ROLES OF CLASS II HISTONE DEACETYLASES IN THE CARDIOVASCULAR SYSTEM

APPROVED BY SUPERVISORY COMMITTEE

Eric N. Olson, Ph.D.

Zhijian J. Chen, Ph.D.

Melanie H. Cobb, Ph.D.

Michelle Tallquist, Ph.D.

To My Grandma and Grandpa,

Zilan Lv and Tianhe Chang (1919-2004)

To My Mom and Dad,

Meiyun Feng, Yunguan Chang

To My Husband,

Shijie Li

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ROLES OF CLASS II HISTONE DEACETYLASES IN THE CARDIOVASCULAR SYSTEM

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SHURONG CHANG

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ROLES OF CLASS II HISTONE DEACETYLASES IN THE CARDIOVASCULAR SYSTEM

Shurong Chang, Ph.D.

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Supervising Professor: **Eric N. Olson, Ph.D.**

Histone acetylation/deacetylation, which is orchestrated by two opposing families of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs), represents one of the fundamental mechanisms to control gene transcription. Class II histone deacetylases regulate developmental and physiological processes through interaction with and repression of a variety of transcription factors, including myocyte enhancer factor 2 (MEF2). Using gene targeting combined with biochemical assays, the function and regulation of class II HDACs are being elucidated.

Here I show that in the absence of HDAC5, the heart becomes profoundly enlarged in response to calcineurin signaling and pressure overload. The cardiac phenotype of *HDAC5* mutant

mice is remarkably similar to that of *HDAC9* mutant mice, strongly suggesting that these two HDACs play comparable roles in the control of cardiac growth. HDAC 5 and 9 also appear to play overlapping roles during heart development, as evidenced by cardiac malformations that occur in mice lacking both genes.

Histone deacetylase 7 (HDAC7) is specifically expressed in the endothelium during early embryogenesis. Disruption of the *HDAC7* gene in mice results in embryonic lethality due to a failure in endothelial cell-cell adhesion and consequent dilatation and rupture of blood vessels. HDAC7 represses *MMP10* gene transcription by associating with MEF2, a direct activator of *MMP10* transcription and essential regulator of blood vessel development.

By in vitro kinase assays, I showed that class II HDACs are substrates for a novel stress-responsive kinase(s) specific for conserved serines that regulate MEF2-HDAC interactions. A eukaryotic expression screen revealed a remarkable variety of signaling pathways that converge on the signal-responsive phosphorylation sites in HDAC5, thereby enabling HDAC5 to connect extracellular signals to the genome.

Microarray analysis was performed to provide a genome-wide molecular description of the target genes of the HDAC5/MEF2 complex in the muscle differentiation pathway. This approach was validated by characterizing the transcriptional regulatory element of a novel gene identified in the microarray analysis, which was confirmed as a direct target of MEF2.

Taken together, this study provided mechanistic insights into the regulatory pathways for class II HDACs and the biological functions of these histone modifying enzymes.

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LIST OF PUBLICATIONS:

1. Zhang CL, McKinsey TA, **Chang S**, Antos, CL, Hill JA and Olson EN (2002) Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy. ***Cell*** 110(4):479-488

2. **Chang S***, McKinsey TA*, Zhang CL, Richardson JA, Hill JA and Olson EN (2004) Histone deacetylases 5 and 9 govern responsiveness of the heart to a subset of stress signals and play redundant roles in heart development. ***Molecular and Cellular Biology*** 24(19):8467-76 (* contributed equally)

3. **Chang S**, Bezprozvannaya S, Li S, Olson EN (2005) An expression screen reveals modulators of class II histone deacetylase phosphorylation. ***Proc Natl Acad Sci U S A*** 102(23):8120-8125

4. Backs J, Song K, **Chang S** and Olson EN (2005) Histone deacetylase 4 serves as a target for CAM kinase II-dependent regulation of cardiomyocyte growth. (Submitted).

5. **Chang S**, Yan Z, Bassel-Duby R and Olson EN (2005) Identification of genes regulated by the association of HDAC5 and MEF2 in skeletal muscle differentiation. (In preparation)

6. **Chang S**, Li S, Richardson JA and Olson EN (2005 Histone deacetylase maintains vascular integrity by repression of MEF2-dependent matrix metalloproteinase expression. (Submitted)

7. Li S, **Chang S**, Qi S, Richardson JA, Olson EN (2005). Myocardin-related transcription factor-A is required for mammary myoepithelial cell development. (Submitted).

