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Role of Hdac3 in Murine Coronary Vessel Development: A Master's Thesis

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Role of Hdac3 in Murine Coronary Vessel Development

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By

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Role of Hdac3 in Murine Coronary Vessel Development

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This work is dedicated to my loving fiancée

Raquel D. Barron

Who has loved and supported me through everything.

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Abstract

Coronary vessel development is a crucial part of heart development requiring the interplay of the epicardial, myocardial and endocardial layers of the heart for proper formation. Coronary vascularization is regulated by a host of transcription factors further regulated by chromatin remodeling enzymes, including Histone Deacetylases (HDACs). To investigate the functions of HDACs in coronary vascular development, we have deleted Hdac3 in endocardial cells using Cre LoxP technology. Endocardial cell-specific deletion of Hdac3 results in aberrant coronary vessel formation and complete postnatal lethality. We have thus shown that Hdac3 is a critical regulator of the coronary vascular development pathway.

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Chapter I Introduction

Vascular development of the murine embryo

Vascular development begins with the specification of hemangioblasts, the precursors of both angioblasts and hematopoetic cells, from mesodermal tissues (Choi et al. 1998). Hemangioblasts coalesce to form the preliminary vascular plexus that will give rise to the major blood vessels of the embryo (Pardanaud and Dieterlen-Lièvre 1993). The vascular plexus forms as early as embryonic day 7.5 (e7.5) as indicated by the formation of the bilateral dorsal aorta. Development continues with the sinus venosus, the conotruncus, and the primitive heart tube forming by embryonic day e8.5 (Baldwin 1996). This has led to the belief that the heart forms initially as a part of this initial vascular plexus formation and becomes surrounded by a myocardial mantle separated by a layer of extracellular matrix known as the cardiac jelly (Baldwin 1996). From the preliminary vascular plexus, the vascular system of the developing embryo expands throughout the whole embryo. Vascularization of the murine embryo continues with formation of blood islands, consist of a ring of angioblasts that will differentiate into endothelial cells surrounding a core of hematopoeitic cells (Risau and Flamme 1995). Angioblasts can come together to form lumen-less chords of cells in response to environmental cues as demonstrated by experiments with chick quail chimeras (Klessinger and Christ 1996). These chords then rearrange themselves to form a vessel lumen by either altering intracellular distance or by arranging intracellular vacuoles to generate space between cells in a process known as tubulogenesis (Iruela-Arispe and Davis 2009). The overall formation of vessels from cellular precursors is termed vasculogenesis. Endodermal and ectodermal tissue layers that lack the ability to

produce hemangioblast progenitor cells are largely vascularized by angiogenesis (Risau and Flamme 1995). It is very common in vascular development for vessels generated by vasculogenic processes to give rise to other vessels through angiogenesis (Siekmann and Lawson 2007).

Angiogenesis is the extension of the vascular network from a pre-established plexus. Endothelial cells making up the lining of the forming blood vessels respond to angiogenic signaling factors including VEGF and Angiopoietin 2 (Ang2) to produce sprouts controlled by Notch and Delta-like ligand 4 (Dll4) signaling cascades (Phng and Gerhardt 2009; Siekmann and Lawson 2007). A "tip" cell designated by expression of Dll4 triggers Notch expression in neighboring cells, inhibiting migration and altering cellular responses to VEGF (Benedito and Hellström 2013). When exposed to VEGF, tip cells express philopedia and migrate towards the VEGF signal. Neighboring "stalk" cells divide in response to VEGF signaling, elongating the sprout as it migrates towards the source of VEGF (Phng and Gerhardt 2009). In addition, stalk cells generate the vessel lumen and express adhesion molecules critical to forming a stable, functioning vessel (Phng and Gerhardt 2009). Sprouting vessels meet through anastomosis, forming new connections between major vessels (Phng and Gerhardt 2009). Tie-2-Angiopoietin 2 signaling also stimulates an angiogenic response, affecting mostly migration and tubulogenesis (Mochizuki et al. 2002). Some studies suggest that angiogenesis and vasculogenesis can occur simultaneously, with angiogenic sprouts connecting networks of formerly avascular vessels to systemic circulation. Once formed, these vessels are stabilized by the association of advential layers of cells and layers of

extracellular matrix that form around the endothelial inner lining of the blood vessels (Orlidge and D'Amore 1987; Gaengel et al. 2009).

Advential cells include mural cells and connective tissue cells that surround mature blood vessels. Mural cells consist of either pericytes or smooth muscle cells that are a part of the advential layer of capillaries, arteries and veins respectively. These cells originate from mesodermal tissues and migrate to vascular endothelial cells in response to a chemotactic signaling gradient including signaling molecules such as PDGFβ expressed by vascular endothelial cells (Gaengel et al. 2009). Mural cell interactions with endothelial cells down regulate proliferative and migratory endothelial signaling pathways while up regulating endothelial cell differentiation and survival signaling pathways (Gaengel et al. 2009). In addition, Tie-2 signaling promotes mural cell adhesion to vascular endothelial cells (Gaengel et al. 2009). Notch signaling pathways down regulate angiogenic process in favor of a quiescent phenotype (Phng and Gerhardt 2009). Fibroblasts make up the connective cells in the advential layer of blood vessels, providing a connective tissue outer layer that provides structural stability (Gaengel et al. 2009). Mural cells also contribute to the structural stability of blood vessels, allowing them to withstand the stresses of systemic blood flow and aiding in maintaining blood pressure (Gaengel et al. 2009). Association of advential cells is a key part of the blood vessel maturation process, with defects in advential layer development leading to leaky vessels and embryonic death (Gaengel et al. 2009).

In addition to advential cells, the proteins comprising the ECM surrounding endothelial cells play a significant role in the development of vascular networks.

Networks of ECM deposited by endothelial cells and surrounding tissue cells contain inactive isoforms of VEGF, which once activated by matrix metaloprotease-dependent cleavage from the ECM trigger angiogenic growth of vascular networks (Akhta et al. 2001). Interaction with isolectins fibronectin and lamanin contribute greatly to vascular network formation (Risau and Lemmon 1988). Fibronectin increases the proliferative and migratory potential of angioblasts while lamanin decreases the proliferative and migratory potential of angioblasts in favor of promoting angioblast differentiation into endothelial cells (Risau and Lemmon 1988). Endothelial cell receptor interactions with integrin and immunoglobulin components of the surrounding ECM environment affect proliferation, differentiation, migration, activation, and gene expression (Baldwin 1996). For instance, the β1 integrin family plays a critical role in endothelial tube development, while α5 integrins have an earlier acting role in endothelial cell fusion to form a functional early vessel network (Baldwin 1996; Drake et al. 1992; Yang et al. 1993). The Immunoglobulin-like cell receptor molecule PECAM-1 is expressed by endothelial cells throughout the vasculature and has a significant role in cell-cell binding interactions between vascular cells and non vascular cells that in the formation of a stable vascular network (DeLisser et al. 1994). ECM proteins and advential cells provide a stable network for angioblast growth and differentiation. Without the signaling and structural support of advential cells and the ECM, the endothelial cells making up the structure of the preliminary blood vessel will regress to be incorporated into other stabilized blood vessels or commit apoptosis (Phng and Gerhardt 2009).

Many transcription factor families play a significant role in the formation of vascular networks through developmental studies in vertebrate model systems. Among the earliest transcription factors to become activated in these processes are the transcription factors Tal1 and Gata2 that distinguish the hemangioblast lineage from the mesodermal stem cells. Activation of Tal1 is sufficient to cause stem cells to commit to an endothelial cell fate (De Val and Black 2009). Genetic analysis of common endothelial cell genes also shows an abundance of Gata2 binding sites, suggesting that Gata2 is a key regulator of endothelial cell fate (De Val and Black 2009). These transcription factors are controlled by the transcription factors Fli-1 and Etv2 (Brown et al. 2000, -1). Among these early transcription factors are the FOX and ETS families of transcription factors that are key players in vasculogenic processes (De Val and Black 2009). Enhancer binding sites for these factors are found among the promoter regions of several key genes in endothelial development including Tal1, Tie-2, Flk-1, and VE-Cadherin (De Val and Black 2009). Many other factors are involved in the later processes of angiogenesis. For example, the aforementioned Notch signaling pathway limits angiogenic activity, seemingly to maintain the order of the vascular network (Siekmann and Lawson 2007; Phng and Gerhardt 2009).

