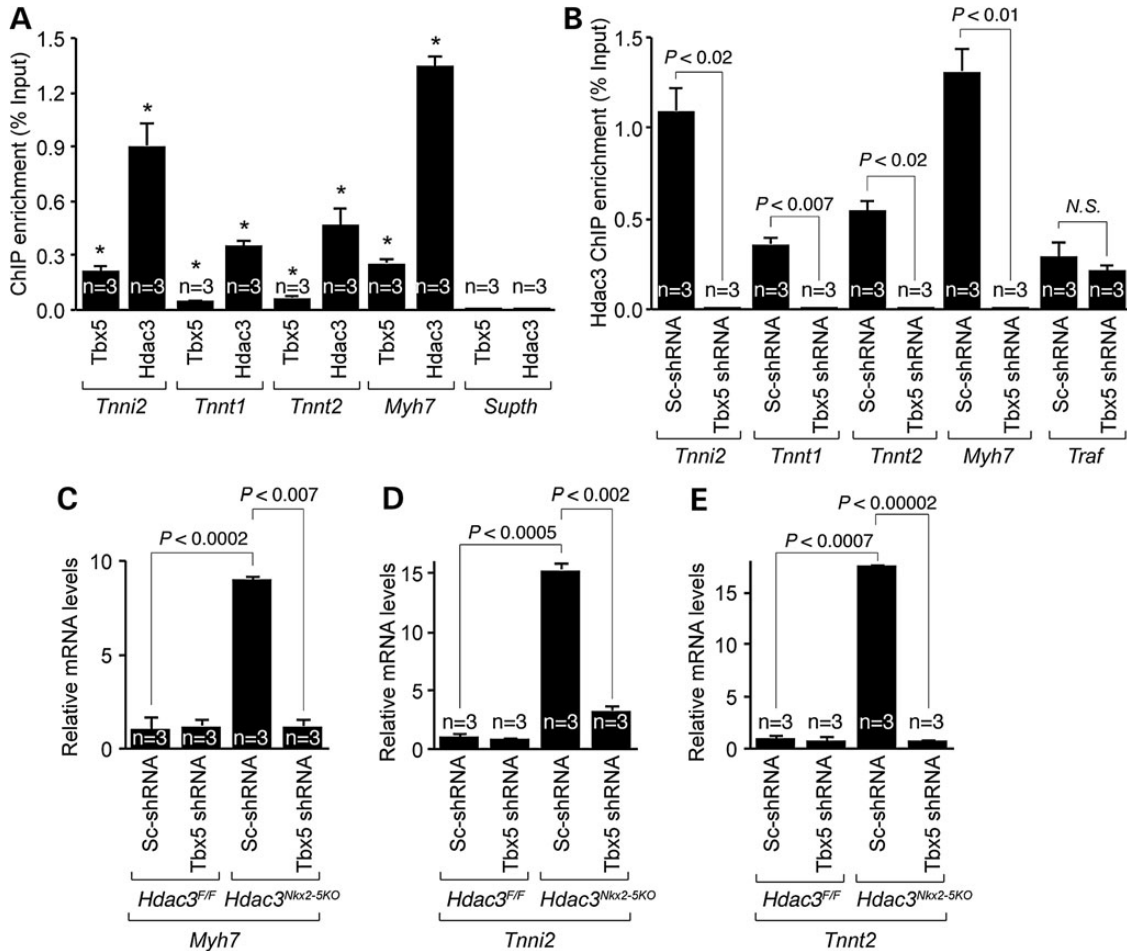
Figure 2. (**A**) Hdac3 localizes to a subset of Tbx5-bound cardiomyocyte-specific enhancers. ChIP-qPCR analysis of Hdac3 and Tbx5 recruitment to promoter-proximal regions of dysregulated cardiomyocyte-specific genes performed in wild-type E8.5 hearts (mean ± SEM, $n = 3$). *Supth* promoter-proximal region served as a control. *$P < 0.05$ by Student's two-tailed t test. (**B**) Tbx5 recruits Hdac3 to enhancer regions of dysregulated cardiomyocyte-specific genes. ChIP–qPCR analysis of Hdac3 recruitment to Tbx5-bound sites interrogated in scramble (Sc) shRNA or Tbx5-shRNA-expressing cultured cardiac cells derived from wild-type E8.5 hearts (mean ± SEM, $n = 3$). *Traf* promoter-proximal region served as a control. N.S., not significant. (**C–E**) Aberrant expression of cardiomyocyte-specific genes in Hdac3-null hearts requires Tbx5 transcriptional activity. Transcripts for *Myh7* (C), *Tnni2* (D) and *Tnnt2* (E) were detected by real-time qPCR in *Hdac3$^{F/F}$* and *Hdac3$^{Nkx2-5KO}$* cultured cardiac cells, derived from E8.5 embryos, expressing scramble (Sc) shRNA or Tbx5-shRNA (mean ± SEM, $n = 3$).

