Histone Deacetylase 3 Coordinates Deacetylase-independent Epigenetic Silencing of Transforming Growth Factor-β1 (TGF-β1) to Orchestrate Second Heart Field Development*

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Background: Histone-modifying genes play critical roles in the pathogenesis of human congenital heart disease.

Results: HDAC3 recruits PRC2 complex to mediate epigenetic silencing of TGF-β1 in a deacetylase-independent manner within second heart field progenitor cells.

Conclusion: HDAC3-mediated epigenetic silencing of TGF-β1 is required for normal heart development.

Significance: This is the first report of HDAC-mediated epigenetic regulation of second heart field development.

About two-thirds of human congenital heart disease involves second heart field-derived structures. Histone-modifying enzymes, histone deacetylases (HDACs), regulate the epigome; however, their functions within the second heart field remain elusive. Here we demonstrate that histone deacetylase 3 (HDAC3) orchestrates epigenetic silencing of Tgf-β1, a causative factor in congenital heart disease pathogenesis, in a deacetylase-independent manner to regulate development of second heart field-derived structures. In murine embryos lacking HDAC3 in the second heart field, increased TGF-β1 bioavailability is associated with ascending aortic dilatation, outflow tract malrotation, overriding aorta, double outlet right ventricle, aberrant semilunar valve development, bicuspid aortic valve, ventricular septal defects, and embryonic lethality. Activation of TGF-β signaling causes aberrant endothelial-to-mesenchymal transition and altered extracellular matrix homeostasis in HDAC3-null outflow tracts and semilunar valves, and pharmacological inhibition of TGF-β rescues these deficits. HDAC3 recruits components of the PRC2 complex, methyltransferase EZH2, EED, and SUZ12, to the NCOR complex to enrich trimethylation of Lys-27 on histone H3 at the Tgf-β1 regulatory region and thereby maintains epigenetic silencing of Tgf-β1 specifically within the second heart field-derived mesenchyme. Wild-type HDAC3 or catalytically inactive HDAC3 expression rescues aberrant endothelial-to-mesenchymal transition and epigenetic silencing of Tgf-β1 in HDAC3-null outflow tracts and semilunar valves. These findings reveal that epigenetic dysregulation within the second heart field is a predisposing factor for congenital heart disease.

Congenital heart disease is the most common of all birth defects, the leading cause of infant mortality (1). Cardiac morphogenesis in the mouse begins at around embryonic day 7.0 (E7.0),2 when a subset of cells derived from the antero-lateral mesoderm forms the cardiac crescent (2). This crescent contains the first and second heart field cardiac progenitor cells (3). The first heart field progenitor cells are the principal contributor to the primary heart tube, the left ventricle, atriointerventricular canal, and atria (4). The second heart field progenitor cells extensively contribute to the outflow tract, semilunar valves, atria, right ventricle, primary atrial septum, and ventricular septum (5–9). Indeed, ablation or genetic manipulation of second heart field progenitor cells and their derivatives leads to outflow tract malrotation, overriding aorta, double outlet right ventricle, aberrant semilunar valve development, and ventricular septal defects (10–12). In humans, about two-thirds of congenital cardiac defects involve the outflow tract, semilunar valves, or ventricular septum (13).

Patterning of the second heart field requires several signaling pathways and their interaction with transcriptional regulators (14). Transforming growth factor-β (TGF-β) family members are among the pioneer signaling molecules that induce endothelial-to-mesenchymal transition (EndMT) to form semilunar valve cushions in the developing outflow tract (15–17). Subsequent elongation, expansion, and extracellular matrix remodeling transform these cushions into the mature semilunar valves (18–20). During these valvular remodeling processes, regulation of TGF-β bioavailability appears to be crucial. For instance, patients with semilunar valve pathologies, such as semilunar valve stenosis, regurgitation, and aberrant deposition of myofibroblasts and extracellular matrix, display elevated TGF-β levels (21–23). Indeed, exogenous TGF-β transforms cultured valvular interstitial cells into myofibroblasts and promotes excessive secretion of extracellular matrix (24–26). Elevated TGF-β level is also implicated in the pathogenesis of connective tissue disorders, such as Marfan syndrome, Ehlers-Danlos syndrome, and Loeys-Dietz syndrome (27–29). Interestingly, patients with these syndromes frequently display con-

2 The abbreviations used are: E, embryonic day n; P0, postnatal day 0; HDAC, histone deacetylase; EndMT, endothelial-to-mesenchymal transition; IPA, Ingenuity Pathway Analysis; qPCR, quantitative PCR; H3K27, histone H3 Lys-27; H3K27me3, H3K27 trimethylation; H3K27ac, H3K27 acetylation.
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genital cardiovascular anomalies, such as aortic dilatation, outflow tract defects, bicuspid aortic valve, semilunar valve stenosis, and ventricular septal defects (30–32). How the second heart field progenitor cells interpret elevated TGF-β at the chromatin level in these connective tissue disorders remains to be elucidated.

Extracellular matrix tightly regulates the bioavailability of active TGF-β (33). For instance, extracellular matrix proteoglycans, such as decorin (DCN), biglycan (BGN), and fibromodulin (FMOD), sequester TGF-β to limit its activity (34). Mice lacking DCN, BGN, or FMOD display elevated TGF-β activity and phenotypes observed in Marfan syndrome or Ehlers-Danlos syndrome (35–37). Indeed, proteoglycan deficiencies have been demonstrated in patients with these syndromes (38). Proteoglycans expressed in the developing outflow tract and semilunar valves also play a key role in the assembly of collagen fibers in the extracellular matrix (37, 39). Patients with collagen deficiency, such as COL3A1 mutations in Ehlers-Danlos syndrome, show elevated TGF-β levels (29). How extracellular matrix homeostasis is regulated remains an area of intense research.

The active form of TGF-β binds to TGFBR2 and TGFBR1, which in turn phosphorylate SMAD2/3 (R-SMADs) to promote assembly of heteromeric complex with SMAD4. Activated SMAD complex accumulates in the nucleus, where it recruits transcription co-factors and chromatin modifiers to regulate the expression of target genes (33). Several factors, in addition to TGF-β, play critical roles to trigger activation and amplification of the intracellular TGF-β signaling pathway at multiple levels (33). For instance, the transcription factor SNAI1 interacts with SMAD3/4 to repress endothelial gene expression and thereby augments TGF-β-mediated mesenchymal transition (40). EndMT, a specialized form of EMT, is the complex biological process in which endothelial cells trans-differentiate into mesenchymal cell types, including smooth muscle-like and fibroblast-like cells. EndMT has been implicated in several pathological processes, including fibrotic disorders and cardiac valvular diseases. A complex orchestration of several signaling pathways, including TGF-β signaling, initiates and promotes EndMT. However, the molecular and epigenetic mechanisms regulating termination of EndMT remain elusive.

Histone deacetylases (HDACs) are chromatin-modifying enzymes that regulate the epigenome (41). The mammalian HDACs are classified into five subfamilies based on their phylogenetic analysis and sequence homology. Class I HDACs (HDAC1, HDAC2, HDAC3, and HDAC8) play critical roles at various stages of development (42). For instance, global loss of HDAC1 or HDAC3 results in early embryonic lethality around E9.5 (42). Similarly, mice lacking HDAC2 or HDAC8 display lethality at birth (43–45). Our group and others, using gene inactivation studies in mice, have demonstrated vital functions for class I HDACs in cardiomyocyte proliferation, differentiation, and hypertrophy (43, 46–49). However, functions of HDACs in second heart field development remain undefined.

HDACs lack intrinsic DNA-binding domains but are recruited to the chromatin via their interaction with transcription factors, co-factors, and large multiprotein transcriptional complexes (41). For instance, HDAC3 is an integral part of NCOR (nuclear receptor corepressor) or its homologue SMRT (silencing mediator of retinoic and thyroid receptors). Interaction of HDAC3 with the deacetylase-activating domain of NCOR/SMRT is required for its enzyme activity. Interestingly, recent evidence suggests that enzymatic activity of HDAC3 is dispensable, but its interaction with NCOR/SMRT is essential for transcriptional repression. However, molecular mechanisms that mediate the deacetylase-independent function of HDAC3 remain to be defined. A recent study shows that HDAC4 controls histone methylation in response to elevated cardiac overload, suggesting that HDACs may recruit histone methyltransferases to the chromatin.

In this study, we demonstrate a deacetylase-independent role for HDAC3 as an epigenetic silencer of Tgf-β1 within the second heart field with direct implications for human TGF-β pathway-associated cardiovascular anomalies. As a part of the NCOR complex, HDAC3 recruits EZH2 (enhancer of zeste homolog 2), the major histone methyltransferase of PRC2 (polycomb repressor complex 2), to mediate epigenetic silencing of Tgf-β1 specifically within the second heart field-derived mesenchymal cells and thereby promotes termination of EndMT. Genetic deletion of Hdac3 in the murine second heart field results in increased TGF-β bioavailability within mesenchymal cells, perpetual activation of mesenchymal cells, aberrant EndMT, and altered extracellular matrix homeostasis, observed in patients with semilunar valve pathologies. Together, these results uncover that epigenetic silencing mediated by HDAC3 in a deacetylase-independent manner orchestrates second heart field development, which may be a molecular target in human cardiovascular anomalies.

Experimental Procedures

Mice—Transgenic Mef2c-AHF-Cre, Cdhr5-Cre, Hdac3flox, and knock-in Isl1-Cre mice were described previously (7, 50–52). Tagln-Cre (Sm22α-Cre), Mvh-Cre (αMhc-Cre), and R26R-LacZ mice were obtained from the Jackson Laboratories. The University of Massachusetts Medical School Institutional Animal Care and Use Committee approved all animal protocols.