Commitment to arterial or venous fate seems to be determined by both genetic and environmental factors. Directional flow of blood plays a significant role in determining arterial or venous fate. Ligation of arteries causes the vessels downstream of the ligation to shift from an arterial to venous fate in order to keep systemic flow of blood undisturbed throughout the organism as a whole (le Noble et

al. 2004). Genetically, several families of transcription factors are responsible for commitment to arterial or venous fate. A reduction in Notch expression causes a loss of arterial blood vessels in addition to severe vascular developmental defects as demonstrated by knockout models of Notch and Notch ligands, including Hey1 and Hey2, in mice and knockdown models of Notch and Notch ligands in Zebrafish (Phng and Gerhardt 2009). Knockout of the FoxC family members FoxC1 and FoxC2 causes almost complete absence of arterial markers in affected vasculature (Seo et al. 2006). Sox7 and Sox18 null mice display severe vascular defects that include complete loss of tail and head circulation. Arterial development is also stunted as a result of Sox7 and Sox 18 deletion in endothelial cells (Cermenati et al. 2008, 18). Conditional deletion of COUP-TFII results in a shift from venous cell markers in veins to arterial markers (You et al. 2005). COUP-TFII functions via suppression of Notch activity, suggesting that arterial and venous identity is a balancing act regulated by relative amounts of Notch activity (Phng and Gerhardt 2009).

Murine coronary vessel development

Coronary development occurs late in heart development. Briefly, the heart forms from the primary and secondary heart fields of the splanchic mesoderm starting around embryonic day 7.5 (e7.5) in the murine embryo (High and Epstein 2008). Over the course of one day, the primary heart field forms the heart tube; a cylindrical beating organ composed of an outer myocardial and an inner endocardial layer. Over the next couple days, the heart undergoes the complex process of looping and partitioning that specifies the two ventricles and two atria and the preliminary outflow tract of the heart by e10.5 (High and Epstein 2008). By e14.5,

the chambers of the heart are fully formed and most major morphological changes are completed with the exception of coronary vascular development (High and Epstein 2008).

Coronary vascular development begins with the formation of the epithelial cell layer of the heart around e9.5 as an outgrowth from the proepicardial organ. The proepicardial organ originates from the coelomic mesothelium, which contributes to the epicardium of the heart as well as the endothelial cells lining multiple tissues (Katz et al. 2012). Cells from the proepicardial organ attach to the atrioventricular region of the heart and proceed to migrate across the surface of the heart, forming the complete epicardial layer around e12.5 (Katz et al. 2012). These cells can be divided into different lineages based on cellular markers that denote their eventual fate. Proepicardial cells expressing Tbx18/WT-1 become smooth muscle cells while Sema3D positive cells become coronary endothelial cells (Katz et al. 2012). Coronary vascular progenitor cells are specified by epithelial mesenchymal transition (EMT) by e11 (Katz et al. 2012). Newly generated mesenchymal cells migrate into the subepicardial space between the epicardial and myocardial muscle layer of the heart, contributing to the coronary veins and coronary arteries (Katz et al. 2012). Endothelial cells from the endocardium also migrate into the myocardium to contribute to the coronary vessel system (Wu et al. 2012).

Vascularization of the heart and vascularization of the embryo as a whole share many of the same signaling processes and events. As previously mentioned, coronary endothelial cells originate from mesenchymal tissues created by EMT of

epithelial cells that make up the epicardium and from migration of endothelial cells from the endocardium (Wu et al. 2012; Pérez-Pomares et al. 2002). Migration of endothelial and mural cell progenitors of the coronary vessel system occurs in response to hypoxic signals from myocardial cells. As the heart increases in mass, the process of diffusion cannot keep up with cellular demand for nutrient and waste exchange. As a result, myocardial cells express signaling factors including Hypoxic Induction Factor 1alpha (HIF-1α) triggering the up regulation of FGF and VEGF signaling in hypoxic regions (Tomanek et al. 2003; Yue and Tomanek 1999). VEGF signaling stimulates the migration of coronary vessel progenitors from the epicardium and endocardium into the myocardium of the heart (Tomanek 2005; Wu et al. 2012). These progenitors co-localize to form the initial coronary vascular plexus, first seen as blood islands interspersed throughout the myocardium and subepicardial space (Wu et al. 2012; Tomanek 2005; Pérez-Pomares et al. 1998). Blood islands, angioblasts, and advential cells come together as described above to form coronary blood vessels.

Upon the establishment of the initial coronary vascular plexus, vessel networks expand towards the aortic lumen driven by angiogenic signaling molecules (Tomanek et al. 2003; Tomanek 2005; Reese et al. 2002). Orifices termed coronary ostia form in the aortic lumen through apoptosis of endothelial and surrounding mural cells and the invasion of coronary vasculature (Tomanek 2005). The mechanisms that govern exact placement of the coronary ostia in the aortic lumen have not been fully described, however a combination of angiogenic signaling gradients and the specific curvature of the aorta at common regions of ostia

formation play a key role in ostial placement (Turner and Navaratnam 1996; Eralp 2005). These observations are further supported by the increase in coronary artery anomalies in murine knockout models and in cases of outflow tract defects. Defects in coronary artery formation include improper coronary artery placement, either in the aortic lumen or in the pulmonary artery lumen (Angelini 2007). Normally, two coronary ostia are present, providing origins for the three major coronary arteries: the left anterior descending artery, the right anterior descending artery, and the right circumflex artery (Tomanek 2005). The left anterior descending artery works its way from the aorta down the left ventricle wall, while the right descending artery makes its way down the right ventricle wall (Tomanek 2005). The right circumflex artery branches from the right anterior descending artery and works its way around to the posterior side of the heart. In the murine heart, the number and branching patterns of the coronary arteries are the main consistent features of coronary vascular development (Reese et al. 2002).

Coronary patterning anomalies restrict the blood flow to the heart by forming stunted and incomplete coronary vascular networks that do not allow for proper systemic flow of blood throughout the heart (Angelini 2007). Defects can manifest as anomalous origin of the coronary arteries, classified as coronary ostia locations in areas seen in less than 1% of human patients and causing clear circulatory defects (Angelini 2007). Anomalous origin of the coronaries can present as ostia formation on the pulmonary artery, ostia formation on a single side of the aorta and or the formation of separate ostia for the right circumflex artery among many other possible origins. Intrinsic structural anomalies such as coronary vessel

stenosis, (narrowing), ectasia (dialation), or atresia (under development or total loss of a coronary vessel) can be observed in the coronary vasculature (Angelini 2007). Defects in coronary vascular structure and origin may result from defective vasculogenic and or angiogenic processes caused by defects at the cellular level including migration, differentiation, proliferation, apoptosis, and defects in cell-cell adhesion. Fatal defects in coronary vascular development have been observed in murine knockout models of key genes involved in each pathway. Defects in migration caused by deletion of myocardial VEGF or endocardial VEGFR2a, result in embryonic lethality (Wu et al. 2012). Likewise deletion of GATA-4 results in absence of the epicardial layer of the heart. GATA-4 null embryos do not develop coronary vasculature, presumably due to the loss of coronary progenitor cells normally provided by the epicardium (Watt et al. 2004, -4). Defective differentiation of endothelial progenitors causes embryonic mortality of WT-1 deficient embryos (Kreidberg et al. 1993). Ablation of VEGF and FGF signaling in postnatal rat hearts results in defects in endothelial proliferation required for postnatal coronary vessel growth (Tomanek et al. 2001). Improper apoptotic signaling during coronary artery formation contributes to a coronary vascular system unconnected to systemic blood flow (Eralp 2005). Improper expression of VE-Cadherin causes vascular fragility in zebrafish embryos when knocked down with morpholinos (Montero-Balaguer et al. 2009).