resulted in a mutant form of TBX5, TBX5$^{K157A-K159A}$, that could only weakly transactivate the *Tbx5*–luciferase reporter (Supplementary Material, Fig. S11C). EP300 failed to enhance TBX5$^{K157A-K159A}$ activity (Supplementary Material, Fig. S11C). Furthermore, co-transfection of TBX5$^{K157A-K159A}$ with EP300 showed significantly reduced acetylation signal compared with wild-type TBX5 (Fig. 5F, Supplementary Material, Fig. S12). We sought to determine whether loss of hyper-acetylation of human mutant TBX5^{G125R} modulates its activity. We generated a mutant form of TBX5, TBX5$^{G125R-K157A-K159A}$, in which lysine residues 157 and 159 are mutated to alanine. This mutant form of TBX5 could not be robustly acetylated by EP300 and failed to transactivate the *Tbx5*–luciferase reporter (Fig. 5F and G, Supplementary Material, Fig. S12). Taken together, these results suggest that Hdac3 functions to deacetylate Tbx5 and thus regulate transcriptional activity and differentiation of cardiac progenitor cells during early cardiogenesis (Supplementary Material, Fig. S13).

DISCUSSION

Recent studies identified a population of multipotent cardiac progenitor cells that progressively become lineage restricted and differentiate into various cardiac cell types in a developmental stage-specific manner (31). However, epigenetic and chromatin modifiers regulating fate specification of cardiac progenitor cells remain elusive. Our present work suggests that ubiquitously expressed Hdac3 plays a critical role in cardiac progenitor cells during early stages of cardiogenesis. Mice lacking Hdac3 in cardiac progenitor cells exhibit complete embryonic lethality and precocious differentiation into the cardiomyocyte lineage. The resulting hearts show hypoplastic ventricular walls and membranous ventricular septal defects. Cardiomyocyte lineage-specific genes are upregulated. Our data suggest that this is due, at least in part, to enhanced Tbx5 transcriptional activity, which in turn is due to Tbx5 hyper-acetylation.