Histology—Tissue samples were fixed in 2% paraformaldehyde at 4 °C overnight, ethanol-dehydrated, embedded in paraffin, and sectioned at 6–8-μm thickness using a microtome.

Antibodies and Reagents—The following antibodies were used in this study: HDAC3 (Abcam and Santa Cruz Biotechnology), phospho-HDAC3 (Ser-424) (Cell Signaling), TGF-β pan-specific polyclonal antibody (R&D Systems), SMAD2/3 (Santa Cruz Biotechnology), phospho-SMAD2/3 (Ser-423/425) (Santa Cruz Biotechnology), vimentin (Santa Cruz Biotechnology), phospho-SMAD2/3 (Ser-423/425) (Santa Cruz Biotechnology), vimentin (Santa Cruz Biotechnology), PECAM1 (BD Pharmingen), troponin T (Developmental Studies Hybridoma Bank, Iowa City, IA), MF-20 (Developmental Studies Hybridoma Bank, Iowa City, IA), cleaved caspase-3 (Cell Signaling), RNA polymerase II (Abcam), EZH2 (Abcam), NCOR1 (Abcam), H3K27ac (Abcam), H3K27me3 (Abcam), EED (Abcam), SUZ12 (Abcam), CREBBP (Abcam), IgG (R&D Systems), GAPDH (R&D Systems), FLAG (Sigma), α-tubulin (Sigma), IRDye-conjugated secondary antibodies (LI-COR), Alexa Fluor® 546-conjugated secondary antibody (Life Technologies), and biotinylated universal pan-specific antibody
(horse anti-mouse/rabbit/goat IgG) (Vector Laboratories). Recombinant TGF-β was purchased from R&D Systems. Alcian blue, alkaline alcohol, orcein, alcoholic hematoxylin, ferric chloride, Lugol’s iodine, woodstain scarlet acid fuchsin, phosphotungstic acid, safron, Bouin’s fixative, Weigert’s iron hematoxylin A, Weigert’s iron hematoxylin B, phosphomolybdic acid-phosphotungstic acid, aniline blue, and Van Gieson’s solution were purchased from Electron Microscopy Sciences. Harris modified hematoxylin, eosin Y, ethanol, xylenes, glacial acetic acid, paraformaldehyde, paraffin, potassium ferricyanide, potassium ferrocyanide, and deoxycholic acid were purchased from Fischer. Polyethyleneimine, linear, was purchased from Polysciences. X-gal was purchased from 5′Prime. Vectashield mounting medium, the Vectastain Elite ABC kit, and the DAB Peroxidase Substrate kit were purchased from Vector Laboratories. The RNeasy minikit and GST bead slurry were purchased from Qiagen. Power SYBR Green PCR Master Mix, Superscript first strand synthesis kit, TOPO-TA cloning Vector Laboratories. The RNeasy minikit and GST bead slurry were purchased from Qiagen. Power SYBR Green PCR Master Mix, Superscript first strand synthesis kit, TOPO-TA cloning kit, DMEM high glucose with sodium pyruvate, penicillin/streptomycin, and horse serum were purchased from Invitrogen. The CellsDirectTM one-step quantitative RT-PCR kit, TRIzol were purchased from Life Technologies, Inc. Rat tail collagen type I was purchased from BD Biosciences. iScript reverse transcription supermix was purchased from Bio-Rad. Collagenase type IV and IVA was purchased from Worthington Biochemicals. The EZ-ChIP assay kit and HDAC assay kit were purchased from Active Motif. The Anti-FLAG M2 mouse monoclonal antibody was purchased from Sigma. Agarose-IgG and IgA bead slurry were purchased from Sigma. The Change II XL site-directed mutagenesis kit was purchased from Stratagene. The 1267-bp TGF-β1 promoter-proximal region immediately upstream of the translational start site of TGF-β1 as a template, and subcloned to CSCGW2. To generate the TGF-β1 luciferase construct, a 1309-bp promoter-proximal region immediately upstream of the translational start site of TGF-β1 was PCR-amplified from mouse genomic DNA, gel-purified, and cloned into pCR2.1 TOPO (Life Technologies). Following the manufacturer’s protocol, the promoter was subcloned from pCR2.1 TOPO to pGL3-basic (Promega). A 42-bp region containing an HDAC3 binding site, identified using published chromatin immunoprecipitation (ChIP)-sequencing data and confirmed by ChIP-qPCR from murine valve tissue, was deleted from the terminal 3′-end of the TGF-β1 promoter-proximal region (−42 to −1 relative to ATG) by PCR amplification using the TGF-β1 luciferase vector as a template. The 1267-bp TGF-β1 deletion promoter was gel-purified, cloned into pCR2.1 TOPO, and subcloned into pGL3-basic. All generated plasmids were verified by restriction analysis and sequencing.

**Immunohistochemistry**—For immunohistochemistry, sections were deparaffinized in xylenes and pretreated using heat antigen retrieval in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6). Immunohistochemistry was conducted using the Vectastain Elite ABC kit and DAB Peroxidase Substrate kit according to the manufacturers’ guidelines. Sec-

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The title suggests that HDAC3 is involved in regulating heart field development, possibly by regulating gene expression through its role in chromatin modification. The text does not provide enough detail to understand the biological mechanism, but it indicates that HDAC3 is involved in early heart development, potentially through its role in histone deacetylation.
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tions were incubated with cleaved caspase-3 antibody (1:100) for 1 h at room temperature or HDAC3 antibody (1:250) or phospho-SMAD2/3 antibody (1:50) overnight at 4 °C. Biotinylated universal pan-specific antibody (horse anti-mouse/rabbit/goat IgG) was used for phospho-SMAD2/3 immunostaining in place of the Vectastain Elite ABC kit secondary antibody according to the manufacturer’s guidelines. For counterstaining, slides were rinsed and then incubated with 30% hematoxylin for 30 s after 3,3′-diaminobenzidine developing. All slides were ethanol-dehydrated, cleared with xylenes, and mounted with Vectashield mounting medium. For immunofluorescent staining, sections were deparaffinized and rehydrated through xylenes and an ethanol gradient. Slides were rinsed in PBS, and antigen retrieval was performed in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for 10 min at 95 °C. After rinsing, sections were blocked in 10% donkey serum, 0.3% Triton X-100 in PBS for 1 h at room temperature. Sections were then washed in PBS and incubated with smooth muscle actin antibody (1:100) in 10% donkey serum and PBS for 1 h at room temperature or with MF-20 (1:50) or troponin (1:25) antibodies in 10% donkey serum in PBS overnight at 4 °C. Finally, slides were washed in PBS, incubated in secondary antibody (donkey anti-mouse 546, 1:500, with Hoechst, 1:1000, in 10% donkey serum, PBS) for 1 h at room temperature, rinsed in PBS, and mounted with Vectashield mounting medium.

Biometric Analysis—Aortic diameter was measured from ×2 images of dissected hearts at three levels (tubular aortic trunk, proximal aortic arch, and intermediate aortic arch) using NIS-Elements analysis software (Nikon). Ventricular wall thickness and valve area measurements were made from ×2 and ×10 images, respectively, of position-matched hematoxylin and eosin-stained sections using NIS-Elements analysis software (Nikon). Nuclei numbers per section in the valves were counted for each sample. The aortic and pulmonic valves were compared separately. The number of valve cusp were manually counted for each sample. The aortic and pulmonic valves were compared separately. The number of cleaved caspase-3-positive nuclei per 1000 nuclei served as an apoptosis index.

Microarray Analysis—Heart tissue was dissected from E9.5 mouse embryos and snap-frozen in liquid nitrogen. RNA was extracted from pools of Hdac3 Isl1KO or Hdac3 F/F control hearts using an RNeasy minikit. Microarray analysis was performed in triplicate from pooled samples by the University of Massachusetts Genomics Core Facility using Affymetrix Mouse Gene 2.0 ST arrays (Affymetrix). Raw microarray data were annotated using the Bioconductor and Oligo packages in R (53–55). Significance of expression differences between Hdac3 Isl1KO and Hdac3 F/F samples was determined using Student’s t test. Heat maps of microarray data were generated using the pheatmap package in R (56). Relative expression of each transcript is reported as log-transformed expression value for each sample, normalized to the median expression value of the transcript across all six samples. MIAME-compliant full microarray data sets can be accessed at the Gene Expression Omnibus (GEO, GSE73666).

Ingenuity Pathway Analysis—Microarray data from E9.5 Hdac3 Isl1KO and control hearts were analyzed using Ingenuity Pathway Analysis (IPA). The IPA Diseases and Functions utility was employed to investigate phenotypes associated with the molecular changes. Hierarchical heat maps were generated using both the Diseases and Biological Functions categories and Tox Functions categories of the Diseases and Functions utility. Plots of phenotypically relevant categories were constructed based on subcategory p values. The Upstream Regulators utility was employed to determine potential regulators of differentially expressed genes. Significant regulators were sorted by significance and by number of associated genes. Differentially expressed genes associated with the upstream regulator TGF-β1 were exported, and a clustered heat map was generated using R software.

Lentiviral Infection—Lentiviral medium was generated by transfecting 100-mm plates of subconfluent 293T cells with 5 μg of lentiviral cDNA, 5 μg of pCMV-dR8.2, and 2.5 μg of pCMV-VSVG, in 10 ml of 2% FBS medium. The medium was changed to a fresh 10 ml of 2% FBS medium 24 h after transfection. Viral medium was collected 24 h later and filtered through a 40-μm cell strainer. Isolated mouse embryonic heart tissue was infected with filtered viral medium supplemented with 10 μg/ml Polybrene reagent. GFP viral medium was used to maintain a constant viral medium volume. Infected cells were harvested for analysis 24 or 48 h after infection.