Establishment of systemic circulation through the coronary vessel system triggers maturation processes that remodel the vasculature to ensure proper flow throughout the coronary vessel systems. Vessels are stabilized by the incorporation

of advential cells; smooth muscle cells, pericytes, and fibroblasts, generated from the epicardial mesenchyme that provide both mechanical stability to vessel walls and promote differentiation and survival of endothelial cells (Tomanek 2005; Mikawa and Gourdie 1996). Advential cells migrate and differentiate into the coronary vessel network through the PDGF, TGFβ signaling pathways as in the murine vasculature as a whole (Hirschi et al. 1998). Unstabilized vessels, vessels lacking advential cell associations, regress by endothelial cell dedifferentiation or apoptosis upon exposure to the hyperoxic conditions generated by establishment of blood flow through the coronary vascular system (Tomanek 2005). Vessels are pruned in this manner postnatally in order to ensure proper communication of blood through arterial and venous networks. Postnatal pruning ensures that blood flow moves in one uniform direction by removing improper connections between coronary arterial and venous networks.

Chromatin dynamics

Chromatin state is an often-overlooked element in the regulation of protein expression. Chromatin is a mixture of DNA and organizational proteins that package the genetic information of the cell. The smallest unit of DNA packaging, the nucleosome, is composed of approximately 147 base pairs of DNA wrapped around a histone octamer. Histone octamers are composed of two sets of the H2A, H2B, H3, and H4 histone subunits consisting of a globular structural domain and a linear tail domain that is largely structureless (Brownell and Allis 1996). Specific residues of the histone tail can play host to posttranslational modifications including phosphorylation, methylation, and acetylation among others that cause changes in

the chromatin structure leading to processes such as DNA replication or transcription or repression of gene expression (Brownell and Allis 1996). Initially, these marks were believed to directly change the chemical interactions between the net positive histone subunits and the net negative DNA backbone, a belief that persists to this day. Recent work however, suggests that these histone tail modifications are more likely to alter chromatin composition through the association of protein complexes that physically unwind or otherwise shift the histone core away from the DNA molecule (Flaus and Owen-Hughes 2001). Once the histone core has been displaced from a particular region, DNA replication or transcription factors can associate and interact with the DNA template, replicating DNA or transcribing an mRNA copy for translation or other processing events (Flaus and Owen-Hughes 2001). The proposed system of histone tail marking by posttranslational modifications creating binding sites for chromatin remodeling enzymes has lead to the distinction of two different types of remodeling enzymes; covalent histone remodeling enzymes that catalyze the posttranslational modifications and ATP-dependent histone remodeling enzymes that unwind the nucleosome and displace the histone core in response to the posttranslational modifications present on histone cores.

Covalent histone remodeling enzymes including phosphorylases, methylases, and acetyltransferases do not catalyze the unwinding of the nucleosome themselves; they mark histone tails to create a target-binding site for active histone remodeling enzymes to form remodeling complexes (Gangaraju and Bartholomew 2007). These marks can be transient or persistent depending on the region of DNA specified.

Regions containing highly transcribed genes tend to have a significant number of acetyl marks consistently present on their neighboring histone tails. Other, less frequently transcribed genes tend to only have transient acetyl marks on nucleosomes surrounding their promoter regions during times of gene expression (Brownell and Allis 1996). In general, acetylation and phosphorylation tend to lead to transcriptional activation and methylation tends to lead to transcriptional repression (Flaus and Owen-Hughes 2001).

The posttranslational state of a given histone tail is largely dependent upon the interactions between enzymes that catalyze the addition of posttranslational modifications and enzymes that remove these posttranslational modifications. As such, phosphorylated histone tails can be dephosphorylated by phosphatases, acetylated histone tails can be deacetylated by deacetylases and methylated histone tails can be demethylated by demethylases. Generally, dephosphorylation and deacetylation leads to transcriptional repression and demethylation leads to transcriptional activation (Flaus and Owen-Hughes 2001). Transcription factors may also be posttranslationally modified to alter their activity. We have shown recently that TBX5 is acetylated in murine cardiomyocyte progenitors, and that deacetylation of TBX5 is a key factor in proper cardiomyocyte differentiation in murine heart development (Lewandowski et al. 2014). Among these modifications, acetylation is among the most common posttranslational modification regulating transcriptional activity, marking regions of transcriptionally active genes. Acetyl marks are deposited on lysine residues present on histone tails and other proteins by acetyltransferases and are removed by lysine deacetylases.

Posttranslational modifications lead to nucleosome re-positioning, moving the DNA molecule away from the histone core to generate enough space for replication and or transcription to occur. Nucleosome unwinding is accomplished by ATP-dependent chromatin remodeling enzymes that use the chemical energy of ATP to catalyze the unwinding of the nucleosome or the removal of the histone core from the DNA molecule. ATP-dependent chromatin remodeling enzymes are classified as members of four main families based upon homology and functionality; the INO80, SWI/SNF, Mi-2 CHD and ISWI families (Bao and Shen 2007). These remodeling complexes all share an ATP-hydrolyzing core that generates superhelical tension in the DNA molecule, permitting local reconstruction of the DNA twist (Flaus and Owen-Hughes 2001). Though there is some commonality in the ATP hydrolyzing domain of ATP-dependent chromatin remodeling enzymes, there are significant functional differences between each family of chromatin remodeling enzymes (Gangaraju and Bartholomew 2007). For example, ISWI requires a nucleosome with histone tails to catalyze enzymatic reactions, while Mi-2 does not require histone tails for reaction catalysis (Flaus and Owen-Hughes 2001). The INO80 family of ATP-dependent remodeling enzymes have a distinct insertion in the otherwise conserved ATPase domain that may contribute to the INO80 family's unique role in H2A.Z histone variant localization, making the INO80 a key factor in double strand break repair and replication fork progression (Watanabe and Peterson 2010). Some ATP-dependent remodelers have domains that allow them to interact with other DNA binding proteins such as transcription factors and or bromodomains that could interact with modified histone tails, suggesting specific interaction with chromatin

molecules at target regions based on protein binding and histone tail modifications (Flaus and Owen-Hughes 2001). For instance, SWI/SNF is recruited to the *HO* genomic region by the transcription factor Swi5p. SWI/SNF then catalyzes nucleosome structural changes that allow for the acetyltransferase SAGA to acetylate H3 and H4 lysine residues with the yeast *HO* genomic region (Cosma et al. 1999; Krebs et al. 1999). As such, some ATP-dependent remodeling complexes seem to play a vital role in modulating chromatin transcriptional accessibility. The specific order of events varies depending on the specific genomic region. In some cases acetylation is required before any histone displacement occurs. In other cases, histone displacement is required for acetylation and subsequent transcription to occur. Histone deacetylases (HDACs) fit into this process by removing and inhibiting the acetylation of histone tails, shifting chromatin from an accessible euchromatic state to a more repressive heterochromatin state through discouragement of ATP-dependent remodeling complex association and binding, or discouragement of enhancer and transcription factor binding.

Transcriptional activation is balanced by the interplay between HATs and HDACs. HATs activate transcriptional activity by adding acetyl groups to lysine residues present in histone tails, while HDACs catalyze the reverse reaction (Brownell and Allis 1996). Interestingly, HDACs and HATs frequently have the same binding partners. A key example related to cardiac development is the MEF2 transcription factor, which interacts with class II HDACs such as HDAC5 and HDAC9 to suppress MEF2 activation of hypertrophic gene pathways. When HDAC5 or HDAC9 are knocked out using null alleles, cardiac response to hypertrophic stimuli

mediated by MEF2 is increased as a result (Chang et al. 2004). HDAC1 represses Notch gene expression in Zebrafish neural development by competitive inhibition of the Notch intracellular domain binding to RBPJ (Cunliffe 2004). Thus, modulation of chromatin state through the balanced interactions of HDACs and HATs plays a significant role in the transcription of genes involved in many developmental processes.