We observed that Hdac3 binds at a subset of Tbx5-bound sites within regulatory regions of cardiomyocyte lineage-specific
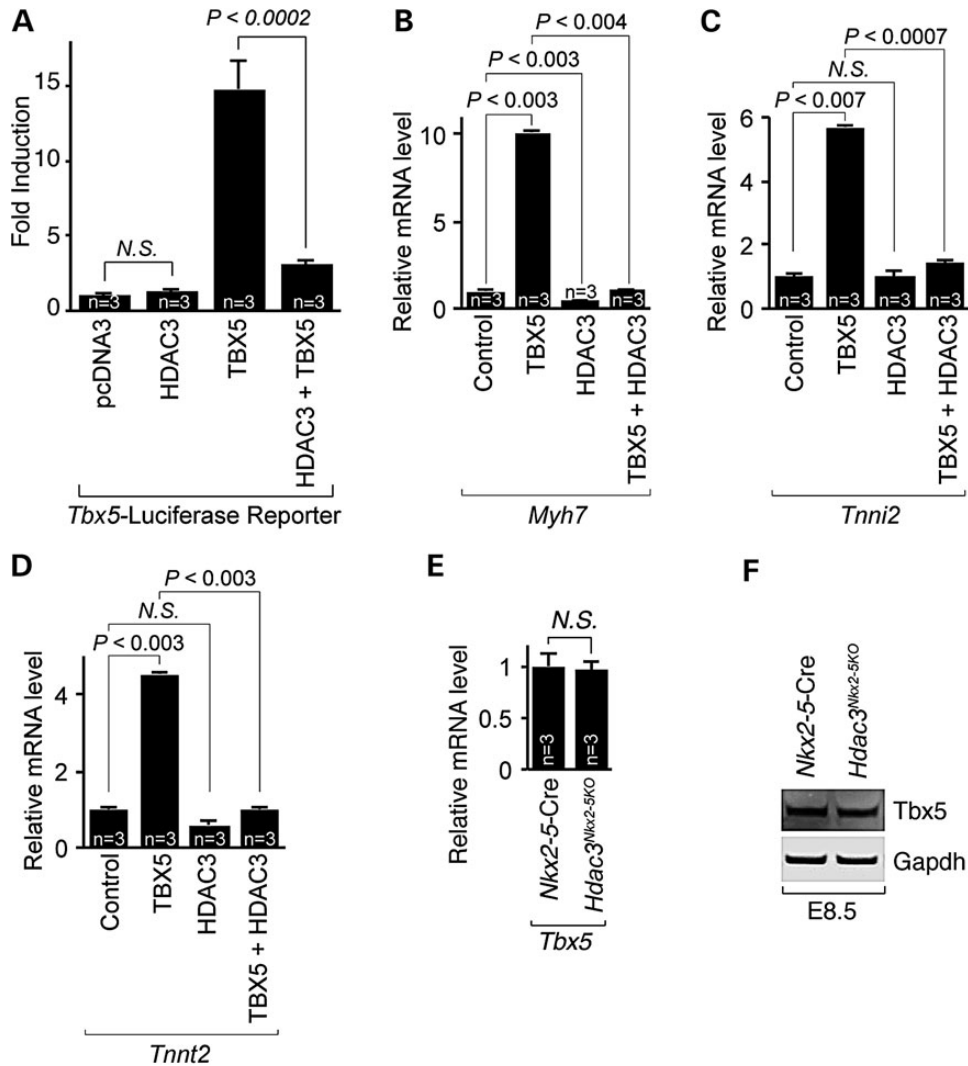
Figure 3. Hdac3 represses Tbx5-dependent transcriptional activity during early cardiogenesis. (**A**) *Tbx5*–luciferase reporter construct was transfected in 293 T cells with or without TBX5 and HDAC3 expression constructs. The induction is represented as fold-induction over the normalized luciferase activity in the control-transfected cells (mean ± SEM, *n* = 3). (**B–D**) Transcripts for *Myh7* (B), *Tnni2* (C) and *Tnnt2* (D) were detected by real-time qPCR in TBX5 and/or HDAC3 cDNA-expressing cultured cardiac cells derived from E8.5 embryos (mean ± SEM, *n* = 3). N.S., not significant. (**E**) *Tbx5* transcripts were detected by real-time qPCR from E8.5 *Nkx2-5*-Cre and *Hdac3^Nkx2-5KO^* hearts (mean ± SEM, *n* = 3). (**F**) Western blot analysis was performed on total lysates from E8.5 *Nkx2-5*-Cre and *Hdac3^Nkx2-5KO^* hearts. Gapdh is shown as a loading control.

genes. Moreover, Tbx5 is able to recruit Hdac3 to chromatin. The available data suggest that several transcription factors synergistically interact with Tbx5 to promote early cardiogenesis (32). For example, Tbx5 associates with Nkx2-5 to promote cardiomyocyte differentiation (33). In contrast, our findings support a model in which Hdac3 interacts with Tbx5 to block the Tbx5-dependent activation of cardiomyocyte lineage-specific genes in cardiac progenitor cells. Consistent with our findings, recent work demonstrates that Tbx5 gain-of-function is sufficient to induce cardiomyocyte lineage specification from cardiac mesoderm (20). Conversely, loss of Tbx5 function results in early embryonic lethality owing to impaired cardiac differentiation (19). Of note, Hdac3 gain-of-function failed to inhibit Nkx2-5-dependent transactivation, suggesting that the functional relationship between Hdac3 and Tbx5 is specific (Supplementary Material, Fig. S5B).