Cell Culture, Transient Transfection, and Luciferase Assay—HEK293T cells and murine endothelial cells were maintained in DMEM with 10% FBS, 100 mg/ml penicillin, and 100 μM/ml streptomycin in a 37 °C incubator with 5% CO2. HEK293T cells were transfected in subconfluent 100-mm plates with 2.5 μg of DNA and 5 μl of polyethyleneimine, linear, in 10 ml of 10% FBS medium. Luciferase assays were conducted by transfecting subconfluent murine endothelial cells in 6-well plates with 1 μg of DNA and 2 μl of polyethyleneimine, linear, in 2 ml of 10% FBS medium. The 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 the Dual-Luciferase reporter assay kit according to the manufacturer’s guidelines. Luciferase activity was measured using a Berthold microplate reader according to the manufacturer’s guidelines.
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**Immunoprecipitation**—Tissue samples were homogenized in immunoprecipitation buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, and 1 mM DTT) containing 1 mM PMSF, phosphate inhibitors (Sigma), and protease inhibitor mixture (Sigma). The homogenized samples were sonicated using a Branson 250 Digital Sonifier with 1-s on and 1-s off pulses at 40% power amplitude for 15 s. Precleared lysates with beads were incubated with primary antibodies for 16 h at 4 °C. After incubation for 1 h at 4 °C with beads, immune complexes were collected, washed four times with immunoprecipitation buffer, and applied to 4–12% SDS-polyacrylamide gels for Western blot analysis.

**Western Blotting**—Tissue lysates were prepared in lysis buffer (20 mM Tris-HCl (pH 7.5), 15 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton X-100, 1 μg/ml leupeptin, 2.5 mM sodium pyrophosphate, 1 mM Na₃VO₄, and 1 mM β-glycerophosphate; 1 mM phenylmethylsulfonyl fluoride was added before use). Protein samples were resolved on 4–12% SDS-PAGE acrylamide gel before transferring to PVDF membranes. We used primary antibodies to HDAC3 (1:1000) and FLAG (1:1000). Primary antibodies were visualized by chemiluminescence using HRP-conjugated secondary antibodies or by infrared with IRDye®-conjugated secondary antibodies. Blots were probed with α-tubulin (1:1000) or GAPDH (1:1000) for loading control.

**ELISA**—Sandwich ELISAs were performed according to the manufacturer’s protocol. Briefly, for the phospho-SMAD2/3 or phospho-HDAC3 ELISA, samples were prepared using 100 μl of 1× cell lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 μg/ml leupeptin). 100 μl of sample diluent was added per 100 μl of sample (1:1 ratio). Samples were incubated overnight at 4 °C. After four washes with 1× wash buffer, samples were incubated with detection antibody for 1 h at 37 °C. Samples were visualized using the HRP detection method at 450 nm. For the phospho-HDAC3 ELISA, a microtiter plate was coated with HDAC3 antibody (5 μg/ml) in carbonate/bicarbonate buffer (pH 9.6). The sandwich ELISA for TGF-β1 was performed according to the manufacturer’s protocol. Briefly, samples were prepared using assay diluent buffer RD1-21. Samples were incubated for 2 h at room temperature. After four washes with 1× wash buffer, samples were incubated with 100 μl of TGF-β1 conjugate for 2 h at room temperature. After four washes with 1× wash buffer, samples were incubated with 100 μl of substrate solution (protected from light) for 30 min at room temperature. After the addition of 100 μl of stop solution, reactions were visualized at 450 and 540 nm. Readings at 540 nm were subtracted from readings at 450 nm to correct for optical imperfections in the plate.

**Outflow Tract Explant Assay**—The outflow tract explant cultures were performed as described previously (57, 58). Briefly, a solution of rat tail collagen type I (1.5 mg/ml) containing NaOH to a final concentration of 15 mmol/liter was dispensed into 24-well microculture dishes. Subsequently, gels were placed in a 37 °C tissue culture incubator at 5% CO₂ and allowed to polymerize. After 30 min, collagen gels were washed several times with DMEM containing 10% fetal bovine serum, 0.1% insulin-transferrin-selenium, 100 mg/ml penicillin, and 100 μg/ml streptomycin. Outflow tracts were carefully dissected from control and Hdacs3−/− E9.5, E10.5, or E14.5 embryonic hearts. The outflow tracts were placed endocardium face down onto collagen gels and allowed to adhere for 8 h at 37 °C in 5% CO₂. Eight hours after attachment, medium was added, and explants were cultured for up to 24, 48, or 72 h. Radial migration was measured from ×4 images of outflow tract explants at 45° intervals using NIS-Elements analysis software (Nikon). Explants were treated with TGF-β1 antibody (1 μg/ml) for 24 or 48 h or with recombinant TGF-β1 (10 ng/ml) for 24 or 48 h. Explants were also detached, trypsinized, and incubated with anti-vimentin (mesenchymal marker) antibody-conjugated or PECA-1 (endothelial marker)-conjugated epoxy M-450 Dynabeads as per the manufacturer’s guidelines to isolate cells.

**ChIP Analysis**—ChIP experiments were performed as described previously (48). Briefly, isolated cardiac tissue or explants from E9.5 mouse embryos were cross-linked for 15 min in cross-linking solution (1% formaldehyde, 1.5 mM ethylene glycol-bis succinimidylsuccinate, 20 mM sodium butyrate, 10% FBS) and then quenched with 125 mM glycine solution for 5 min, followed by two washes with 1× PBS. Chromatin fragmentation was carried out by sonication in ChIP SDS lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, 1× protease inhibitors), using the Branson Sonifier 250 (40% power amplitude, 110 s). Proteins were immunoprecipitated in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris- HCl, pH 8.0, 167 mM NaCl, 20 mM sodium butyrate, 1× protease inhibitor) using IgG, HDAC3, NCOR1, EZH2, H3K27me3, H3K27ac, polymerase II, EED, SUZ12, or CREBBP antibody. Specificity of HDAC3, NCOR1, EZH2, H3K27me3, H3K27ac, polymerase II, EED, SUZ12, or CREBBP antibody at the relevant loci was determined using IgG control antibody. Specificity of HDAC3 antibody at the relevant loci was also determined using HDAC3-null hearts compared with wild-type control. Immunoprecipitated antibody–chromatin complexes were washed twice with low salt wash buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, and 1% Triton X-100) followed by two washes with lithium chloride wash buffer (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 1 mM EDTA, 1% deoxycholate, and 1% Nonidet P-40) and TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). After removing wash buffer, cross-linking was reversed at 65 °C overnight in Proteinase K buffer (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 50 mM NaCl, 1% SDS, 20 mM sodium butyrate, 50 μg/ml Proteinase K). The following day, DNA was purified using phenol/ chloroform/isoamyl alcohol. Purified precipitated DNA was analyzed by quantitative RT-PCR as described previously. Enrichment of target sequences was determined by quantitative RT-PCR using primers designed against HDAC3 consensus binding sites within the proximal 10 kb upstream of the transcription start site of genes of interest. Tgf-β1, Kpun1, Sumo1, and Smad4. Primer sequences are available upon request.

**HDAC Activity Assay**—HDAC activity was measured by using an HDAC activity assay kit for fluorometric detection as per the manufacturer’s protocol. Briefly, 293T cells were transfected with or without plasmid containing either HDAC3-
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A Hx3 Cx1 R26R LacZ
B Hx3 Cx1 R26R LacZ
C 20X
D Hx3
E Hx3
F Hx3
G Hx3 Hx3
H Aorta diameter (m)
I Aorta diameter (m)
J Hx3 Hx3
K Hx3 Hx3
L Control Hx3
M Mer2C Cre Hx3
N Acute diameter (m)
O Hx3
P Mer2C Cre Hx3

E9.5 E13.5 E18.5 E18.5
FLAG or HDAC3ΔH134AΔH135A-FLAG. Using anti-FLAG antibody, total lyase was immunoprecipitated and then incubated with HDAC assay substrate for 60 min at 37 °C. After the addition of diluted activator reagent, samples were incubated at room temperature for 10 min and analyzed by a fluorescent microplate reader at excitation of 360 nm and emission of 450 nm.

Statistical Analysis—Statistical significance between groups was determined using two-tailed Student’s t test or χ² test. A p value of <0.05 was considered significant.

Results

HDAC3 Is Required in the Second Heart Field for Cardiac Development—Germ line deletion of ubiquitously expressed HDAC3 results in embryonic lethality at E9.5 (59, 60). To determine the function of HDAC3 in the second heart field, we deleted HDAC3 in the second heart field progenitor cells using two Cre lines, Isl1-Cre (Hdac3Δ Isl1KO) (Fig. 1, A–C) and Mef2C-Cre (Hdac3Δ Mef2CKO) (7, 50). Hdac3Δ Isl1KO embryos were identified at E18.5 (Table 1). However, Hdac3Δ Isl1KO pups displayed complete lethality at birth (P0) (p < 0.01; Table 1). Hdac3Δ Isl1KO embryos were characterized by malrotation of outflow tract, overriding aorta, double outlet right ventricle, membranous ventricular septal defect, ascending aortic dilation (21–35%, p < 0.02), and normal outflow tract septation at E18.5 (Table 2 and Fig. 1, D–I). Hdac3Δ Isl1KO embryos displayed aberrant distribution and expression of collagen in all layers of ascending aortic and pulmonary arterial walls (Fig. 1, J–L). In addition, elastin fibers appeared fragmented, probably explaining aortic dilation in Hdac3Δ Isl1KO embryos (Fig. 1, K and L). Isl1-Cre is a knock-in allele; however, we did not observe genetic interaction between HDAC3 and ISL1 (Tables 1 and 2). Hdac3Δ Mef2CKO embryos revealed similar developmental cardiac defects, including double outlet right ventricle, ascending aortic dilation (22–32%, p < 0.002), and perinatal lethality (Tables 2 and 3 and Fig. 1, M and N). Primary atrial septum and right ventricle, in part derived by the second heart field progenitor cells, appeared normal in both Hdac3Δ Isl1KO and Hdac3Δ Mef2CKO hearts (Fig. 1, O and P) (data not shown). Cardiomyocyte differentiation was unaffected in early Hdac3Δ Isl1KO embryos (data not shown).