Histone Deacetylases

To date, there are 18 HDACs that make up four different classes based on homology to yeast deacetylase proteins and protein activity (Haberland et al. 2009b). Classical HDACs make up HDAC class I, class II, and class IV, while non-classical HDACs known as Sirtuins make up class III HDACs. Sirtuins differ from classical HDACs in that they require NAD+ for their catalytic activity (Michan and Sinclair 2007). Classical HDACs are further broken down based on homology with yeast deacetylases. Ubiquitously expressed class I HDACs Consisting of HDAC1, HDAC2, HDAC3, and HDAC8 share homology with the yeast HDAC RPD3 (Haberland et al. 2009b). Class I HDACs are commonly found in protein complexes such as the NURD CoREST complex for HDAC1 and HDAC2 and NCOR/SMRT complex for HDAC3 with the exception of HDAC8 (Haberland et al. 2009b). Class II HDACs are split into two subgroups: class IIa, made up of HDAC4, HDAC5, HDAC7, and HDAC9 and class IIb, made up of HDAC6 and HDAC10. Class II HDACs are homologous to yeast HDAC Hda1 and differ from class I HDACs in several respects (Haberland et al. 2009b). Class II HDACs as a whole have a more tissue-specific expression pattern. Also, class II HDACs contain nuclear import and export signals, which allow them to

localize to the nucleus and the cytosol depending on association with protein chaperones such as 14-3-3 and MEF2 which localize class II HDACs to the cytoplasm and the nucleus respectively (Lahm et al. 2007). Class II HDACs also have decreased HDAC activity due to a residue substitution in the catalytic active site of the HDAC domain that substitutes a tyrosine residue with a histidine residue, resulting in a 1000-fold decrease in protein activity (Lahm et al. 2007). HDAC6 and HDAC10 are most homologous to one another. HDAC11 is the sole class IV HDAC. Though HDAC11 has been determined to have histone deacetylase activity, no biological function has been determined for this HDAC (Haberland et al. 2009b).

HDAC1 and HDAC2

Hdac1 is critical in embryonic development. Deletion of Hdac1 in the murine embryo results in embryonic death prior to e10.5 as a result of proliferation defects. Observed proliferation defects are a result of increased expression of CDK inhibitors p21 and p27, believed to be a response to increased global acetylation caused by loss of Hdac1 activity (Lagger et al. 2002, 1). Conditional deletion of the Hdac1 allele in the cardiac myocyte linage using a *αMHC*-Cre driver did not result in a perceivable phenotype. Our prior studies suggest that deletion of Hdac2 results in partial lethality due to increased cardiomyocyte hyperplasia. Surviving Hdac2-null mice displayed resistance to hypertrophic stimuli through the modulation of the Gsk3β pathway (Trivedi et al. 2007). Hdac2 also interacts with Hopx to regulate cardiomyocyte proliferation by modulating Gata4 transcriptional activity (Trivedi et al. 2010).

HDAC3

Murine embryos homozygous for an Hdac3-null allele do not survive past e9.5 as a result of defects in gastrulation (Montgomery et al. 2008, 3). Defects in gastrulation may be a result of defects in S phase DNA repair, as seen in Mouse Embryonic Fibroblasts (MEFs) upon deletion of Hdac3 (Bhaskara et al. 2008). Cardiomyocyte-specific deletion of Hdac3 results in metabolic defects in cardiomyocytes, including lipid accumulation and defective utilization of glucose in cardiomyocyte cells (Montgomery et al. 2008). Liver specific deletion of Hdac3 causes a similar effect, with lipid accumulation in liver tissue leading to a hypertrophic growth response.[13] Previously, we have shown that Hdac3 promotes cardiomyocyte proliferation by repressing cyclin-dependent kinase inhibitors (Trivedi et al. 2008). Recently, we have shown that Hdac3 deacetylates Tbx5 in order to control differentiation of cardiomyocyte progenitor cells (Lewandowski et al. 2014). Hdac3 plays a significant role in endothelial cell progenitor cell differentiation. Activation of Hdac3 is required in Sca1+ progenitor cells to produce endothelial cells (Xiao 2006). Shear stress induces Hdac3 activity in stem cells through the Flk-1-PI3K-Akt pathway, which leads to activation of p21 and differentiation into endothelial cells (Zeng et al. 2006, 3). Arterial injury was reduced when endothelial cells differentiated from embryonic stem cells by Hdac3 stimulation were introduced in injury models (Xiao 2006, -1; Zeng et al. 2006). Hdac3 inhibits angiogenesis in tumor cells by reducing VEGF signaling and down regulating plasmogen activation inhibitor-1 (PAI-1) expression (Park et al. 2013). Isoforms of HDAC3 regulate endothelial mesenchymal transition through altering HDAC3-Akt activity (Zeng et al. 2013, 2). In addition, Hdac3 also deacetylates Mef2D,

a transcription factor with known involvement in cardiac hypertrophy (Grégoire et al. 2007).

HDAC8

HDAC8 is not associated with any known protein complexes unlike the other three members of the Class I HDAC family (Haberland et al. 2009b). Also, Hdac8 does not cause cardiovascular defects upon deletion, suggesting that Hdac8 may not play a major role in cardiovascular development. Hdac8 global deletion causes perinatal lethality in mice due to unstable skull development as a result of mis-regulation of homeobox transcription factors (Haberland et al. 2009a).

Class IIa HDACs

Deletion of Hdac4 in mice results in premature ossification of skeletal tissue. Resulting mutant mice die of asphyxiation due to inhibition of rib cage expansion required for proper breathing (Vega et al. 2004). Hdac4 inhibits the Runx2 transcription factor, which promotes chondrocyte hypertrophy and differentiation. Over expression of Hdac4 in transgenic murine models has shown the inverse effect, resulting in mice with under ossified skeletons (Vega et al. 2004, 4). Deletion of Hdac5 and Hdac9 individually in murine models displays increased cardiac hypertrophic phenotypes in response to hypertrophic stimuli (Zhang et al. 2002; Chang et al. 2004). Of further interest, simultaneous deletion of Hdac5 and Hdac9 results in mice with ventricular septal defects and thin ventricle walls (Chang et al. 2004). Hdac7 deletion in murine models causes embryonic lethality by e11, displaying vascular dilation in major arteries (Chang et al. 2006). Over-expression of Matrix Metaloprotease 10 (MMP10), an ECM degrading protein was linked to the

observed vascular dilation phenotype. MEF2 activation of MMP10 is inhibited by interactions with Hdac7, suggesting that Hdac7 plays a key role in vascular integrity by transcriptionally repressing the expression of MMP10 (Chang et al. 2006).

HDAC6

Hdac6 is unique among the classical HDACs. Structurally, Hdac6 has two HDAC domains; one functional and one unfunctional (Haberland et al. 2009b). Unlike the other HDACs, which are active mainly in the nucleus, observed Hdac6 activity occurs in the cytoplasm in association with microtubule proteins (Hubbert et al. 2002). As such, Hdac6 is a microtubule deacetylase. However, Hdac6 associates with ubiquitination machinery suggesting a role in protein turnover (Sadoul et al. 2008). Deletion of Hdac6 causes hyperacetylation of skeletal microtubules in mice but does not result in lethality (Hubbert et al. 2002, 6).

Class III HDACs

Class III HDACs are made up of the NAD+ dependent Sirtuin family that share homology with yeast HDAC Sir2 (Rajendran et al. 2011). The Sirtuin family of HDACs comprises seven members, each sharing a common deacetylase domain, but differentiated by unique N and C terminal sequences (Michan and Sinclair 2007). Sirtuins deacetylate their targets by a two-step mechanism in which NAD+ is hydrolyzed to nicotinamide, leading to the generation of an acetyl-ADP-ribose metabolite (Landry et al. 2000; Michan and Sinclair 2007). Sirtuins are also distinct in their sub-cellular localization, with Sirt1 Sirt6 and Sirt7 predominantly localized in the nucleus associated with euchromatin, heterochromatin, and the nucleolus respectively (Michan and Sinclair 2007). Sirt2 is the predominant cytoplasmic

Sirtuin while Sirt3, Sirt4, and Sirt5 localize to the mitochondria (Michan and Sinclair 2007). Sirt1 plays a role in the formation of heterochromatin, and gene expression, targeting lysine residues in H3 and H4 histone tails (Michan and Sinclair 2007; Vaquero et al. 2004). Sirt7 is an activator of RNA-Pol I-mediated transcription through interaction with RNA Pol I machinery and the expression of ribosomal RNA genes (Michan and Sinclair 2007). Sirtuins have been shown to interact with transcription factors such as MyoD and Mef2 in order to modulate muscle cell differentiation and proliferation (Michan and Sinclair 2007; Fulco et al. 2003; Zhao et al. 2005). Sirt1 interacts with CDK pathway gene p53 and the FOXO transcription factor family, as a potential means to regulate cell proliferation and differentiation in response to metabolic and apoptotic signaling (Chen et al. 2005; Nakae et al. 2006).