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ANF	atrial natriuretic factor
BNP	b-type natriuretic peptide
bHLH	basic helix-loop-helix
CaMK	calcium, calmodulin-dependent protein kinase
CDK	cyclin-dependent kinase
cDNA	complementary DNA
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
EDG	endothelial differentiation gene
ET-1	Endothelin-1
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GST	glutathione S-transferase
HAT	histone acetyltransferase
HDAC	histone deacetyltransferase
IP	immunoprecipitation
LPA	lysophosphatidic acid
MADS box	MCM1, Agamous, Deficiens, and SRF box
MAP (kinase)	mitogen activated protein kinase
MEF2	myocyte enhancer factor 2

MMP	matrix metalloproteinase
NFAT	nuclear factor of activated T-cells
NLS	nuclear localization signal
PAMH	pyridine activator of myocyte hypertrophy
PBS	phosphate-buffer saline
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PKC	protein kinase C
PKD	protein kinase D
RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
S1P	sphingosine 1-phosphate
SM	smooth muscle
SMC	smooth muscle cell
SRF	serum response factor
TAB	thoracic aortic banding
TAD	transcriptional activation domain
Tg	transgenic
TIMP	tissue inhibitor of metalloproteinase
WT	wild-type

Chapter I

Introduction: Transcriptional Control of Cardiovascular Muscle Development

Introduction

The development of the three types of muscles in vertebrates, skeletal muscle, cardiac muscle and smooth muscle, which share common properties, including providing contractility and excitability, and express overlapping sets of muscle structural genes, is largely controlled at the transcriptional level. Many muscle genes are controlled by common transcription factors. Recently significant progress has been made in determining key transcription factors during muscle development.

Histone acetylation/deacetylation represents one of the fundamental mechanisms to control gene transcription. Acetylation is a dynamic process that is dictated by two opposing types of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). Histone acetyltranferases (HATs) initiate gene transcription by acetylating core histone tails, which results in relaxation of chromatin and facilitates recruitment of transcription factors to DNA target sequences. The stimulatory effect of HATs is counteracted by histone deacetylases (HDACs), which remove acetyl group from histones and promote chromatin condensation and thereby repress gene transcription (24).

Here I will review the latest progress about transcriptional control of cardiac development, skeletal muscle development and vascular formation. Then, I will give a general review about histone deacetylases (HDACs).

I. Transcriptional Control of Cardiac Development and Growth

Transcriptional control of cardiac development

The circulatory system, consisting of a heart, blood cells and an intricate plexus of blood vessels, is the first functional unit, and the heart is the first functional organ in the developing embryo. Studies in model organisms, such as fly, zebrafish, xenopus and mouse, have revealed an evolutionarily conserved program that controls the genesis of cardiomyocytes from mesodermal stem cells and the subsequent activation of genes responsible for cardiac contractility and morphogenesis. Cardiomyocytes originate in the anterior lateral mesoderm soon after gastrulation (51). They are produced in response to protein factors, including bone morphogenetic proteins (BMPs) and Wnts, which are secreted from adjacent endoderm (61). Soon after their specification, cardiac muscle cells converge along the ventral midline of the embryo to form a beating linear heart tube composed of distinct myocardial and endocardial layers separated by an extracellular matrix. The linear heart tube then undergoes rightward looping, which is essential for proper orientation of the pulmonary (right) and systemic (left) ventricles, and for alignment of the heart chambers with the vasculature. Each cardiac chamber balloons out from the outer curvature of the looped heart tube in a segmental fashion. The four chambers then form and are separated by atrial and ventricular septa (48, 65).

Homeobox proteins The homeobox gene *tinman* is required for the formation of the primitive heart in the fruit fly. A mammalian ortholog of *tinman*, called *Nkx2-5* or *Csx*, is expressed in cardiac muscle cells from the onset of embryonic heart formation until adulthood. *Tinman* in flies is necessary for specification of cardiac lineage and directly activates *D-mef2* expression (7, 15). But in contrast to *tinman*, *Nkx2.5* in mice, although highly conserved and restricted to the cardiac lineage, is not necessary for cardiac

specification, instead it is required for proper cardiac looping and left ventricle development (34, 69), suggesting that other homeobox genes may play redundant roles .

GATA factors Tinman and Nkx2.5 interact with zinc finger transcription factors of the GATA family to activate cardiac gene expression (11). These two classes of transcription factors also regulate each other's expression through mutually reinforcing positive feedback loops (62). The fly GATA gene *pannier* is required for cardiogenesis (16). In mice, loss of *GATA-4* leads to bilateral heart tubes and a reduced number of cardiomyocytes (28). The less severe phenotype in mice can be partly attributed to the expression of other GATA factors (GATA 5 and 6) in the mouse heart.

MEF2 proteins Another family of transcription factors that plays essential roles in caridac muscle development is the MEF2 family of proteins. In vertebrates, there are four MEF2 genes, MEF2 A-D, which are highly enriched in all three muscle lineages (6, 42). MEF2 proteins have been found to interact with Nkx2.5 and GATA factors to synergistically activate expression of a variety of cardiac genes (6). MEF2 proteins belong to the MADS box family of transcription factors. The MADS box of MEF2 proteins binds to a consensus DNA sequence YTA(A/T)$_4$TAR, termed the MEF2 site, which has been found in a variety of muscle-specific and growth gene promoters, and in many cases to be essential for muscle-specific expression. The definitive proof of the requirement of MEF2 for muscle development comes from the inactivation of *D-mef2*, the only *MEF2* gene in flies. In the *D-mef2* mutant fly, the precursor cells for all three muscle lineages are specified and positioned normally, but failed to form normal differentiated muscles, suggesting an obligatory role of MEF2 in muscle differentiation (29). This is partly recapitulated by the deletion of the *MEF2*