HDAC3 Is Essential for Semilunar Valve Development—The second heart field progenitor cells are required for construtural cushion development, which contributes to the semilunar valves and outflow tract septum (5, 6, 8–10). Hdac3Δ Isl1KO embryos demonstrated dysmorphic, thickened (1.59–3-fold, p < 0.02), and hyperplastic (1.31–3.14-fold, p < 0.02) semilunar valves at E18.5 (Table 2 and Fig. 2, A–N). Interestingly, aortic valves were bicuspid in Hdac3Δ Isl1KO hearts (Table 2 and Fig. 2E). The outflow tract septum and atrioventricular valves appeared normal in these embryos at E18.5 (Figs. 1G and 2O). Similarly, Hdac3Δ Mef2CKO embryos revealed defects in semilunar valve development, but not in atrioventricular valve development, at E18.5 (Table 2 and Fig. 2, P–T).

The mature semilunar valves are composed of extracellular matrix, valvular interstitial cells, and endothelial cells. Remodeling of valvular interstitial cells and extracellular matrix, mainly collagens, proteoglycans, and elastin, is essential for maturation of the semilunar valves (61). Hdac3Δ Isl1KO hearts displayed loss of extracellular matrix triaminar stratification and valvular interstitial cell compartmentalization in the semilunar valves at various stages of development (Fig. 3, A–F, L, and M). Specifically, loss of HDAC3 resulted in aberrant expression of collagen and proteoglycan in all layers, probably explaining enlarged and thickened semilunar valves (Fig. 3, A–F, L, and M). Elastin fibers appeared disorganized and fragmented in Hdac3Δ Isl1KO semilunar valves (Fig. 3, A, B, E, F, L, and M). Similarly, Hdac3Δ Mef2CKO embryos revealed aberrant expression of collagen and proteoglycan in all layers of the semilunar valves (Fig. 3, G and H). Remodeling of the semilunar valves is associated with an increase in apoptosis (18). Hdac3Δ Isl1KO semilunar valve interstitial cells displayed a 55–65% decrease in apoptosis compared with controls (p < 0.02) during valve remodeling (Fig. 3, I–K).

HDAC3 Regulates TGF-β Signaling Pathway—Transcriptional analysis of early embryonic Hdac3Δ Isl1KO hearts revealed significant expression changes indicative of the

### Table 1

| Genotyping of Isl1-Cre; Hdac3Δ Isl1KO | Observed (Expected) |
|--------------------------------------|---------------------|
| E18.5 | P0 |
| Hdac3Δ Isl1KO | 9 (9) | 18 (11) |
| Hdac3Δ Isl1KO | 17 (18) | 22 (22) |
| Hdac3Δ Isl1KO | 11 (9) | 12 (11) |
| Isl1-Cre; Hdac3Δ Isl1KO | 13 (9) | 17 (11) |
| Isl1-Cre; Hdac3Δ Isl1KO | 15 (18) | 19 (22) |
| Isl1-Cre; Hdac3Δ Isl1KO | 7 (9) | 0 (11) |
| Total | 72 | 88 |
| N<sub>S</sub> | p < 0.01 |

*Not significant.

### Figure 1

HDAC3 functions within the second heart field to regulate cardiac morphogenesis. A–C, Hdac3 immunostaining of LacZ-stained Hdac3Δ Isl1KO; R26R-LacZ<sup>+</sup> and control embryos shows that HDAC3 protein expression (black arrows) is lost (gray arrows) in the Isl1-Cre expression domain in E9.5 (A) and E13.5 (B and C) Hdac3Δ Isl1KO hearts, including in semilunar valves (C), D, dissected and fixed E18.5 hearts show malrotation of the outflow tract in Hdac3Δ Isl1KO hearts. E, tracings of outflow tract vessels demonstrate abnormal, parallel orientation of the aorta and pulmonary artery in Hdac3Δ Isl1KO hearts. F, hematoyxin- and eosin-stained sections demonstrate double outlet right ventricle, with membranous ventricular septal defect (arrow) and an overriding aorta in Hdac3Δ Isl1KO E18.5 hearts. G, hematoyxin- and eosin-stained cross-sections show malrotation but complete septation of the aorta and pulmonary artery in E18.5 Hdac3Δ Isl1KO hearts. H and J, schematic diagram of proximal aorta showing three levels: level 1 (tubular aortic trunk), level 2 (proximal aortic arch), and level 3 (intermediate aortic arch) (H). Shown is a comparison of aorta size in Hdac3Δ Isl1KO and control hearts, measured at three levels from dissected, fixed hearts (J). M, Masson’s trichrome-stained sections of aorta and pulmonary artery demonstrate disorganized collagen (blue) in the vessel walls of Hdac3Δ Isl1KO E18.5 hearts. K, Verhoeffen-Van Gieson-stained sections of aorta and pulmonary artery demonstrate disorganized collagen (arrows, red) and fragmented elastin (arrows, black) in the vessel walls of Hdac3Δ Isl1KO E18.5 hearts. L, schematic model of disorganized outflow vessel wall morphology. M, hematoyxin- and eosin-stained sections demonstrate double outlet right ventricle with aortic valve overriding a membranous ventricular septal defect (arrow) in Hdac3Δ Isl1KO E18.5 hearts. N, quantification of aortic diameter shows dilation of E18.5 Hdac3Δ Isl1KO aortas at three levels: level 1 (tubular aortic trunk), level 2 (proximal aortic arch), and level 3 (intermediate aortic arch). O and P, hematoyxin- and eosin-stained sections show normal primary atrial septum (arrow) in Hdac3Δ Isl1KO (O) and Hdac3Δ Mef2CKO E18.5 hearts (P). RA, right atrium; LA, left atrium; atr; AV, aortic valve; PA, pulmonary artery; PV, pulmonic valve; RV, right ventricle; LV, left ventricle; BA, brachiocephalic artery; LCA, left common carotid artery; PAS, primary atrial septum.
HDAC3 Orchestrates Second Heart Field Development

**TABLE 2**

Phenotypes of mice, age E18.5–P0

| Genotype                          | Incidence | Total | $\chi^2$ test | $p$  |
|----------------------------------|-----------|-------|---------------|-----|
| Outflow tract malrotation        |           |       |               |     |
| Hdac3$^{f/f}$                     | 6         | 6     |               |     |
| Hdac3$^{M/F}$                     | 8         | 9     |               |     |
| Hdac3$^{M/F}$                     | 3         | 3     |               |     |
| Hdac3$^{M/F}$; Isl1-Cre           | 0         | 4     |               |     |
| Double outlet right ventricle     |           |       |               |     |
| Hdac3$^{M/F}$                     | 5         | 6     |               |     |
| Hdac3$^{M/F}$                     | 3         | 3     |               |     |
| Hdac3$^{M/F}$                     | 3         | 3     |               |     |
| Hdac3$^{M/F}$; Isl1-Cre           | 0         | 4     |               |     |
| Ventricular septal defect        |           |       |               |     |
| Hdac3$^{M/F}$                     | 6         | 6     |               |     |
| Hdac3$^{M/F}$                     | 5         | 5     |               |     |
| Hdac3$^{M/F}$                     | 3         | 3     |               |     |
| Hdac3$^{M/F}$; Isl1-Cre           | 0         | 4     |               |     |
| Overriding aorta                 |           |       |               |     |
| Hdac3$^{M/F}$                     | 5         | 6     |               |     |
| Hdac3$^{M/F}$                     | 3         | 3     |               |     |
| Hdac3$^{M/F}$                     | 3         | 3     |               |     |
| Hyperplastic aortic valve        |           |       |               |     |
| Hdac3$^{M/F}$                     | 12        | 12    |               |     |
| Hdac3$^{M/F}$                     | 9         | 9     |               |     |
| Hdac3$^{M/F}$                     | 3         | 3     |               |     |
| Hyperplastic pulmonic valve      |           |       |               |     |
| Hdac3$^{M/F}$                     | 12        | 12    |               |     |
| Hdac3$^{M/F}$                     | 9         | 9     |               |     |
| Hdac3$^{M/F}$                     | 3         | 3     |               |     |
| Bicuspid aortic valve            |           |       |               |     |
| Hdac3$^{M/F}$                     | 4         | 4     |               |     |
| Hdac3$^{M/F}$                     | 3         | 3     |               |     |
| Hyperplastic aortic valve        |           |       |               |     |
| Hdac3$^{M/F}$                     | 14        | 16    |               |     |
| Hdac3$^{f/f}$                     | 6         | 6     |               |     |
| Tgf$\beta$-mediated EndMT and Extracellular Matrix Remodeling in Hdac3$^{f/f}$KO Heart—TGF-$\beta$ signaling and its downstream target genes, such as Snai1, are critical factors that induce EndMT in the developing outflow tract cushions (40). EndMT is characterized by loss of endothelial markers, such as TEK, and gain of mesenchymal markers, such as TAGLN (33). Hdac3$^{f/f}$KO hearts exhibited robust down-regulation of Tek and up-regulation of Snai1 and Tagln (Figs. 4F and 5A). Hdac3$^{f/f}$KO outflow tract cushion explants showed ~3-fold induction of EndMT ($p < 0.005$; Fig. 5, B and C), and these changes were largely abolished by TGF-$\beta$-neutralizing antibody ($p < 0.009$; Fig. 5, B–F). Aberrant TGF-$\beta$ signaling activates valvar interstitial cells, which in turn, express smooth muscle genes and produce extracellular matrix scaffold proteins to remodel valvar matrix (24). Hdac3$^{f/f}$KO hearts revealed significant expression changes in smooth muscle and extracellular matrix genes (Fig. 5, A and G–M). Smooth muscle genes, including Tagln, Myh11, and Cnn2, were up-regulated in Hdac3$^{f/f}$KO hearts and semilunar valves (Fig. 5, A, G, and H). Simultaneously, expression of proteoglycans, including Dcn, Bgn, Fmod, and Vcan, and collagens, including Col2a1, Col11a2, Col9a2, Col1a2, and Col3a1, were significantly altered in Hdac3$^{f/f}$KO hearts and semilunar valves (Fig. 5, A and I–M). We next examined the requirement of TGF-$\beta$ for aberrant expression of extracellular matrix genes in Hdac3$^{f/f}$KO valvar interstitial cells. Loss of HDAC3 resulted in 6–10-fold expression changes in proteoglycans, collagen, and smooth muscle genes ($p < 0.006$), and these changes were largely abol-