In respect to cardiovascular development, Sirt1 protects cardiomyocytes from apoptosis in a p53 dependent manner upon serum starvation or DNA damage (Kolthur-Seetharam et al. 2006; Pillai et al. 2014, 2005). Sirt1 also promotes the degradation of the H2A.Z histone variant known to promote cardiac hypertrophy (Chen et al. 2006). Sirt3, Sirt6, and Sirt7 act in addition to Sirt1 to inhibit cardiac hypertrophy. Sirt7 also deacetylates p53 to inhibit cardiomyocyte apoptosis and inhibit cardiac hypertrophy (Vakhrusheva et al. 2008, 7). Sirt3 represses cardiac hypertrophy by inhibition of Akt signaling and by activating FoxO3a antioxidant signaling pathways (Sundaresan et al. 2009, 3; Sebastian et al. 2012). Sirt6 represses Akt signaling by repressing NF-κB and cJun transcriptional activity (Sundaresan et al. 2012, 6; Yu et al. 2013, 6). In the vascular system, Sirt1 plays a critical role in

tissue revascularization after ischemic injury (Potente et al. 2007; Oellerich and Potente 2012). Sirt1 counteracts the anti-angiogenic signaling of FOXO1 through deacetylation of the FOXO1 protein (Potente et al. 2007). In addition to developmental roles, Sirt1 plays a significant role in the physiological relaxation of cerebral blood vessels by regulating the endothelium dependent reaction to NO signaling (Tajbakhsh and Sokoya 2012). NO regulation may be the result of Sirt1-dependent activation of eNOS in response to increased flow (Chen et al. 2010). The ability of Sirt1 to deacetylate eNOS leads to identification of Sirt1 as a key factor in maintaining vascular health and responsiveness and reducing the effects of aging on vascular systems (Oellerich and Potente 2012). Sirt1 also regulates endothelial growth by degrading PHD2, an inhibitor of HIF-2α and activating HIF-2α through deacetylation dependent processes (Rane et al. 2009; Dioum et al. 2009).

Chapter II

Endothelial specific deletion of HDAC3 in mice results in aberrant coronary vasculature and postnatal lethality

Rationale and Significance

Hdac3 functions as a key epigenetic regulator of differentiation and proliferation processes essential to the development of the cardiovascular system (Haberland et al. 2009b; Bhaskara et al. 2008, 3). However, the role of Hdac3 in the endothelial cells of the developing embryo has not been examined *in vivo.* Conditional Hdac3 mice provide an opportunity to study Hdac3 in vascular development, providing some much-needed elucidation of the role of this Hdac in mammalian developmental processes.

Results

Deletion of Hdac3 in endothelial cells results in Postnatal Lethality

To determine the role of Hdac3 in endothelial cell during development, we deleted Hdac3 using endothelial cell specific Cdh5-driven Cre recombinase (termed as $Hdac3^{EKO}$). $Hdac3^{EKO}$ pups were recovered at postnatal day 0 (p0) in near-Mendelian ratios (Table 1). $Hdac3^{EKO}$ pups were not observed beyond p14 (Table 1).

Loss of Hdac3 results in coronary vessel defects

Gross analysis of the peripheral vessels of $Hdac3^{EKO}$ embryos appeared normal at e14.5 and e16.5 (Figure 1, 2). We began evaluating the phenotype of $Hdac3^{EKO}$ pups by dissecting out the hearts at p0, p6 and p7. Three wild type ($Hdac3^{F/F}$, or $Hdac3^{+/+}$) and three knockout hearts were examined at each time point. All $Hdac3^{EKO}$ hearts displayed aberrant coronary vessels (Figure 3-5).

$Hdac3^{EKO}$ *embryos display occasional ventricular septal defects*

The Endocardium plays a significant role in heart development, contributing to valvular and septal development in addition to coronary vessel development (Lin et al. 2012). The tricuspid and mitral valve appeared normal (Figure 6). $Hdac3^{EKO}$ embryos displayed occasional ventricular septal defect (Figure 7).

Methods

Generation of $Hdac3^{EKO}$ mice.

The Hdac3 floxed allele mouse and the VE Cadhernin Cre (Cdh5 Cre) mouse have been previously described (Mullican et al. 2011; Alva et al. 2006). Breedings between *$hdac3^{flox/+}$* mice and *$hdac3^{flox/+}$; cdh5 cre* mice were established and pups were collected at p0 and p14 for survival tables. Survival of *$hdac3^{flox/flox}$; cdh5 cre*

(Hdac3^{EKO}) was measured against expected Mendelian ratios by Chi square analysis. Significance was verified using Student's Two-tailed T test. Embryos and pups collected for histology were the result of breedings between either *hdac3$^{flox/+}$* mice and *hdac3$^{flox/+}$; cdh5 cre* or *hdac3$^{flox/flox}$* and *hdac3$^{flox/+}$; cdh5-cre* mice. Approximate age of collected embryos was determined by observation of vaginal plug by probe. The date of the observed plug was marked as e0.5. At the appropriate embryonic day, pregnant mothers were euthanized as per animal protocol. Pups were harvested at postnatal day 0 (p0), p6 and p7 as determined by date of birth, marked as p0. Embryos and pups were genotyped using PCR amplification of Hdac3 and Cdh5 Cre genomic DNA. PCR products of amplifications were run on agarose gels with known controls. The University of Massachusetts Medical School Institutional Animal Care and Use Committee approved all animal protocols.

Histological analysis of embryos and postnatal pup hearts

Embryos and hearts where photographed upon harvesting with a dissection microscope. Once harvested, embryos and hearts were fixed overnight in 2% PFA. Tissues were dehydrated using a graded series of ethanol washes. Embryos and hearts were stored in 100% ethanol at -20^{O}C. Hearts and embryos were embedded in Paraffin and 6-8 micron sections were cut with a microtome and adhered to glass microscope slides. Slides were dried overnight and stored at 4^{O}C until use. Every third or fourth slide was stained with Hemotoxylin and Eosin.

Immunostaining:

Deletion of Hdac3 was verified by HRP staining for Hdac3 on adjacent slides. Sections were washed with xylenes for three minutes a total of three times. Slides

were rehydrated through washes in a graded ethanol series (100%, 95%, 70%, 50%) slides were washed in distilled water for five minutes, then washed in PBS for 10 minutes. Antigen retrieval was conducted in 10mM Sodium Citrate buffer (pH6.0) for 10 minutes at 95^{0}C. Slides were cooled to room temperature and incubated in blocking buffer consisting of 1% horse serum 1% BSA and 0.3% Triton X100 for one hour at room temperature. Slides were washed with PBS for five minutes a total of three times. Hdac3 primary antibody (Santa Cruz sc-11417) was diluted in PBS containing 2.5% horse serum to a final dilution of 1:200. Slides were incubated with primary antibody for one hour at room temperature. Slides were washed in PBS as detailed above. Next, slides were incubated with a universal biotinylated secondary antibody (Vectastain ABC kit, Vector) for one hour at room temperature. PBS washes were conducted as detailed above. Then, slides were incubated with Vectastain ABC reagent (Vector) for one hour at room temperature. Slides were washed in PBS as above. Development was conducted using a DAB substrate kit as per manufacturer's instructions (Vector). Slides were dipped in ice-cold distilled water and washed in tap water for five minutes. Slides were dehydrated through a graded ethanol series (30%, 50%, 70%, 95%, 100%) and washed three times for five minutes with Xylenes. Slides were mounted with VectaMount Permanent mounting medium (Vector). Brightfield microscope images of stained slides were taken and tissues were compared on similar levels.

Discussion

Coronary vascular anomalies make up approximately 33% of cardiovascular anomalies that result in sudden death (Angelini 2007). These anomalies are often

known as the “silent killers” of the cardiovascular anomaly world, displaying no overt symptoms in a majority of cases with most being diagnosed when other cardiovascular problems arise or post-mortem (Hauser 2005). We have shown that deletion of endothelial Hdac3 results in post-natal lethality in mice by generation of coronary vessel defects. The observed phenotype of post-natal death caused by coronary vessel defect is novel to the mouse model system. Prior study of coronary defects using mouse models has resulted in embryonic lethality (Wu et al. 2012; Mellgren et al. 2008; Singh et al. 2011; Zhou et al. 2009, -2). Thus, work with Hdac3 may lead toward the establishment of the first model system for coronary vessel induced postnatal lethality.