TBX5, the causative gene in Holt–Oram syndrome, was the first identified single-gene mutation giving rise to congenital heart defects (CHDs) (18,34). Several pathogenic mutations of *TBX5*, located within the highly conserved T-box domain, have been reported in patients with or without Holt–Oram syndrome (26). Further studies demonstrated that several of these mutations cause, at least in part, defective interactions between TBX5 and NKX2-5 or GATA4 to affect cardiac gene expression and lead to CHDs (19,35). Our studies revealed that the human TBX5^G125R^ mutation, located within the T-box domain, disrupts interaction between TBX5 and HDAC3. Furthermore, HDAC3 fails to repress the transcriptional activity of TBX5^G125R^, resulting in activation of cardiomyocyte lineage-specific genes in cardiac progenitor cells. Consistent with our findings, TBX5^G125R^ mutation results in gain-of-function in human patients with Holt–Oram syndrome (27).
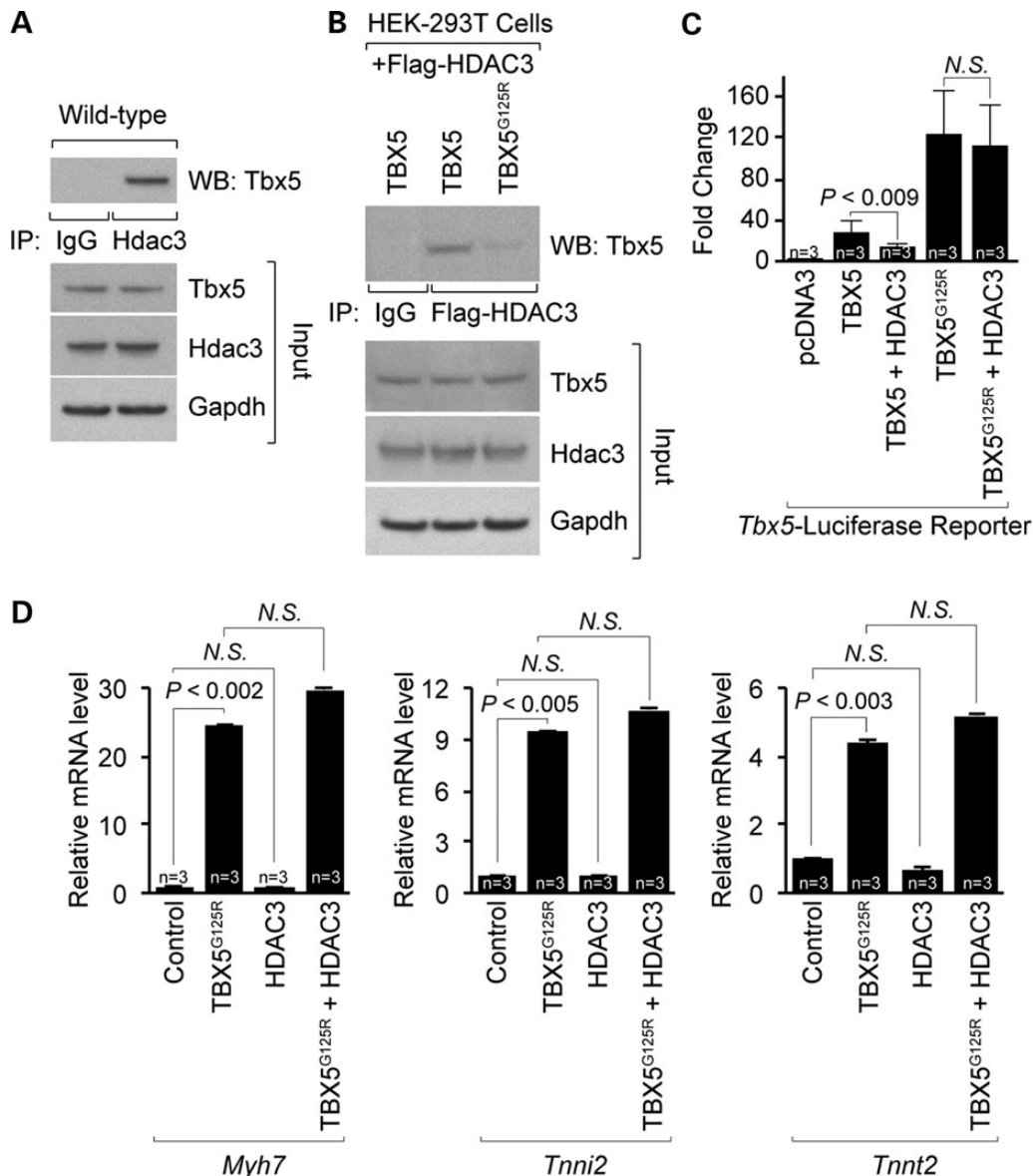
Figure 4. Hdac3 interacts with Tbx5. (**A**) Total lysates from E8.5 wild-type hearts were immunoprecipitated by Hdac3 antibody, and western blot was performed using Tbx5 antibody. Tbx5, Hdac3 and Gapdh are shown as an input control. (**B**) Total lysates from Flag-HDAC3 and TBX5 or TBX5^{G125R} cDNA-expressing 293 T cells were immunoprecipitated by Flag antibody to immunoprecipitate HDAC3, and western blot was performed with Tbx5 antibody to detect TBX5. (**C**) *Tbx5*–luciferase reporter construct was transfected in 293 T cells with or without TBX5, TBX5^{G125R} and HDAC3 expression constructs. The induction is represented as fold-induction over the normalized luciferase activity in the control-transfected cells (mean $\pm$ SEM, $n = 3$). (**D**) Transcripts for *Myh7*, *Tnni*2 and *Tnnt2* were detected by real-time qPCR in TBX5^{G125R} and/or HDAC3 cDNA-expressing cultured cardiac cells derived from E8.5 embryos (mean $\pm$ SEM, $n = 3$). N.S.*,* not significant.