**TABLE 3**

Genotyping of Mef2c-Cre; Hdac3$^{f/f} \times$ Hdac3$^{f/f}$

| Genotype                          | Incidence | Total | $\chi^2$ test | $p$  |
|----------------------------------|-----------|-------|---------------|-----|
| E18.5                            |           |       |               |     |
| Hdac3$^{f/f}$                     | 14 (8)    | 10 (12)|               |     |
| Hdac3$^{f/f}$                     | 12 (16)   | 23 (24)|               |     |
| Hdac3$^{f/f}$                     | 6 (8)     | 12 (12)|               |     |
| Tgf$\beta$-mediated EndMT and Extracellular Matrix Remodeling in Hdac3$^{f/f}$KO Heart—TGF-$\beta$ signaling and its downstream target genes, such as Snai1, are critical factors that induce EndMT in the developing outflow tract cushions (40). EndMT is characterized by loss of endothelial markers, such as TEK, and gain of mesenchymal markers, such as TAGLN (33). Hdac3$^{f/f}$KO hearts exhibited robust down-regulation of Tek and up-regulation of Snai1 and Tagln (Figs. 4F and 5A). Hdac3$^{f/f}$KO outflow tract cushion explants showed ~3-fold induction of EndMT ($p < 0.005$; Fig. 5, B and C), and these changes were largely abolished by TGF-$\beta$-neutralizing antibody ($p < 0.009$; Fig. 5, B–F). Aberrant TGF-$\beta$ signaling activates valvar interstitial cells, which in turn, express smooth muscle genes and produce extracellular matrix scaffold proteins to remodel valvar matrix (24). Hdac3$^{f/f}$KO hearts revealed significant expression changes in smooth muscle and extracellular matrix genes (Fig. 5, A and G–M). Smooth muscle genes, including Tagln, Myh11, and Cnn2, were up-regulated in Hdac3$^{f/f}$KO hearts and semilunar valves (Fig. 5, A, G, and H). Simultaneously, expression of proteoglycans, including Dcn, Bgn, Fmod, and Vcan, and collagens, including Col2a1, Col11a2, Col9a2, Col1a2, and Col3a1, were significantly altered in Hdac3$^{f/f}$KO hearts and semilunar valves (Fig. 5, A and I–M). We next examined the requirement of TGF-$\beta$ for aberrant expression of extracellular matrix genes in Hdac3$^{f/f}$KO valvar interstitial cells. Loss of HDAC3 resulted in 6–10-fold expression changes in proteoglycans, collagen, and smooth muscle genes ($p < 0.006$), and these changes were largely abol-

congenital heart defects observed in late stage Hdac3$^{f/f}$KO embryos (Fig. 4, A and B). Upstream regulator analysis identified marked changes in TGF-$\beta$ pathway genes (Fig. 4, C–E). Positive regulators of the TGF-$\beta$ pathway, including Smo1, Npr2, Smad4, Snai1, Tgf-$\beta$, and Kpn1 were 3–9-fold up-regulated ($p < 0.02$) in Hdac3$^{f/f}$KO hearts (Fig. 4F). ChIP-qPCR analysis confirmed chromatin occupancy of HDAC3 (0.15–0.5%, $p < 0.002$) on identified sites in the conserved noncoding regions within 10 kb upstream of TGF-$\beta$ pathway genes, including Smad4, Sumo1, Tgf-$\beta$, and Kpn1 (Fig. 4, G–I). We also observed ~3.5-fold higher TGF-$\beta$ levels in Hdac3$^{f/f}$KO outflow tracts compared with control ($p < 0.015$; Fig. 4K). Loss of HDAC3 resulted in ~2.7-fold activation of SMAD2/3 phosphorylation ($p < 0.02$), suggesting enhanced TGF-$\beta$ signaling (Fig. 4, L and M). These results suggest that HDAC3 represses TGF-$\beta$ signaling pathway during early cardiogenesis.
ished by TGF-β-neutralizing antibody (Fig. 5, H–M). Taken together, these results suggest that aberrant EndMT and extracellular matrix remodeling in \textit{Hdac3}^{MxKO} hearts are mediated by TGF-β signaling.

\textbf{HDAC3 Functions within Mesenchymal or Smooth Muscle Cells to Regulate Semilunar Valve and Outflow Tract Development—} The second heart field progenitor cells give rise to cardiomyocytes, endothelial cells, and mesenchymal cells or smooth muscle cells (62, 63). Genetic deletion of \textit{Hdac3} using \textit{Cdh5-Cre} (\textit{Hdac3\textit{Cdh5KO}}), which is expressed in differentiated endothelial and endocardial cells at E8.0 (52), and \textit{Myh6-Cre} (\textit{Hdac3\textit{Myh6KO}}), which is expressed in differentiated car-

\textbf{A} \hspace{1cm} \textbf{B} \hspace{1cm} \textbf{C} \hspace{1cm} \textbf{D} \hspace{1cm} \textbf{E}

\begin{figure}[h]
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\caption{Aortic valve}
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\caption{Pulmonic valve}
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\caption{Tricuspid valve}
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\caption{Mitrval valve}
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HDAC3 Orchestrates Second Heart Field Development

diomyocytes at E8.5 (64), did not reveal malrotation of outflow tract, overriding aorta, double outlet right ventricle, or valve defects observed in Hdac3<sup>Tgαlox/lox</sup> or Hdac3<sup>Mo2C/Cko</sup> embryos (Fig. 6, A–O). Importantly, genetic deletion of Hdac3 using Tagl-Cre (Hdac3<sup>Tgαlox/lox</sup>), which is expressed in differentiated mesenchymal or smooth muscle cells at E8.5 (65), recapitulated outflow tract malrotation, overriding aorta, double outlet right ventricle, aberrant semilunar valve development, membranous ventricular septal defect, disorganized collagen, and fragmented elastin in great vessel walls and showed hyperplastic semilunar valves with expanded proteoglycans and aberrant collagen deposition, as observed in Hdac3<sup>Tgαlox/lox</sup> and Hdac3<sup>Mo2C/Cko</sup> embryos (Fig. 7, A–L). Expression of positive regulators of TGF-β pathway genes, smooth muscle markers, proteoglycans, and collagens were 4–20-fold up-regulated in Hdac3<sup>Tgαlox/lox</sup> semilunar valves (p < 0.03; Fig. 7M). Hdac3<sup>Tgαlox/lox</sup> outflow tract cushion explants displayed an ~3-fold increase in EndMT (p < 0.03) and ~20–40-fold expression changes in proteoglycans and smooth muscle genes (p < 0.004; Fig. 7, N–Q). Importantly, these changes were largely abolished by TGF-β-neutralizing antibody (p < 0.004), suggesting that aberrant EndMT and extracellular matrix remodeling in Hdac3<sup>Tgαlox/lox</sup> hearts are mediated by TGF-β signaling (Fig. 7, N–Q). Together, these data suggest a primary role of HDAC3 in the second heart field-derived mesenchymal or smooth muscle cells during early cardiac development.

HDAC3 Epigenetically Silences TGF-β1 within Mesenchymal Cells by Recruiting EZH2 to the NCOR Complex—During EndMT, endothelial cells lose their endothelium-specific markers and morphology and acquire a mesenchymal cell-like phenotype (33). In this process, endothelium-derived mesenchymal cells displayed ~90% repression of TGF-β1 expression compared with endothelial cells (p < 0.003); however, this repression was completely absent in mesenchymal cells lacking HDAC3 (Fig. 8, A and B). We observed ~0.6–1.8% enrichment of HDAC3 at a regulatory region upstream of TGF-β1 within mesenchymal cells compared with endothelial cells (p < 0.02; Fig. 8, C and D). We evaluated chromatin occupancy of various histone marks to determine the regulatory mechanism of the HDAC3-enriched region upstream of TGF-β1 within mesenchymal cells. ChIP-qPCR analysis revealed loss of trimethylation of Lys-27 on histone H3 (H3K27me3) and ~8% enrichment of acetylation of Lys-27 on histone H3 (H3K27ac) at the TGF-β1 regulatory region in H9252 semilunar valves compared with control (p < 0.04, Fig. 8, E and F). Consistent with these findings, Hdac3<sup>Tgαlox/lox</sup> semilunar valves displayed ~0.9–3% enrichment of RNA polymerase II and CREBBP (p < 0.006) and abolished occupancy of EZH2, EED, and SUZ12, the H3K27 methyltransferase components of PRC2 complex, at the TGF-β1 regulatory region (Fig. 8, G–K). NCOR1 was required for recruitment of HDAC3 to the TGF-β1 locus (Fig. 8L). Interestingly, NCOR1 occupancy remained unchanged at this locus in Hdac3<sup>Tgαlox/lox</sup> semilunar valves, suggesting that HDAC3 is not required for NCOR1 recruitment to the TGF-β1 regulatory region (Fig. 8M). Co-ChIP-qPCR analysis revealed co-occupancy of HDAC3 with either EZH2, H3K27me3, or NCOR1 at the TGF-β1 locus in wild-type semilunar valves (Fig. 8N). HDAC3 interacts with EZH2 within mesenchymal cells of semilunar valves (Fig. 8O). Importantly, EZH2 and NCOR1 are required for deposition of the H3K27me3 mark at the TGF-β1 locus within valvular mesenchymal cells (Fig. 8P). These data suggest that HDAC3 is required to mediate epigenetic silencing of TGF-β1 within mesenchymal cells of semilunar valves.