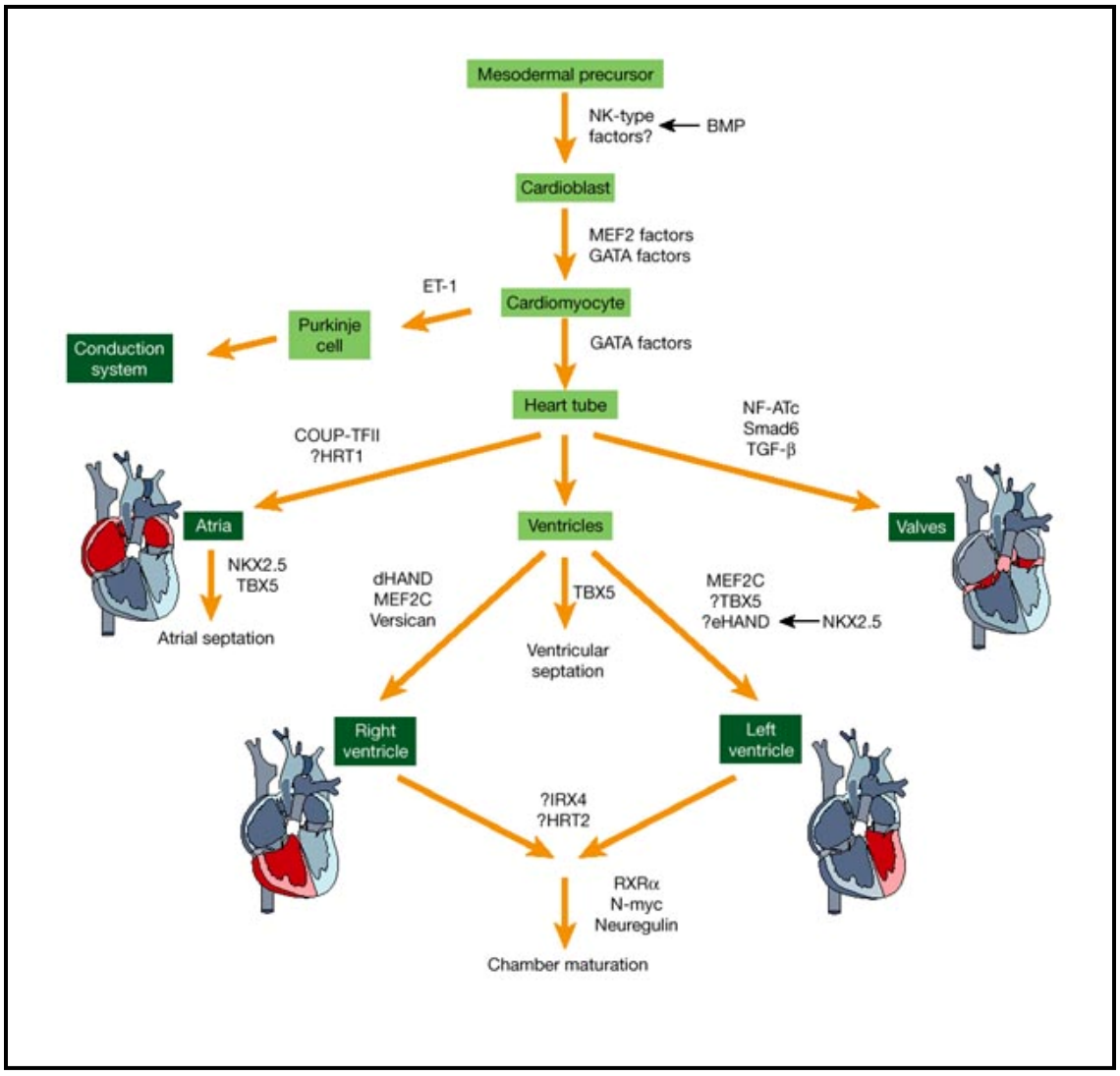

Fig. 1.1. A genetic blueprint for heart development. This schematic shows particular steps in cardiac morphogenesis, focusing on mesodermal contributions. Genetic pathways leading to linear heart-tube formation are partly conserved between Drosophila and mouse. The formation of valves, ventricles, atria and the conduction system are under the control of groups of regulatory proteins that may act independently or in a common pathway. Cardiac regions affected by specific pathways are highlighted in dark green boxes with white type. Factors necessary for distinct steps during cardiogenesis in model organisms or humans are indicated beside the arrows. Regulatory factors suspected to have region-specific roles are indicated with a '?'. Cell types or regions of the heart are indicated in boxes. Distinct processes during cardiogenesis (atrial septation, ventricular septation and chamber maturation) are indicated. (Adapted from Srivastava D. and Olson EN, 2000).

gene in mice, which develop hypoplasia of the right and left ventricles and vascular defects, leading to early embryonic lethality (31). Deletion of the *MEF2A* gene in mice leads to a mitochondrial deficiency and cardiac sudden death (43).

HAND proteins During mouse heart development, the related bHLH transcription factors dHAND/HAND2 and eHAND/HAND1 are expressed predominantly in the right and left ventricle, respectively (64). Deletion of *dHAND/HAND2* in the heart results in hypoplasia of the right ventricle (66). *eHAND/HAND1* has also been implicated in left ventricle development, although early placenta defects precluded a detailed analysis of its role in the heart (12, 52). This has been shown by a conditional knockout of *eHAND* in the heart, which displayed defects in the left ventricle and endocardial cushions, and exhibited dysregulated ventricular gene expression (36). The role of HAND proteins in heart development is further confirmed in zebrafish. Loss of the only *HAND* gene in zebrafish abolishes ventricle development (71).