Recent reports demonstrate that acetylation of cardiac transcription factors modulate their activity (28). Acetylation of Tbx5 has not been reported; however, there are reports suggesting cooperation between Tbx5 and histone acetyl-transferases (29,30). We show that EP300 directly acetylates TBX5 to enhance its transcriptional activity. Further, we identified conserved acetylation sites of TBX5, Lys157 and Lys159, which are important for EP300-mediated acetylation and transcriptional activation. Although Lys157 and Lys159 are conserved among various TBX genes, functional acetylation targets could be different within TBX family. Indeed, we observed that Lys234, conserved among TBX family, was not required

for TBX5 acetylation or activity (not shown). Future mass spectrometry analysis will be needed to identify all the acetylated lysine residues within TBX5.

HDACs regulate gene expression by deacetylating histone and non-histone proteins (10). Our data suggest that Tbx5 is a novel non-histone catalytic target of Hdac3 in the embryonic heart. Overall, our findings support a model in which Hdac3 deacetylates Tbx5 and represses Tbx5-dependent transcriptional activity to maintain the multipotent state of cardiac progenitor cells. Loss of Hdac3 removes this brake to precociously activate cardiomyocyte lineage-specific genes in progenitor cells, likely explaining precocious differentiation of cardiomyocytes in
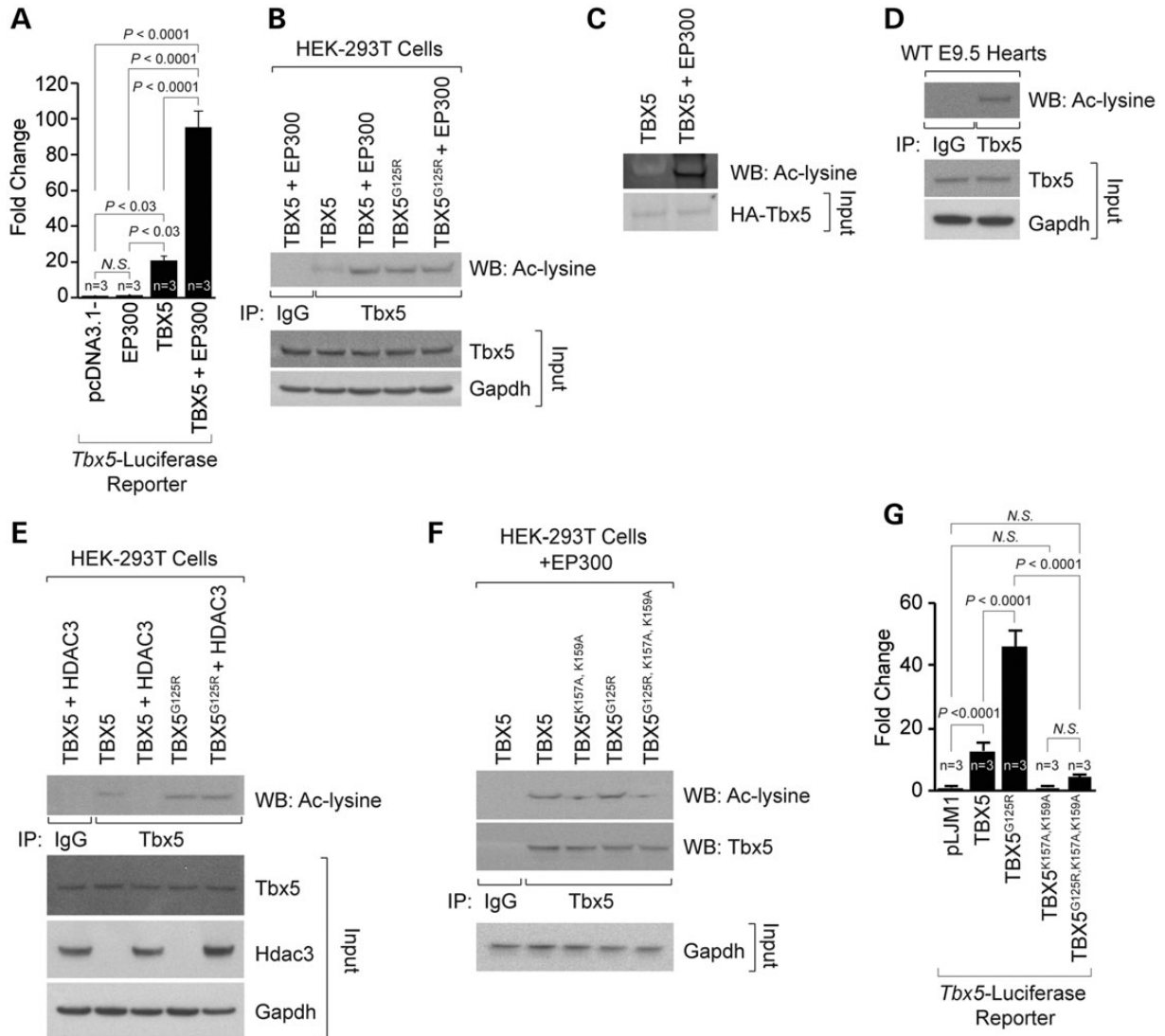
Figure 5. Hdac3 regulates Tbx5 acetylation. (**A**) EP300 induces TBX5-dependent transactivation. *Tbx5*–luciferase reporter construct was transfected in 293 T cells with or without TBX5 and EP300 expression constructs. Induction is represented as fold-induction over the normalized luciferase activity in the control-transfected