Observed coronary defects are similar to defects seen with in previously described knockout models. Deletion of Vegf in the myocardium or Vegfr2a in the endocardium results in hemorrhaging and loss of coronary vessel surface area coverage (Wu et al. 2012). Sox18 and Sox7 deficiency mouse model causes a hemorrhagic phenotype with stunted coronary vessel growth (Downes and Koopman 2001; Cermenati et al. 2008). These phenotypes are similar to the observed phenotypes in our knockout Hdac3 mice (Figure 5), albeit at earlier time points. Thus, Hdac3 may be a crucial regulator of transcription factor activity during postnatal coronary vessel growth.

Our findings dovetail into recent work showing that the endocardium contributes endothelial cells to the coronary vessel system. Fate mapping studies using the Endocardial specific Nfatc1 Cre mark approximately 80% of coronary artery endothelial cells with trace contributions to coronary veins (Wu et al. 2012).

As such, Hdac3 may play a role in the migration of endothelial cells from the Endocardial layer of the heart into the myocardium and subepicardium where coronary vessels form. Further experimentation will be required to determine the molecular role of Hdac3 in coronary vessel development.

The role of epigenetic factors in cardiac development continues to be advanced. Here we have begun to unravel the contributions of Hdacs in coronary vascular development. Future work will determine the molecular basis of Hdac3 activity in coronary vessel development. Prior work has demonstrated a role for Hdac3 in negative regulation of angiogenic process (Park et al. 2013). In addition, we have shown Hdac3 involvement in cardiomyocyte differentiation, while others have shown a requirement for Hdac3 in differentiation of stem cells and vascular progenitor cells (Lewandowski et al. 2014; Xiao 2006, 1; Zeng et al. 2006, 3). Thus Hdac3 may play a key role in regulating endothelial cell dedifferentiation towards a migratory progenitor cell state for incorporation into the coronary vessel system.

Figures

Table 1: Genotypes of crosses between *Hdac3$^{F/+}$; Cdh5-Cre* mice and *Hdac3$^{F/+}$* mice.

	64 mice aged p0		67 mice aged p14	
Genotype	Observed	Expected	Observed	Expected
Hdac3$^{+/+}$	9	8	10	8
Hdac3$^{F/+}$	21	16	25	17
Hdac3$^{F/F}$	7	8	4	8
Hdac3$^{+/+}$; Cdh5-Cre	9	8	10	8
Hdac3$^{F/+}$; Cdh5-Cre	12	16	18	17
Hdac3$^{F/F}$; Cdh5-Cre	6*	8	0$^{\$}$	8
p value	p > 0.05		p < 0.05	

*Two *Hdac3$^{F/F}$; Cdh5-Cre* mice died before reaching p1
$Four dead *Hdac3$^{F/F}$; Cdh5-Cre* mice were recovered at p7

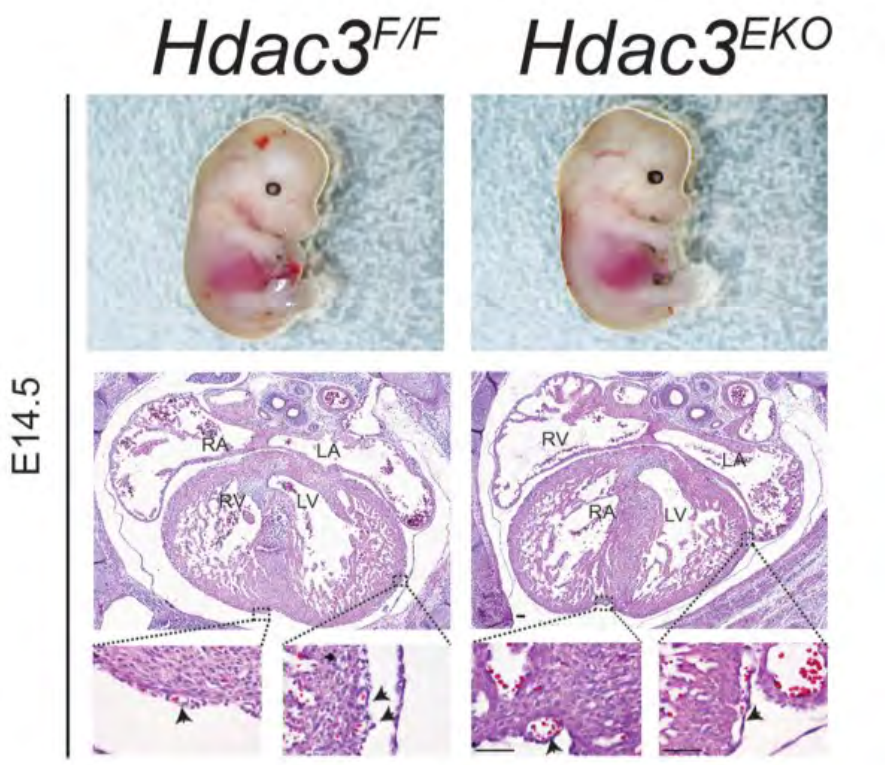

Figure 1: Gross observation and histological analysis of E14.5 embryos. Embryo pictures were taken in PBS post-dissection at approximately 0.8X. Sections were stained with Hematoxalin and Eosin. RA = right atria, LA = left atria, RV = right ventricle, LV = left ventricle. Arrows denote coronary vessels. Scale bar = 50 µM

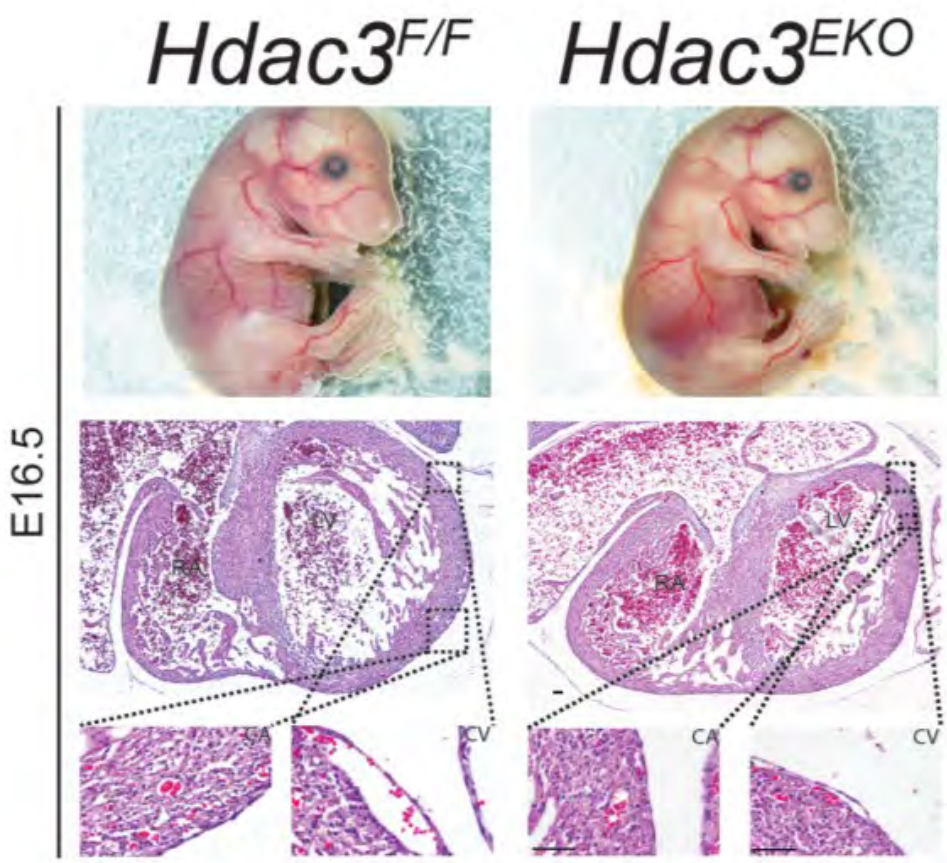

Figure 2: Gross observation and histological analysis of E16.5 embryos. Embryo pictures were taken in PBS post-dissection at approximately 0.8X. Sections were stained with Hematoxalin and Eosin. RA = right atria, LA = left atria, RV = right ventricle, LV = left ventricle, CA = coronary artery, CV= coronary vein. Scale bar = 50 µM.