T-box proteins Members of T-box family of transcription factors have been indicated in human congenital cardiac malformations, such as Holt-Oram syndrome and DiGeorge syndrome (3, 4, 23). T-box proteins share high homology at the DNA binding domain called T-box, which binds consensus sequence of GGTGT in the regulatory region of many cardiac genes, including *ANF*, *Connexin 40* and *Fgf10*. There are 8 T-box proteins in fruit flies and 18 T-box proteins in mice, of which Tbx1, Tbx2, Tbx3, Tbx5, Tbx18 and Tbx20 are expressed in developing cardiac structures (49). Genetic analysis in human patients and in model organisms revealed that T-box proteins are essential for early cardiac lineage determination, chamber formation, valvuloseptal development and diversification of

the specialized conduction system. T-box proteins interact and synergize with GATA factors and Nkx2.5 to fully activate their target genes (49).

More and more transcription factors have now been implicated in the network of regulating cardiac gene expression (Figure 1.1) (65). It is increasingly evident that it is the combination of different factors that determines the specificity and intensity of gene expression. However, so far no single factor or a combination of factors is able to activate the entire cardiac gene program in non-muscle cells.

Molecular pathways of cardiac hypertrophy

In response to a variety of extrinsic and intrinsic stimuli that impose increased biomechanical stress, such as aortic hypertension and valvular diseases, the heart undergoes hypertrophic growth, which is defined by an increase in cardiomyocyte size, enhanced protein synthesis, and a higher organization of the sarcomere, and is accompanied by induction of fetal gene program. While cardiac hypertrophy is initially beneficial since it normalizes the increase in wall tension and sustains cardiac output, prolonged cardiac hypertrophy is associated with increased risk of sudden death or progression to heart failure, suggesting that cardiac hypertrophy is a maladaptive process instead of being a compensatory response. Thus, to prevent or reverse cardiac hypertrophy to circumvent the subsequent development of heart failure could be an effective way to prevent cardiac demise. Many cardiomyocyte-automonous and endocrine/paracrine pathways have been implicated in regulating cardiomyocyte hypertrophic growth (14). Here I will briefly review recent progresses in understanding molecular pathways involved in hypertrophic growth.

Calcinerin/GSK-NFAT-MCIP Calcineurin, also called protein phosphatase 2B, is a Ca2+/calmodulin - dependent protein phosphatese (10). Calcineurin dephosphorylates members of the NFAT (nuclear factor of activated T-cells) family of transcription factors, and thereby unmasking nuclear localization signals, which in turn results in translocation of NFAT proteins to the nucleus and activation of immune response genes and cardiac growth genes. Overexpression of constitutively active calcineurin in the heart stimulates cardiac hypertrophic growth and eventually heart failure (41). A similar phenotype was observed in mice overexperssing constitutively nuclear NFAT3, indicating that NFAT is the major downstream target of calcineurin in the heart (46, 63). Meanwhile, glycogen synthase kinase-3 (GSK-3) phosphorylates NFAT proteins and counteracts calcineurin by inducing nuclear export of NFAT proteins. Overexpression of constitutively active GSK-3β in the heart diminishes cardiac hypertrophy induced by calcineurin overexpression, thoracic aortic banding and β-adrenergic stimulation (46, 63).

The newly identified family of calcineurin inhibitors RCAN (regulator of calcineurin, formerly MCIP) is highly enriched in striated muscles (55, 56). RCAN1 is a direct target of NFAT, and the expression of MCIP1 is correlated to calcineurin activity, which forms a feedback inhibition loop to regulate cardiomyocyte growth. Overexpression of RCAN1 in the heart blunts cardiac growth triggered by overexpression of calcineurin, β-adrenergic stimulation and exercise. Taken together, these data support a notion that calcineurin/NFAT signaling is sufficient and necessary to induce cardiac hpertrophic growth (57, 69).

CaMK-HDAC-MEF2 Ca2+/calmodulin-dependent kinases (CaMKs) stimulate myogenesis and hypertrophic growth of cardiomyocyte by phosphorylating class II

histone deacetylases (HDACs) and thereby de-repressing MEF2 activity (34). This will be discussed in more detail in part IV of the introduction.

II. Transcriptional Control of Skeletal Muscle Development

During vertebrate embryogenesis, the paraxial mesoderm will give rise to somites, blocks of mesodermal cells on both sides of the neural tube, which will produce many of the connective tissues of the body, including bone, muscle, cartilage and demis. Muscle cells come from two cell lineages in the somite, the hypaxial myotome and the epaxial myotome. Paracrine factors instruct these myotome cells to become muscles. Wnts proteins (Wnt1 and Wnt3a) from dorsal neural tube, in combination with low concentrations of Sonic hedgehog from the notochord and floor plate, induce the epaxial myotome. While Wnt proteins from the epidermis, in conjunction with BMP4 and FGF5 from the lateral plate mesoderm, are thought to induce the hypaxial myotome (9, 50). The myotome cells produce myogenic bHLH transcription factors and become committed muscle cell precursors - myoblasts. These cells then align with each other and fuse to form the multinucleated myotubes characteristic of muscle tissue. Thus the multinucleated myotube cells are the product of several myoblasts joining together and fusion of their cell membranes (26, 40). The transcription factors that play important roles during myogenesis are discussed below (Figure 1.2).