cells (mean ± SEM, $n = 3$). (**B**) EP300 acetylates TBX5 *in vivo*. Total lysates from TBX5, TBX5^{G125R} and/or EP300 cDNA-expressing 293 T cells were immuno-precipitated by TBX5 antibody. Acetylated TBX5 was detected by western blot analysis using acetyl lysine antibody. (**C**) EP300 acetylates TBX5 *in vitro*. Purified GST/HA-tagged TBX5 was subjected to *in vitro* acetylation using purified EP300. Western blot analysis was performed to determine acetylated TBX5 using acetyl lysine antibody. HA-TBX5 is shown as a loading control. (**D**) Tbx5 is acetylated in developing myocardium. Total lysates from E9.5 wild-type hearts were immunoprecipitated by Tbx5 antibody, and western blot was performed using acetyl lysine antibody to detect acetylated Tbx5. Western blot for Tbx5 and Gapdh is shown as an input control. (**E**) HDAC3 deacetylates TBX5. Total lysates from TBX5, TBX5^{G125R} and/or HDAC3 cDNA-expressing 293 T cells were immunoprecipitated by TBX5 antibody, and acetylated TBX5 was detected by western blot analysis using acetyl lysine antibody. (**F**) EP300 acetylates TBX5 at Lys157 and Lys159. Total lysates from TBX5, TBX5^{G125R}, TBX5$^{K157A, K159A}$, TBX5$^{G125R, K157A, K159A}$ and/or EP300 cDNA-expressing 293 T cells were immunoprecipitated by TBX5 antibody, and acetylated TBX5 was detected by western blot analysis using acetyl lysine antibody. (**G**) Lys157 and Lys159 acetylation modulate TBX5-dependent transactivation. *Tbx5*–luciferase reporter construct was transfected in 293 T cells with or without TBX5, TBX5^{G125R}, TBX5$^{K157A, K159A}$ and TBX5$^{G125R, K157A, K159A}$ expression constructs. The induction is represented as fold-induction over the normalized luciferase activity in the control-transfected cells (mean ± SEM, $n = 3$).