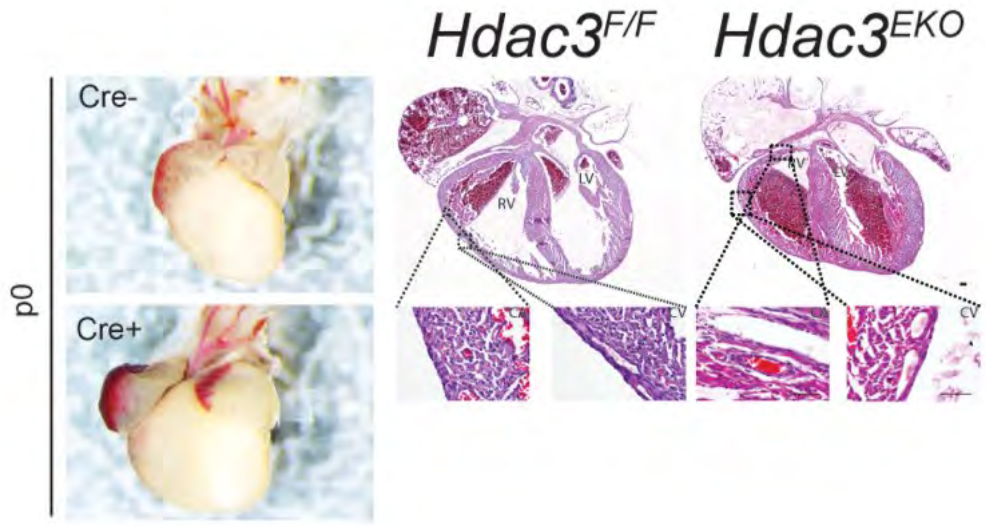

Figure 3: Gross observation and histological analysis of P0 hearts. Heart pictures were taken in 100% ethanol post-dissection at approximately 2.5X. Sections were stained with Hematoxalin and Eosin. RA = right atria, LA = left atria, RV = right ventricle, LV = left ventricle, CA = coronary artery CV = coronary vein. Arrows denote coronary vessels. Scale bar = 50 µM

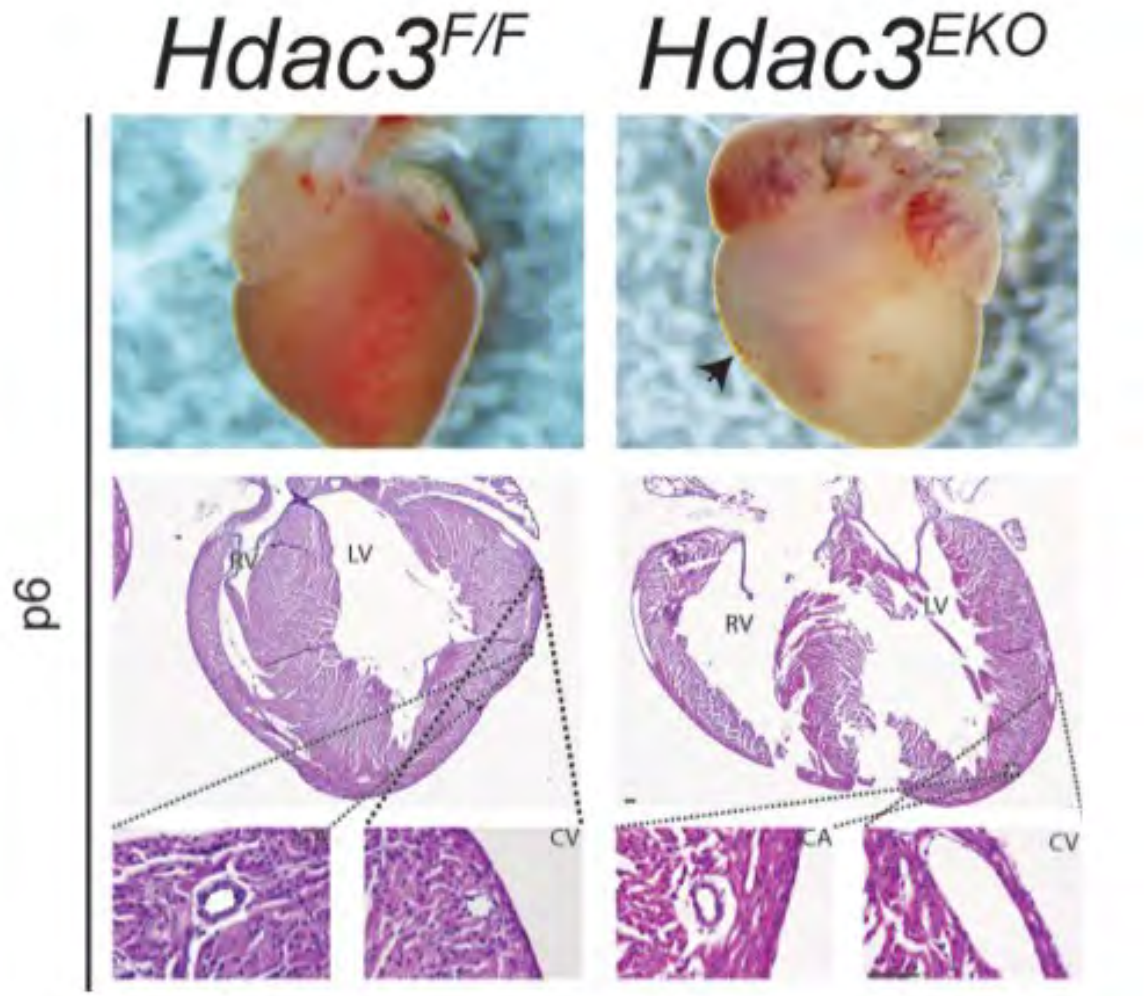

Figure 4: Gross observation and histological analysis of P6 hearts. Heart pictures were taken in 100% ethanol post-dissection at approximately 2.5X. Sections were stained with Hematoxalin and Eosin. RA = right atria, LA = left atria, RV = right ventricle, LV = left ventricle, CA = coronary artery CV = coronary vein. Arrows denote coronary vessels. Scale bar = 50 μM. Arrows denote observed hemorrhaging.