Myogenic bHLH proteins The myogenic regulatory factors (MRFs) are part of a superfamily of basic helix-loop-helix (bHLH) transcription factors. The MRF subfamily consists of MyoD, myogenin, myf5 and MRF4, which are expressed exclusively in skeletal muscle (45). The MRF proteins contain a conserved basic DNA-binding domain and a helix-loop-helix motif required for heterodimerization. These proteins form heterodimers with

ubiquitously bHLH proteins known as E-proteins, to bind to a consensus DNA sequence CANNTG (E-box), which is found in the control regions of most skeletal muscle genes. These myogenic proteins have the striking ability to activate the entire skeletal muscle differentiation program when introduced into a variety of non-muscle cells, including cells from all three germ layers. Thus, they are considered to be the "master regulators" of skeletal muscle differentiation. Their roles in muscle development have been confirmed by gene knockout studies. MyoD and myf5 play redundant roles in the generation of myoblasts, whereas myogenin, as well as MRF4 control myoblast differentiation (47).

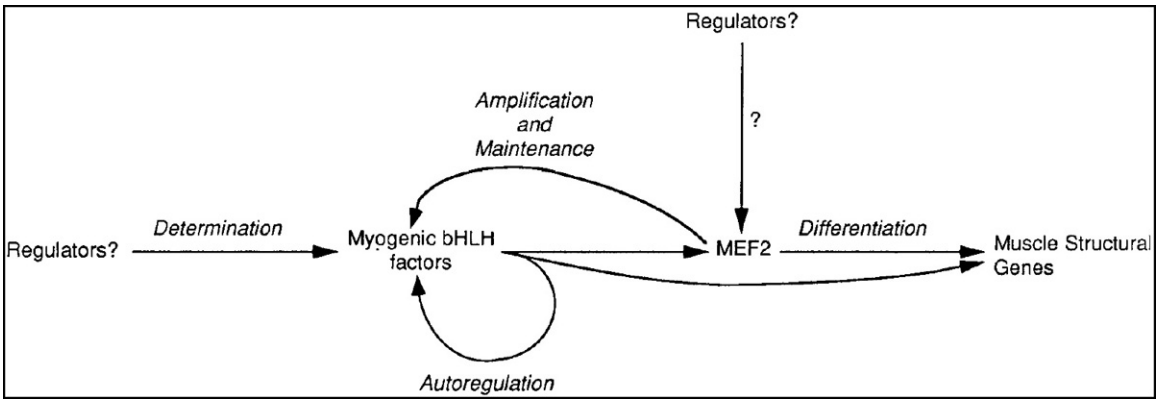

Fig. 1.2. Myogenic bHLH proteins and MEF2 factors in the skeletal muscle lineage. Early mesodermal regulators induce the expression of myogenic bHLH factors during myoblast determination. Myogenic bHLH factors autoregulate their expression and induce the expression of MEF2, which binds the promoters of several myogenic bHLH genes, amplifying and maintaining their transcription. MEF2 and Myogenic bHLH factors collaborate to induce muscle structural genes during differentiation. While MEF2 can be induced by myogenic bHLH factors in tissue culture, this has not yet been demonstrated in vivo. Other regulators might also initially induce MEF2 expression in skeletal muscle cells independent of myogenic bHLH factors. (Adapted from Olson, et al., 1995)

MEF2 proteins MEF2 proteins physically interact with myogenic bHLH proteins and the interaction precisely correlates with their myogenic activity (25). Myogenic bHLH proteins upregulate their own expression and that of MEF2 factors, and MEF2 proteins then

feedback on myogenic bHLH factors and themselves to amplify and maintain their expression in committed muscle cells, thereby establishing a mutually reinforcing regulatory circuit for skeletal myogenesis (47).

III. Transcriptional Control of Vasculogenesis and Angiogenesis

Although the heart is the first functional organ of the body, it does not even begin to pump until the vascular system of the embryo has established its first circulatory loops of blood vessels. Blood vessels are formed by two processes, vasculogenesis and angiogenesis. During vasculogenesis, blood vessels are created de novo from the lateral plate mesoderm. The splanchnic mesoderm cells are specified to become hemangioblasts, the precursors of both the blood cells and the blood vessels. These cells condense into aggregations that are often called blood islands. The inner cells of these blood islands become hematopoietic stem cells, while the outer layer cells become angioblasts, the progenitor cells of the blood vessels. The angioblasts then multiply and differentiate into endothelial cells, which form the lining of the blood vessels. Finally the endothelial cells form tubes and connect to form the primary capillary plexus (54). This process of primary vascular network formation occurs both within the embryo and in extra-embryonic tissue, the yolk sac (20, 53, 54).