**Deacetylase Activity of HDAC3 Is Dispensable for EndMT and Epigenetic Silencing of TGF-β1**—Approximately 10% of mammalian enzymes are catalytically inactive (66). These “dead” enzymes or pseudoenzymes are biologically functional; however, their enzyme-independent functions remain largely undefined. A recent study (67) describes a deacetylase-independent function of HDAC3 in lipid metabolism. To determine whether deacetylase activity of HDAC3 is required to regulate EndMT, expression of Tgf-β1, and recruitment of EZH2 to the TGF-β1 locus, we generated a previously described mutant form of HDAC3 in which two highly conserved tandem His residues, 134 and 135, are mutated to alanine (HDAC3<sup>H134A,H135A</sup>). These mutations do not affect its expression (Fig. 9, A and B), chromatin recruitment, and interaction with NCOR1 (67); however, they render HDAC3 completely inactive (Fig. 9C). HDAC3-null outflow tract cushion explants showed an ~2-fold increase in EndMT (p < 0.05), and these changes were largely rescued by either wild-type HDAC3 or HDAC3<sup>H134A,H135A</sup> expression (p < 0.04; Fig. 9, D and E). Similarly, either wild-type HDAC3 or HDAC3<sup>H134A,H135A</sup> rescued aberrant Tgf-β1 transcription within cultured HDAC3-null semilunar valve mesenchymal cells (p < 0.0004; Fig. 9F). Hence, we generated a mutant TGF-β1 promoter luciferase reporter lacking the HDAC3 enriched regulatory region (~42 to ~1 upstream of ATG).
FIGURE 3. **HDAC3 is required for extracellular matrix homeostasis and remodeling of semilunar valves.**

A, Movat’s pentachrome staining of remodeling aortic valve shows an increase in proteoglycans (arrows, blue) from E13.5 to E18.5 in **Hdac3**KO aortic valves with a reduction and remodeling of proteoglycans in control valves. B, Movat’s pentachrome staining of remodeling pulmonic valve shows an increase in proteoglycans (arrows, blue) from E13.5 to E18.5 in **Hdac3**KO pulmonic valves with a reduction and remodeling of proteoglycans in control valves. C, Masson’s trichrome staining demonstrates disorganized collagen expression (arrow, blue) in **Hdac3**KO E18.5 aortic valve. D, Masson’s trichrome staining demonstrates disorganized collagen expression (arrow, blue) in **Hdac3**KO E18.5 pulmonic valve. E, Verhoeff-Van Gieson (VVG) staining shows disorganized collagen expression (arrow, red) in **Hdac3**KO E18.5 aortic valve. F, Verhoeff-Van Gieson staining shows disorganized collagen expression (arrow, red) in **Hdac3**KO E18.5 aortic valve. G, Movat’s pentachrome-stained sections show increased proteoglycans (blue) in aortic valve cusps of E18.5 **Hdac3**KO hearts. H, Movat’s pentachrome staining reveals increased proteoglycans (blue) in E18.5 **Hdac3**KO aortic valve. I, representative images of cleaved caspase-3 immunostaining in E13.5 semilunar valves. Arrows, positive staining. J, quantitative analysis of cleaved caspase-3-positive cells in E13.5 control and **Hdac3**KO aortic valves. **K**, quantitative analysis of cleaved caspase-3-positive cells in E13.5 control and **Hdac3**KO pulmonic valves. **L**, schematic model depicting disorganized extracellular matrix and reduced apoptosis in **Hdac3**KO E13.5 semilunar valves. **M**, schematic model showing hyperplastic, enlarged, and disorganized **Hdac3**KO E18.5 semilunar valves.
FIGURE 4. HDAC3 is a critical regulator of TGF-β signaling pathway. A–E, IPA of microarray data from E9.5 Hdac3 Isl1KO hearts. A, cardiovascular development and function subcategories significantly dysregulated in E9.5 Hdac3 Isl1KO hearts, sorted by significance, from IPA diseases and function analysis. B, classes of congenital heart anomalies affected in E9.5 Hdac3 Isl1KO hearts, sorted by significance, from IPA diseases and function analysis. C, 30 most significant upstream regulators from IPA upstream analysis of E9.5 Hdac3 Isl1KO microarray data. D, clustered heat map of differentially expressed genes related to upstream regulator TGF-β1 from IPA upstream analysis of Hdac3 Isl1KO microarray data. E, top 30 upstream regulators, based on number of associated genes differentially expressed in E9.5 Hdac3 Isl1KO hearts from IPA upstream analysis. F, transcripts for Sumo1, Nrp2, Smad4, Snai1, Tgf-β1, and Kpnb1 were detected by real-time qPCR in E9.5 Hdac3 Isl1KO hearts (mean ± S.E., n = 3). G–I, ChIP-qPCR analysis of HDAC3 recruitment to promoter-proximal regions of TGF-β pathway genes performed in wild-type E9.5 outflow tract (mean ± S.E., n = 3). J, ELISA for TGF-β1 (K) and phospho-SMAD2/3 (L) was performed in E9.5 Hdac3 Isl1KO hearts (mean ± S.E., n = 3 (K) and n = 4 (L)). M, phospho-SMAD2/3 immunostaining (arrows) in E9.5 Hdac3 Isl1KO; R26R-LacZ Hearts.
FIGURE 5. TGF-β mediates aberrant expression of EndMT and extracellular matrix genes in Hdac3<sup>S11KO</sup> heart. A, heat map of microarray data shows differential expression of extracellular matrix, smooth muscle, and endothelium-related genes in Hdac3<sup>S11KO</sup> E9.5 hearts. B, EndMT assay of TGF-β antibody-treated or IgG antibody-treated outflow tract cushion explants 24 h after isolation from Hdac3<sup>S11KO</sup> and Hdac3<sup>F/F</sup> E10.5 hearts. C, quantification of average radial migration, measured in eight directions, from TGF-β antibody-treated or IgG antibody-treated outflow tract cushion explants from Hdac3<sup>S11KO</sup> and Hdac3<sup>F/F</sup> E10.5 hearts, measured 24 h after isolation. D–F, transcripts for Snai1 (D), Tagln (E), and Tek (F) were detected by real-time qPCR in IgG or TGF-β antibody-treated control and Hdac3<sup>S11KO</sup> outflow tract explants derived from E9.5 hearts (mean ± S.E., n = 3). G, immunostaining for smooth muscle actin in Hdac3<sup>S11KO</sup> and control E18.5 aortic valves (AV). H–M, transcripts for Tagln (H), Dcn (I), Bgn (J), Lum (K), Col1a2 (L), and Col2a1 (M) were detected by real-time qPCR in IgG or TGF-β antibody-treated control and Hdac3<sup>S11KO</sup> proximal outflow tract explants with semilunar valves derived from E14.5 embryos (mean ± S.E., n = 3).
Transfection of HDAC3 or HHDAC3H134A,H135A resulted in ~90% repression of the wild-type TGF-β1 reporter (p < 0.002); however, it failed to repress the mutant TGF-β1 reporter (Fig. 9G). The mutant TGF-β1 promoter luciferase reporter displayed transcriptional activity similar to control (Fig. 9G). Interestingly, valvular mesenchymal cells expressing HDAC3H134A,H135A showed similar levels of H3K27ac at the TGF-β1 locus compared with those expressing wild-type HDAC3, although HDAC3H134A,H135A lacks the ability to actively deacetylate histones (Fig. 9H). Either wild-type HDAC3 or HDAC3H134A,H135A expression restored chromatin occupancy of EZH2, EED, and SUZ12 and thereby deposition of the H3K27me3 mark at the TGF-β1 promoter luciferase reporter (Fig. 9). Interestingly, valvular mesenchymal cells expressing HDAC3H134A,H135A displayed transcriptional activity similar to control (Fig. 9G). Taken together, these findings demonstrate that HDAC3-mediated epigenetic silencing of TGF-β1 within second heart field-derived mesenchymal cells is essential for termination of EndMT (Fig. 10, A and B). In the absence of HDAC3, aberrant expression of TGF-β within mesenchymal cells leads to perpetual activation of mesenchymal cells to myofibroblasts, altered extracellular matrix homeostasis, and enhanced EndMT, which in turn, leads to congenital cardiac anomalies resembling those seen in patients with mutations causing activation of the TGF-β signaling pathway (Fig. 10, A and B).

Discussion

Previous studies from our group and others have demonstrated that HDACs are critical regulators of various developmental processes, including cardiogenesis (48, 49). Among class I HDACs (HDAC1, -2, -3, and -8), global loss of HDAC2 or HDAC8 in mice does not affect morphogenesis of second heart field-derived structures at birth (43, 45, 46). Interestingly, murine embryos lacking HDAC1 within the second heart field (Hdac1Is1KO) display normal cardiogenesis, including development of the outflow tract and semilunar valves. Similarly, second heart field-derived structures appear normal in mice lacking class II HDACs, such as HDAC4, -5, -6, or -9 at birth (42). Taken together, our data reveal a unique and specific role for HDAC3 in regulation of second heart field morphogenesis.