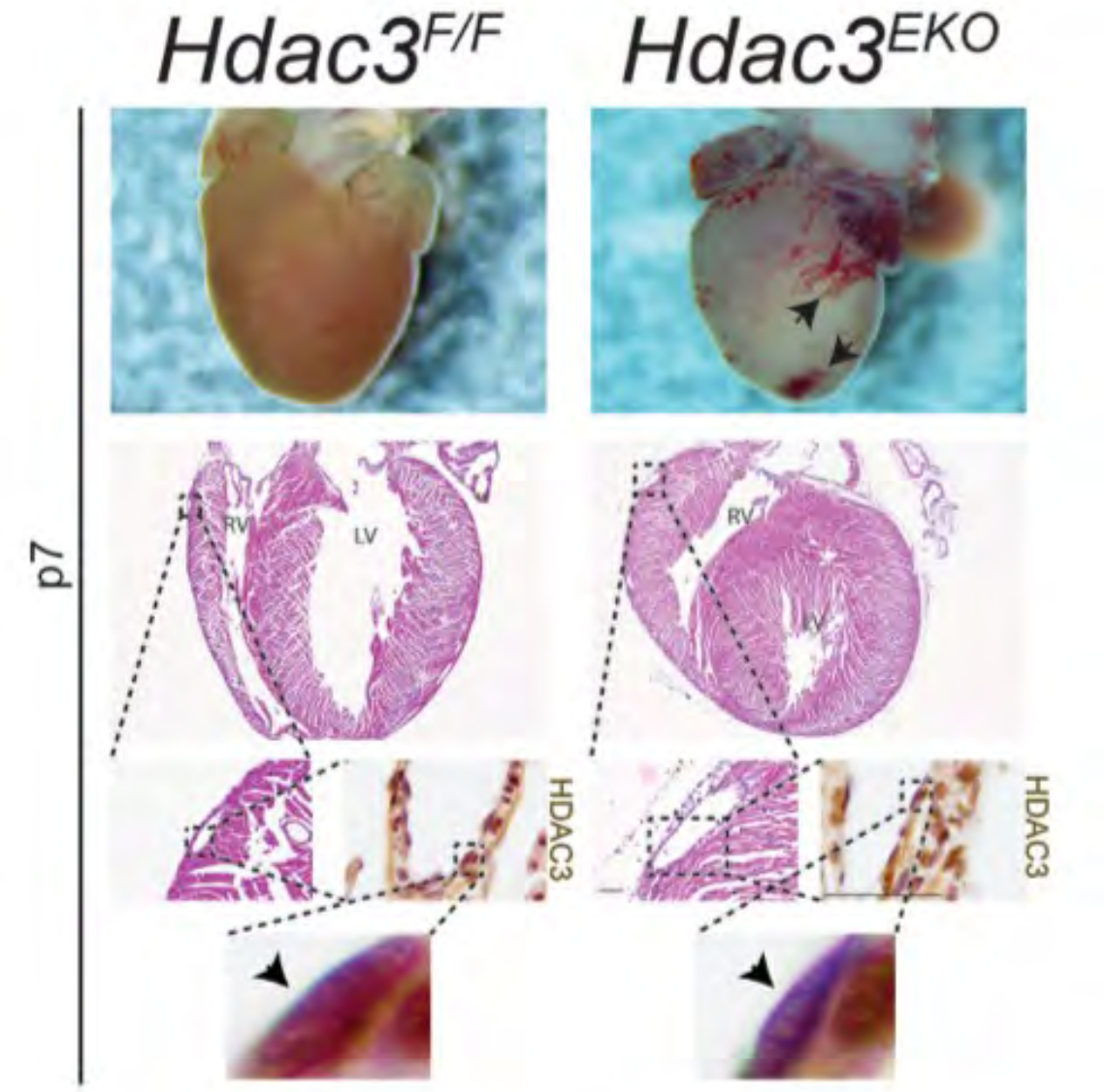

Figure 5: Gross observation and histological analysis of P7 hearts. Heart pictures were taken in 100% ethanol post-dissection at approximately 2.5X. Sections were stained with Hematoxalin and Eosin. RA = right atria, LA = left atria, RV = right ventricle, LV = left ventricle, CA = coronary artery CV = coronary vein. Arrows denote coronary vessels. Scale bar = 50 µM. Arrows denote observed hemorrhaging Arrowheads denote endocardial endothelial cell nuclei.

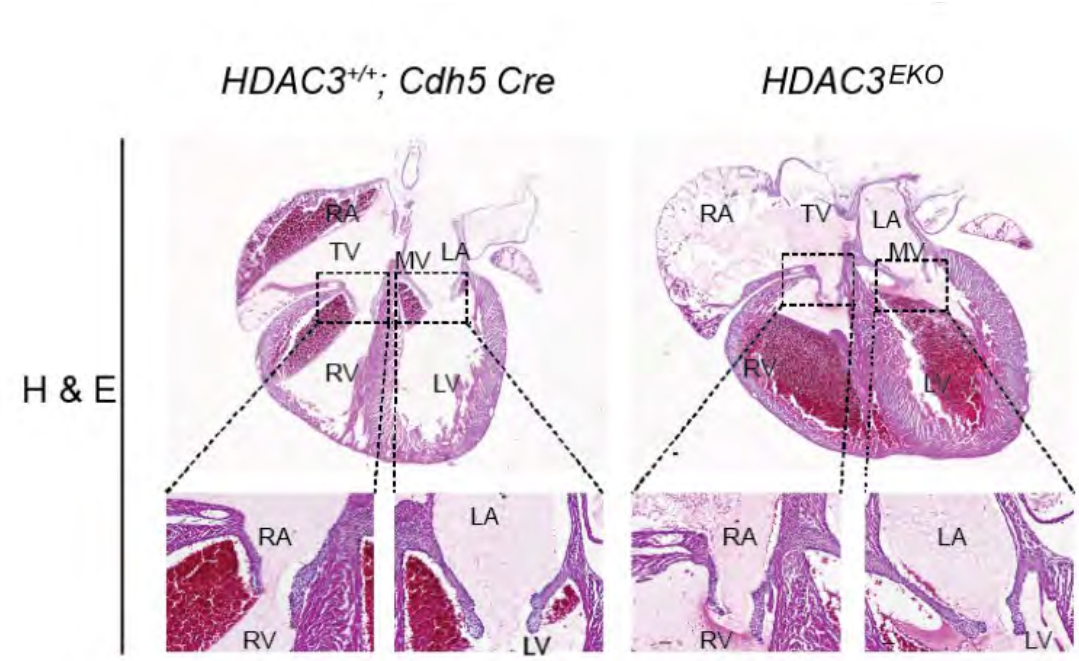

Figure 6: Mitral and Tricuspid valves of HDAC3^{EKO} mice. Hearts were dissected out of p0 pups, sectioned and stained with Hemotoxylin and Eosin. Top panels are taken at 2X magnification while lower panels are taken at 10X magnification. RA = right atria, LA = left atria, RV = right ventricle, LV = left ventricle, TV = tricuspid valve, MV = mitral valve.

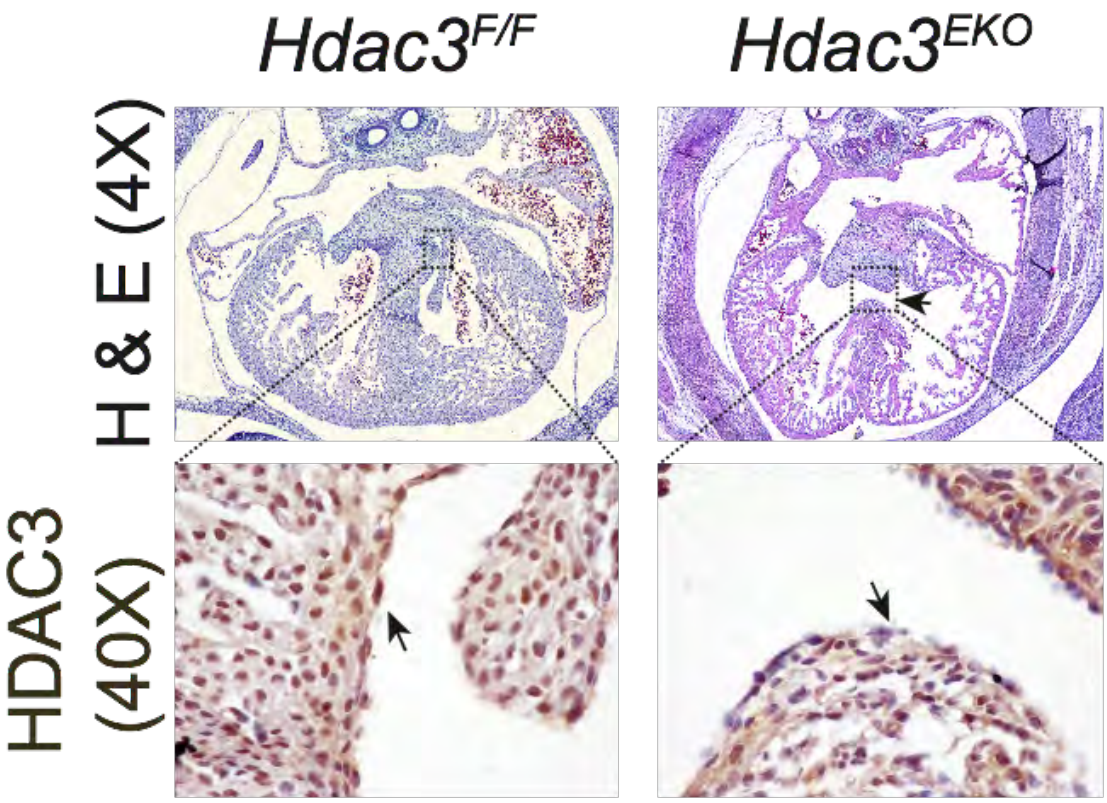

Figure 7: Ventricular septal defects observed in *Hdac3^{EKO}* mice. E14.5 embryos were sectioned and stained with H & E. Deletion of HDAC3 was confirmed with HDAC3 HRP staining. Arrowhead denotes malformation in the ventricular septum. Arrows denote endocardial endothelial cells

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