After the phase of vasculogenesis, angiogenesis begins. New vessels form by sprouting from the capillaries or splitting an existing vessel. The capillaries also fuse with each other to form wider vessels, the arteries and veins. Vessel maturation requires the subsequent recruitment of surrounding mesenchymal cells and their differentiation into vascular smooth muscle cells. This process has been shown to involve the interaction of

endothelial cells with mesenchymal cells and the release of specific growth factors such as platelet-derived growth factor (21, 22, 54).

Therefore, endothelial differentiation is an early event followed by the formation of primitive tubes. The subsequent recruitment and differentiation of vascular smooth muscle cells is a later event leading to the formation of stable blood vessels. Growth factors including platelet-derived growth factor (PDGF), β fibroblast growth factor (βFGF), vascular endothelial growth factor (VEGF), angiopoietin-1, and transforming growth factor-ß (TGF-ß) are key mediators of these events promoting proliferation, differentiation and migration of these cells (32). A series of transcription factors are either key regulators of the expression of either the growth factors or their receptors, or mediators of the cellular responses to these growth factors (44), as summarized in Figure. 1.3.

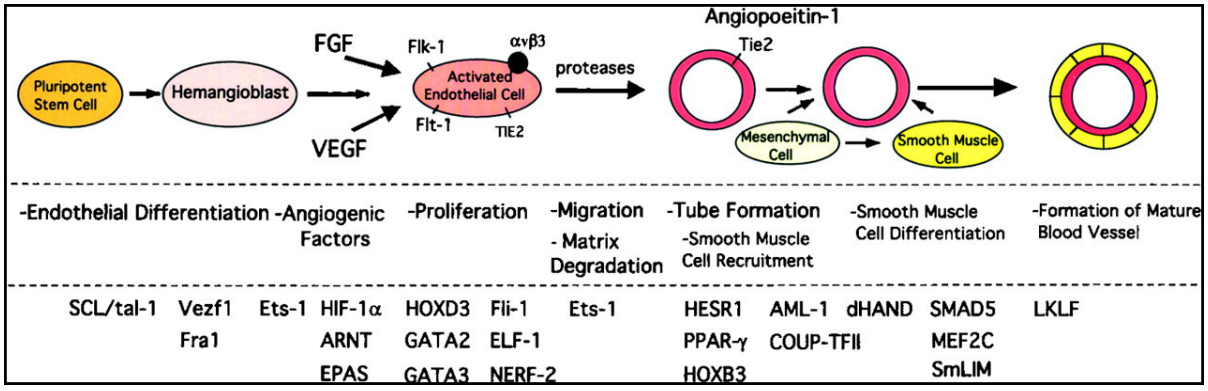

Fig. 1.3. Role of transcription factors during different stages of vascular development. (Adapted from Oettgen, 2001).

MEF2 Proteins MEF2C is highly expressed in the developing vascular endothelium, and MEF2C knockout mice die at E9.5 from severe vascular abnormalities that

have been attributed in part to a failure of the endothelium (30, 31). Endothelial cells were present and were able to differentiate, but failed to organize normally into a vascular plexus, and smooth muscle cells did not differentiate in *MEF2C* mutant embryos (30). The essential role of MEF2 during cardiovascular development is further supported by the abnormalities of endothelial cells in both *ERK5/BMK1* and *MEKK3* mutant mice. ERK5, which is a downstream target of MEKK3, phosphorylates MEF2 proteins at the transcriptional activation domain and activates transcriptional activity of MEF2. Targeted deletion of ERK5 or MEKK3 in mice leads to embryonic lethality at around E10 due to cardiovascular abnormalities including defects in vascular angiogenesis/maturation, which is seen in MEF2C null mice, indicating that MEF2C is the major target of ERK5/MEKK3 during vascular development.

Ets transcription factors Members of the Ets family of transcription factors, including Ets-1, Egr1 and Fli1 are highly expressed in endothelial cells during angiogenesis. The downstream targets from Ets family members in endothelial cells include genes that are essential for vascular development, such as Tie-1 and -2, and VEGFR-1 and -2. A growing volume of data supports the notion that Ets proteins are involved in regulation of endothelial cell adhesion, spreading and motility by regulating the expression of many extracellular matrix-related genes, including VE-cadherin, integrins, intracellular adhesion molecules and matrix metalloproteinases. In vivo experiments in Xenopus suggest that Egr1 and Fli1 are able to induce ectopic endothelial cell differentiation.

MMPs and TIMPs Matrix metalloproteinases (MMPs) are endoproteinases which degrade components of extracellular matrix, and tissue inhibitors of metalloproteinase

(TIMPs), which are highly expressed in endothelial cells, bind to MMPs and inhibit their activity. During angiogenesis, MMP activity is up-regulated to degrade the extracellular matrix between smooth muscle cells and endothelial cells and to cleave the adhesions proteins between adjacent endothelial cells, thereby to allow smooth muscle cells (SMCs) to detach from endothelial cells and to permit endothelial cells to migrate into surrounding tissues (58, 60).