Hdac3$^{Nkx2-5KO}$ embryos. This is consistent with the model where transient binding of HDACs maintains a low level of acetylation and prevent activation of primed genes in pluripotent cells (36). However, alternate functions of Hdac3, related to proliferation, histone deacetylation or chromatin remodeling, remain as plausible causes of precocious differentiation observed in *Hdac3$^{Nkx2-5KO}$* heart. Indeed, transient proliferation arrest and reduced population of undifferentiated cardiac cells could

explain, in part, precocious differentiation and hypoplastic ventricular walls in E8.5 *Hdac3$^{Nkx2-5KO}$* hearts (Supplementary Material, Fig. S2). Recent studies have suggested that functions unrelated to catalytic activity of Hdac3 may also exist and may require nuclear compressor corepressor NCoR (37,38). Thus, it will be critical in future experiments to determine whether the catalytic activity of Hdac3 and NCoR is required for its normal function during early cardiogenesis.

MATERIAL AND METHODS

Mice

Transgenic *Nkx2-5* Cre and *Hdac3*[Flox] mice have been previously described ([39,40]). *Myh6*-Cre (αMHC-Cre) mice were obtained from The Jackson Laboratory. The University of Massachusetts Medical School Institutional Animal Care and Use Committee approved all animal protocols.

Cell culture, transient transfection and luciferase assays

HEK-293 T cells were maintained in DMEM with 10% FBS, 100 μg/ml penicillin and 100 μM/ml streptomycin in a 37°C incubator with 5% CO_2. Hearts from E8.5 mouse embryos were collected in DMEM with 10% FBS and plated on gelatin-coated dishes. Subconfluent HEK-293 T cells were transfected in 100-mm plates with 12.5 μg of DNA and 25 μl of polyethylenamine, linear, in 10 ml of 10% FBS medium. Luciferase assays were conducted by transfecting subconfluent HEK-293 T cells in 6-well plates with 1 μg of DNA and 2 μl of polyethylenamine, linear, in 2 ml of 10% FBS media. DNA amount was maintained constant using pcDNA3.1(−) or pLJM1-EGFP DNA. Cells were lysed with passive lysis buffer 16 h after transfection, and lysates were analyzed using a dual luciferase reporter assay kit according to the manufacturer's guidelines. Luciferase activity was measured using an Omega microplate reader according to manufacturer's guidelines.

GST protein purification

Cultures of transformant *Escherichia coli* were grown to an optical density at 600 nm (OD_{600}) between 0.6 and 0.8. Transformants were induced upon reaching appropriate OD_{600} with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 2 h at 37°C. Fusion proteins were bound to GST bead slurry and eluted by Thrombin cleavage for 16 h at 22°C with 10 units of Thrombin enzyme.

In vitro acetylation assay

TBX5 protein, purified from *E. coli*, was incubated with 600 ng P300-HAT domain, 1 mM Acetyl-CoA, 50 μM TSA and 50 mM Nicotinamide in HAT Buffer (50 nM Tris−HCl, pH8, 0.1 mM DTT, 10% glycerol) for 1 h at 30°C. Acetylated protein was resolved by SDS−PAGE and analyzed by western blot.

Statistical analysis

Statistical significance between groups was assessed using two-tailed Student's *t* test or χ^2 test. A *P*-value of <0.05 was considered significant.

An expanded methods section is available in Supplementary Material.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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