Second heart field progenitor cells progressively and restrictively differentiate into various cardiac cell types, including cardiomyocytes, endothelial cells, and mesenchymal or smooth muscle cells (3). Genetic ablation of HDAC3 in the second heart field progenitor cells using either Isl1-Cre or Mef2c-AHF-Cre resulted in ascending aortic dilatation, outflow tract malrotation, overriding aorta, double outlet right ventricle, semilunar valve stenosis, bicuspid aortic valve, and membranous ventricular septal defects that closely resembled those seen in patients with congenital cardiovascular diseases. Strikingly, genetic deletion of HDAC3 in mesenchymal or smooth muscle cells (Hdac3TaglnKO) recapitulated the cardiovascular anomalies observed in Hdac3H9252KO and Hdac3Mef2CKO embryos; however, genetic deletion of HDAC3 in differentiated cardiomyocytes (Hdac3Myh6KO) or endothelial cells (Hdac3Cdh5KO) did not recapitulate these defects. These observations define a spatiotemporal function of HDAC3 within mesenchymal or smooth muscle cells of second heart field-derived structures that is required for arterial pole morphogenesis (Fig. 10, A and B).

The anterior part of the second heart field contributes to the arterial pole of the heart, myocardial, and endothelial layers of the outflow tract, right ventricle, ventricular septum, and endocardial cushion mesenchyme of the outflow tract. The posterior part of the second heart field contributes to the venous pole of the heart, atria, dorsal mesenchymal protrusion, and atrial septum (4). Isl1Cre marks both the anterior and posterior part of the second heart field (3). However, genetic deletion of ubiquitously expressed HDAC3 in the Isl1Cre expression domain only affected morphogenesis of the anterior heart field-derived structures. Indeed, embryos lacking HDAC3 in the anterior second heart field (Mef2C-AHF-Cre) recapitulated these defects. Thus, HDAC3 mainly functions within the anterior part of the second heart field. Interestingly, embryos lacking HDAC3 in the second heart field display normal ventricular myocardium (data not shown). Previously, we demonstrated that HDAC3 is required in the primary heart field (Nkx2-5 enhancer-Cre) for development of the ventricular myocardium at the early stages of cardiogenesis (48). Taken together, these findings suggest that once the primary heart field progenitors have adopted a cardiac fate to form the nascent heart tube, the second heart field-derived ventricular myocardium can develop independently of HDAC3. This is consistent with prior evidence that the ventricular myocardium remains unaffected after genetic manipulations or ablation of the second heart field progenitor cells (10, 11).

Outflow tract malrotation in tandem with ventricular septal defect is one of the most common cyanotic congenital heart defects at birth (13). Proper alignment, orientation, and septation of the cardiac outflow tract into the aorta and the pulmonary artery require intricate coordination and interaction among multiple cell types, including neural crest and second heart field (68). Cardiac neural crest cell-dependent processes, such as outflow tract septation and distal outflow tract morphogenesis, appeared normal in embryos lacking HDAC3 in the second heart field (Fig. 1G). Previously, we demonstrated that neural crest-specific genetic deletion of HDAC3 resulted in anomalous outflow tract septation and distal outflow tract mor-

3 Z. Milstone and C. M. Trivedi, unpublished results.
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...dilatation. Consistent with these observations, significantly reduced expression of Col1a2 and Col3a1 in Hdac3<sup>Isl1KO</sup> embryos might explain bicuspid aortic valves and ascending aortic dilatation.

Recent elegant studies (74) clearly demonstrate that in addition to their complex roles in early development, TGF-β signaling pathways are involved in many human diseases as a result of mutations in components of the pathways or aberrant regulation of signaling. Our study identifies HDAC3 as a novel and specific regulator of the TGF-β signaling pathway at the extracellular, membrane, cytoplasmic, and nuclear levels. For instance, our data demonstrate that loss of HDAC3 augments expression of extracellular agonist ligands of the TGF-β pathway, such as TGF-β1. Simultaneously, expression of extracellular antagonists of these ligands, such as decorin, biglycan, fibromodulin, and collagen, is strikingly diminished in the absence of HDAC3, probably explaining increased TGF-β bioavailability in Hdac3<sup>Isl1KO</sup> embryos. Similarly, gain of NRP2, SUMO1, KPN1, and SMAD4 expression in Hdac3<sup>Isl1KO</sup> embryos would probably explain amplification of TGF-β signaling at the membrane, cytoplasmic, and nuclear levels.

FIGURE 7. HDAC3 functions within mesenchymal or smooth muscle cells to regulate outflow tract and semilunar valve development. A, dissected, fixed P0 hearts show malrotation of the aorta and pulmonary artery in Hdac3<sup>TaglnKO</sup> hearts. B, hematoxylin- and eosin-stained sections show double outlet right ventricle with aortic valve (AV) overriding a ventricular septal defect (arrow) in Hdac3<sup>TaglnKO</sup> P0 hearts. C, Masson’s trichrome staining of P0 aortic wall shows collagen on the outer surface of control aortic walls (white arrow, blue staining) and aberrant collagen expression throughout the vessel wall (black arrow, blue staining) in Hdac3<sup>TaglnKO</sup> aortic walls. D, Verhoeven-Van Giesen (VVG) staining of P0 aortic wall shows thick, continuous elastin fibers (white arrow, gray staining) in control aortas and thin, fragmented elastin fibers (black arrow, gray staining) in Hdac3<sup>TaglnKO</sup> aortas. E, Masson’s trichrome staining of P0 pulmonic artery wall shows collagen on the outer surface of control pulmonic artery walls (white arrow, blue staining) and aberrant collagen expression throughout the vessel wall (white arrow, blue staining) in Hdac3<sup>TaglnKO</sup> pulmonic artery walls. F, Verhoeven-Van Giesen staining of P0 pulmonic artery wall shows thick, continuous elastin fibers (white arrow, gray staining) in control aortas and thin, fragmented elastin fibers (white arrow, gray staining) in Hdac3<sup>TaglnKO</sup> pulmonic arteries. G, Movat’s pentachrome staining of P0 aortic valve shows expanded proteoglycans (arrow, blue) in Hdac3<sup>TaglnKO</sup> aortic valve compared with HDAC3<sup>+/-</sup> control. H, Movat’s pentachrome staining of P0 pulmonic valve shows expanded proteoglycans (arrow, blue) in Hdac3<sup>TaglnKO</sup> pulmonic valve compared with HDAC3<sup>+/-</sup> control. J, Masson’s trichrome staining of P0 aortic valves shows disorganized collagen expression (arrow, blue) in Hdac3<sup>TaglnKO</sup> aortic valve. J, Masson’s trichrome staining of P0 pulmonic valves shows disorganized collagen expression (arrow, blue) in Hdac3<sup>TaglnKO</sup> pulmonic valve. K, Verhoeven-Van Giesen stain of P0 aortic valves demonstrates disorganized collagen expression (arrow, red) in Hdac3<sup>TaglnKO</sup> aortic valve. L, Verhoeven-Van Giesen stain of P0 pulmonic valves demonstrates disorganized collagen expression (arrow, red) in Hdac3<sup>TaglnKO</sup> pulmonic valve. M, relative mRNA levels of Tgfl, Snai1, Smad4, Sumo1, Tagln, Lmna, and Col2a1 show increased expression in Hdac3<sup>TaglnKO</sup> > E14.5 hearts. N, EndMT assay of TGF-β antibody-treated or IgG antibody-treated outflow tract cushion explants from Hdac3<sup>TaglnKO</sup> and Hdac3<sup>+</sup> E10.5 hearts, 24 h after isolation. O, quantification of average radial migration, measured in eight directions, from TGF-β antibody-treated or IgG antibody-treated outflow tract cushion explants from Hdac3<sup>TaglnKO</sup> and Hdac3<sup>+</sup> E10.5 hearts, measured 24 h after isolation. P and Q, transcripts for Tagln (P) and Lum (Q) were detected by real-time qPCR in IgG- or TGF-β antibody-treated control and Hdac3<sup>TaglnKO</sup> outflow tract cushion explants derived from E10.5 hearts (mean ± S.E. (error bars), n = 3).
Our findings demonstrate that HDAC3 is required to maintain quiescence of valvular mesenchymal cells, the most prevalent cells of valvular cusps. Pathologic conditions promote activation and differentiation of valvular interstitial cells into myofibroblasts, smooth muscle-like fibroblasts that are observed in patients with semilunar valve disease (25). Myofibroblasts express smooth muscle-specific genes and secrete strikingly higher levels of TGF-β, proteoglycans, and collagen that alter the composition of the extracellular matrix (19). Hence, semilunar valve pathologies augment TGF-β bioavailability, which in turn promotes aberrant EndMT and transcriptional activation of smooth muscle and extracellular matrix genes within the myofibroblasts (24). TGF-β activation and aberrant extracellular matrix further promote the activation and differentiation of new valvular mesenchymal cells derived via EndMT (25). Consistent with these observations, our data dem-