IV. Histone Deacetylases

Histone modification, including acetylation, phosphorylation, methylation and ubiquitination of histone tails, forms a dynamic "histone code" that changes chromatin configuration and thereby regulating gene transcription, DNA replication, DNA recombination, DNA repair, and chromosome segregation (24). Of these modifications, acetylation at the lysine residues in histone tails is the best studied. Lysine is acetylated through the transfer an acetyl moiety from acetyl-coenzyme A to the ε-amino group. Histone acetylation/deacetylation is a dynamic process that is orchestrated by two opposing families of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). Histone acetyltransferases (HATs) stimulate gene transcription by acetylating nucleosomal histones which relaxes chromatin structure and facilitates recruitment of transcription factors to DNA target sequences. Conversely, histone deacetylation by HDACs results in chromatin condensation and transcriptional repression (24).

Based on the homology to yeast RPD3 or HDA1, mammalian HDACs are grouped into two classes, class I and class II (17-19), as summarized in Figure 1.4. Class I HDACs (HDAC-1, -2, -3, -8, and -11), which are ubiquitously expressed, share high homology to

RPD3 and contain only a histone deacetylase catalytic domain. In contrast, class II HDACs (HDAC-4, -5, -6, -7, -9), are highly homologous to yeast HDA1 and are highly expressed in heart, brain and skeletal muscle (17-19). In addition to their carboxyl-terminal catalytic domain, class II HDACs contain an amino-terminal extension that interacts with other transcriptional cofactors and confers responsiveness to a variety of external signals (8, 33, 34, 39). The SIR complex is another protein complex in yeast that is required for silencing at telomeric DNA regions and at the silent mating type loci. One of the components, SIR2, was found to have NAD-dependent histone deacetylase activity. Based on the homology to yeast SIR2 protein, seven human SIR2-like proteins, SIRT 1 – 7, were identified. These SIRTs form the third class of histone deacetylases (1, 5, 59).

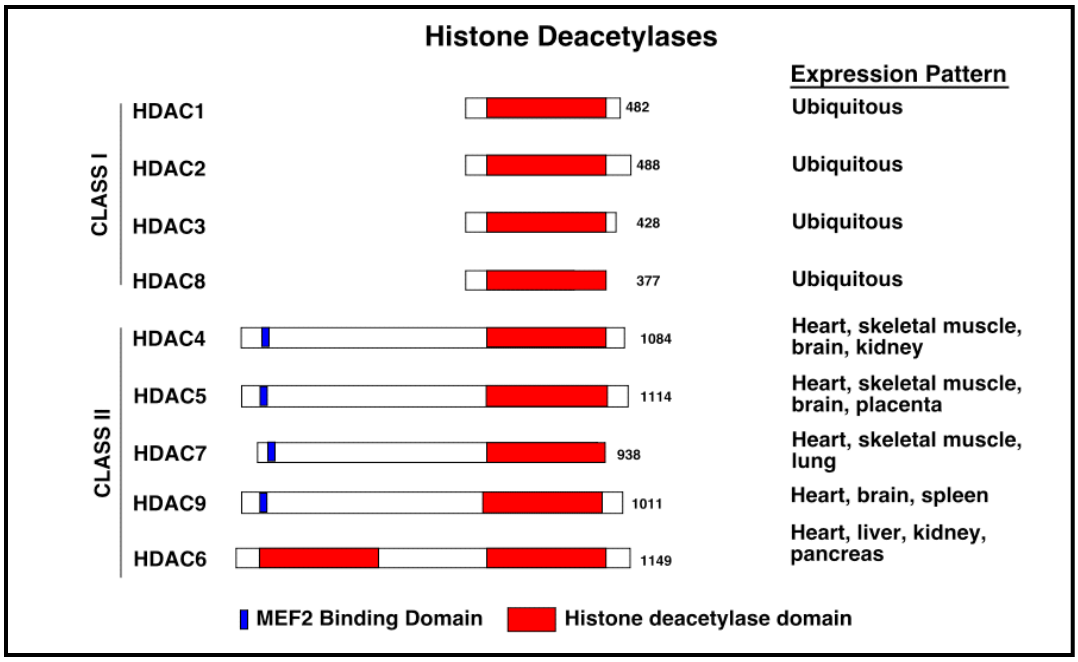

Fig. 1.4. Schematic diagrams of class I and class II histone deacetylases. Class II HDACs have a bipartite structure, with a C-terminal catalytic (HDAC) domain and an N-terminal extension with a MEF2-binding domain, while class I HDACs contain only histone deacetylase catalytic domain.

Class I HDACs The stimulatory effect of class I HDACs on cardiac hypertrophic growth was demonstrated by the treatment of cardiomyocytes with HDAC inhibitors, trichostatin A (TSA) and sodium butyrate (NaBu), which inhibit class I HDACs efficiently but not class II HDACs (13). Both TSA amd NaBu impose a dose-dependent blockade to hypertrophy and fetal gene activation, which seems paradoxical to the inhibitory effect of class II HDACs in cardiomyocyte hypertrophic growth, and argues that class I HDACs and class II HDACs may target to completely different sets of genes, which regulate cardiac hypertrophy in opposite ways (2). In addition, in vivo experiments revealed that TSA attenuates cardiac hypertrophy induced by β-adrenergic stimulation and overexpression of HOP in the heart. These findings point to a possibility that HDAC inhibitors may be used as therapeutics for cardiac hypertrophy and heart failure (27).