![FIGURE 8. HDAC3 epigenetically silences TGF-β1 within valvular mesenchymal cells by recruiting PRC2 complex to the NCOR complex. A, relative mRNA levels of TGF-β1 in isolated cardiac endothelial cells (EC) or cardiac mesenchymal cells (MC) from E10.5 HDac3<sup>-/-</sup> outflow tract cushion explants, infected either with CRE or GFP control lentivirus. B, relative mRNA levels of TGF-β1 in isolated cardiac endothelial cells or dissected semilunar valves (SL) derived from HDac3<sup>-/-/KO</sup> and HDac3<sup>-/-</sup> E14.5 hearts. C, ChIP-qPCR analysis of HDAC3 occupancy upstream of TGF-β1 in isolated cardiac endothelial cells or cardiac mesenchymal cells from E10.5 outflow tract cushion explants. D, ChIP-qPCR analysis of HDAC3 occupancy upstream of TGF-β1 in isolated cardiac endothelial cells or dissected semilunar valves from E14.5 hearts. E-K, ChIP-qPCR analysis of H3K27 trimethylation (E), H3K27 acetylation (F), RNA polymerase II (G), CREBBP (H), EZH2 (I), EED (J), and SUZ12 (K) upstream of TGF-β1 in HDac3<sup>-/-/KO</sup> and control E14.5 semilunar valves. L, ChIP-qPCR analysis of HDAC3 occupancy upstream of TGF-β1 in E14.5 valvular mesenchymal cells infected with either control shRNA (sc-shRNA), EZH2 shRNA, or NCOR1 shRNA. M, ChIP-qPCR analysis of NCOR1 upstream of TGF-β1 in HDac3<sup>-/-/KO</sup> and control E14.5 semilunar valves. N, Co-ChIP for HDAC3 and either EZH2, NCOR1, H3K27me3, H3K27ac, or polymerase II upstream of TGF-β1 in E14.5 wild-type dissected semilunar valves. O, total lysates from E14.5 wild-type pooled semilunar valves were immunoprecipitated (IP) by EZH2 antibody, and Western blot was performed using HDAC3 antibody. α-Tubulin is shown as an input control. HDAC3 was quantified and normalized to total input α-tubulin using ImageJ software (mean ± S.E. (error bars), n = 3). P, ChIP-qPCR analysis of H3K27me3 upstream of TGF-β1 in E14.5 valvular mesenchymal cells infected with either control shRNA, EZH2 shRNA, or NCOR1 shRNA.

![Image of a diagram showing HDAC3 activity in valvular mesenchymal cells.]

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FIGURE 9. HDAC3 functions in a deacetylase-independent manner to regulate EndMT and epigenetic silencing of TGF-β1. A, HDAC3-FLAG and HDAC3 H134A, H135A-FLAG expression constructs were transfected in HEK-293T cells. Expression was detected by Western blot from whole cell lysates using FLAG antibody. GAPDH is shown as a loading control. B, HDAC3-FLAG and HDAC3 H134A, H135A-FLAG expression was quantified and normalized to total input GAPDH using ImageJ software (mean ± S.D. (error bars), n = 3). C, HDAC activity of HDAC3-FLAG and HDAC3 H134A, H135A-FLAG expression was quantified against a pseudosubstrate. D, EndMT assay of control- or Cre-infected E10.5 Hdac3 F/F outflow tract cushion explants co-infected with GFP, HDAC3-FLAG, or HDAC3 H134A, H135A-FLAG lentiviruses, imaged 24 h after isolation. E, quantification of average radial migration, measured in eight directions, of control- or Cre-infected E10.5 Hdac3 F/F outflow tract cushion explants co-infected with GFP, HDAC3-FLAG, or HDAC3 H134A, H135A-FLAG lentiviruses, measured 24 h after isolation (mean ± S.E. (error bars), n = 3). F, relative mRNA levels of Tgf-β1 in control- or Cre-infected E14.5 Hdac3 F/F valvular mesenchymal cells co-infected with control, HDAC3-FLAG, or HDAC3 H134A, H135A-FLAG lentiviruses (mean ± S.E., n = 3). G, a 1309-bp TGF-β1 promoter luciferase reporter (WT) or a truncated, 1267-bp TGF-β1 promoter luciferase reporter, lacking an HDAC3-enriched region (mutant) were transfected in murine endothelial cells with and without an HDAC3-FLAG or HDAC3 H134A, H135A-FLAG expression plasmid. Induction is represented as a ratio of firefly and Renilla luciferase activity. H–L, ChIP-qPCR analysis of H3K27 acetylation (H), H3K27 trimethylation (I), EZH2 (J), EED (K), and SUZ12 (L) upstream of TGF-β1 in control- or Cre-infected E14.5 Hdac3 F/F valvular mesenchymal cells co-infected with control, HDAC3-FLAG, or HDAC3 H134A, H135A-FLAG lentiviruses (mean ± S.E., n = 3).
A loss of HDAC3 in second heart field progenitor cells leads to outflow tract and semilunar valve pathologies. Strikingly, genetic deletion of HDAC3 in differentiated mesenchymal and smooth muscle cells (Hdac3TaglnKO) recapitulates the majority of these phenotypes. However, deletion of HDAC3 in differentiated cardiomyocytes (Hdac3Myh6KO) or endothelial cells (Hdac3Cdh5KO) did not recapitulate the cardiovascular defects observed in Hdac3Isl1KO embryos.

Hdac3Isl1KO hearts exhibit disorganized collagen and elastin within dilated aortic walls and hyperplastic semilunar valves containing activated myofibroblasts and disorganized extracellular matrix. In both control and Hdac3Isl1KO cardiac endothelial cells, the upstream regulatory region of TGF-β1 is occupied by RNA polymerase II and CREBBP and exhibits H3K27 acetylation concomitant with TGF-β1 expression. In control semilunar valves, endothelial cells undergo EndMT to become mesenchymal cells. In these mesenchymal cells, NCOR1, HDAC3, and PRC2 complex (EZH2, EED, and SUZ12) are recruited to the upstream regulatory region of TGF-β1, which becomes trimethylated on histone H3 Lys-27, and TGF-β1 expression is epigenetically silenced. In Hdac3Isl1KO hearts, EZH2, EED, and SUZ12 are not recruited to the TGF-β1 regulatory region, RNA polymerase II and CREBBP are present, and histone H3 Lys-27 remains acetylated, favoring aberrant expression of TGF-β1 in mesenchymal cells. TGF-β1 activates mesenchymal cells to become myofibroblasts, which perpetuate EndMT and activation of mesenchymal cells through continued induction of TGF-β1 and aberrant expression of extracellular matrix, including proteoglycans and collagen.
onstrate that loss of HDAC3 leads to strikingly higher expression of TGF-β1, smooth muscle genes, and aberrant expression of extracellular matrix genes within the valvular mesenchymal cells, and these changes were largely abolished by TGF-β-neutralizing antibody. Taken together, our findings support a model in which HDAC3-mediated repression of TGF-β1 within valvular mesenchymal cells is required to prevent perpetual activation of EndMT and to maintain extracellular matrix homeostasis in the semilunar valves.

Catalytically inactive enzymes or “pseudoenzymes” are clearly widespread, occurring in most enzyme families, including HDACs (66). For instance, class II HDACs, such as HDAC4, -5, -7, and -9, lack deacetylase activity due to a His substitution at the key Tyr residue (75). These pseudoenzymes function as scaffolding proteins to recruit various co-factors in a signal-dependent manner (42). Similarly, HDAC1 and -3, class I HDACs, and their deacetylase-dead mutants have the same degree of in vivo effect on cardiomyopathy (76). During endothelial-to-mesenchymal transition, epigenetic mechanisms regulating gene activation and silencing remain largely undefined (77). The present study provides the mechanistic basis of a deacetylase-independent function of HDAC3 as a scaffold to recruit methyltransferase components of PRC2 (polycomb repressive complex 2) to epigenetically silence TGF-β1. Repressor complex-mediated gene silencing is critical to maintain cellular identity of differentiated cells through multiple divisions (78). Recent evidence suggests that PRC2-mediated epigenetic silencing is maintained for many cell generations. Ezh2, the major H3K27 methyltransferase of the PRC2 complex, supported by EED and SUZ12, catalyzes methylation of H3K27 to mediate chromatin compaction and thereby regulate differentiation and cell identity (79). Consistent with this model, our data demonstrate that HDAC3 recruits Ezh2, EED, and SUZ12 to the NCOR complex in a deacetylase-independent manner and thereby mediates epigenetic silencing of TGF-β1 within valvular mesenchymal cells. In the absence of HDAC3, the NCOR complex fails to recruit PRC2 complex, resulting in aberrant recruitment of CREBBP, which catalyzes acetylation of H3K27 to mediate activation of TGF-β1 expression, which in turn promotes perpetual transdifferentiation of valvular mesenchymal cells to myofibroblasts. Subsequently, activated myofibroblasts secrete higher levels of extracellular matrix proteins and promote aberrant EndMT. Future investigations dissecting the modes of interaction among HDAC3, Ezh2, EED, and SUZ12 will be important to determine PRC2-independent functions of HDAC3. Similarly, murine models expressing catalytically inactive HDAC3 would further define deacetylase-independent functions of HDAC3 during cardiogenesis.

Elucidation of the role of HDAC3 in the second heart field is directly relevant to human congenital heart disease. Marfan syndrome, Ehlers-Danlos syndrome, and Loeys-Dietz syndrome, caused by mutations and activation of TGF-β signaling genes, are associated with congenital cardiovascular anomalies resembling those seen in Hdac3<sup>−<sub>1</sub>KO</sup>, Hdac3<sup>−<sub>2</sub>CKO</sup>, or Hdac3<sup>−<sub>Tag</sub>CKO</sup> embryos. Our data suggest that many of these cardiovascular defects, such as aortic dilatation, outflow tract defects, bicuspid aortic valve, semilunar valve stenosis, and membranous ventricular septal defects, are likely to be caused by defective morphogenesis of the second heart field and aberrant extracellular matrix as a result of anomalous epigenetic silencing of TGF-β1.

Author Contributions—S. L. L., H. P. J., and C. M. T. designed, performed, and analyzed the experiments. S. L. L. and C. M. T. coordinated the study and wrote the paper. C. M. T. conceived the study, acquired funding, and supervised S. L. L. and H. P. J. All authors reviewed the results and approved the final version of the manuscript.

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