Class II HDACs Class II HDACs interact with MEF2 proteins and silence the expression of MEF2 target genes (33, 34). During myoblast differentiation when MEF2 activity is required, HDAC5:MEF2 complex is disrupted and HDAC5 is translocated from the nucleus to the cytoplasm. Upon maturation of myotubes, HDAC5 is relocated to the nucleus to interact with and inhibit MEF2 activity (37). The nuclear/cytoplasmic shuttling is regulated by phosphorylation of class II HDACs at two conserved serine residues located at the amino-terminal region of HDACs, which recruits 14-3-3 chaperone proteins and results in nuclear export of HDAC/14-3-3 complex in a Crm1-dependent manner with consequent derepression of specific target gene (37, 38). Ca2+/calmodulin - dependent kinase I (CaMKI) is the first kinase identified that phosphorylates class II HDACs and derepresses MEF2

activity, which at least partially revealed the mechanism by which CaMK stimulates myogenesis and cardiac growth (33, 34). Recently, PKD (protein kinase D, also called PKCμ) has also been shown to be able to phosphorylate class II HDACs and regulate cardiomyocyte hypertrophic growth (67). However, the identity of the physiological kinase for class II HDACs remains unclear.

While the biochemical functions of HDACs have been extensively analyzed, their functions *in vivo* are only beginning to be elucidated. The functions of class II HDACs appear to be remarkably tissue-specific and dedicated to the control of tissue growth and development. HDAC5 and HDAC9 have been implicated in the repression of cardiomyocyte growth in the adult heart. Mutant mice lacking HDAC9 are viable, but develop extremely enlarged hearts in response to pathological signals (72). Conversely, signal-resistant mutants of HDAC5 and HDAC9 lacking the conserved phosphorylation sites, prevent cardiomyocyte growth (72).

HDAC4 is expressed in prehypertrophic chondrocytes of the developing skeleton and negatively regulates the activity of runt-related transcription factor-2 (Runx2), which is required for chondrocyte hypertrophy. Mice homozygous for an *HDAC4* mutation exhibit lethal ossification of endochondral cartilage due to precocious and ectopic hypertrophy of chondrocytes, whereas ectopic expression of HDAC4 in proliferating chondrocytes inhibits hypertrophic growth and differentiation. Thus, the repression of Runx2 activity by HDAC4 in the pre-hypertrophic zone of developing bones determines the timing and extent of endochondral bone formation (68). These studies demonstrate a common role of HDAC4, 5 and 9 in regulating cellular hypertrophy.

In light of the importance class II HDACs in regulating myogenesis and cardiac growth, the specific aims for my study are:

1) **investigate the in vivo function of HDAC5 by gene targeting;**
2) **examine the in vivo function of HDAC7 by gene targeting;**
3) **dissect the regulatory pathways for class II HDACs;**
4) **identify the target genes of HDACs during myogenesis.**

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Chapter II

Histone deacetylases 5 and 9 govern responsiveness of the heart to a subset of stress signals and play redundant roles in heart development

Abstract

The adult heart responds to stress signals by hypertrophic growth, which is often accompanied by activation of a fetal cardiac gene program and eventual cardiac demise. We showed previously that histone deacetylase (HDAC) 9 acts as a suppressor of cardiac hypertrophy and that mice lacking HDAC9 are sensitized to cardiac stress signals. Here we report that mice lacking HDAC5 display a similar cardiac phenotype and develop profoundly enlarged hearts in response to pressure overload resulting from aortic constriction or constitutive cardiac activation of calcineurin, a transducer of cardiac stress signals. In contrast, mice lacking either HDAC5 or HDAC9 show a hypertrophic response to chronic β-adrenergic stimulation identical to that of wild type littermates, suggesting that these HDACs modulate a specific subset of cardiac stress response pathways. We also show that compound mutant mice lacking both HDAC5 and HDAC9 show a propensity for lethal ventricular septal defects and thin-walled myocardium. These findings reveal central roles for HDACs 5 and 9 in the suppression of a subset of cardiac stress signals, as well as redundant functions in the in the control of cardiac development.

Introduction

Post-natal growth of the heart occurs primarily through hypertrophy, in which cardiac myocytes increase in size but not in number (reviewed in 34). Hypertrophy can occur in response to physiological stimuli, such as exercise, or pathological stimuli, such as myocardial infarction, hypertension, aortic stenosis or valve dysfunction. While stress-induced hypertrophy serves initially to normalize ventricular wall stress, this form of hypertrophy, when prolonged, can progress to dilated cardiomyopathy and sudden death. Pathological cardiac hypertrophy is a major predictor of human morbidity and mortality and a major cause of heart failure (17, 18, 23).

Numerous intracellular signaling pathways have been implicated in the transduction of hypertrophic signals from the cardiomyocyte cell surface to the nucleus (reviewed in 2, 8, 22, 34). Many hypertrophic agonists acting through cell surface receptors coupled to Gαq mobilize intracellular calcium, which activates downstream kinases and the calcium, calmodulin-dependent phosphatase calcineurin. Activation of these effectors is sufficient and, in many cases, necessary for hypertrophic growth of the heart (14, 31, 35, 50). Elevation of cAMP in response to β-adrenergic agonists also stimulates cardiac hypertrophy via protein kinase A (PKA) and other downstream effectors (36). The identification of nodal points in hypertrophic signaling pathways and the mechanisms that link signaling in the cytoplasm with changes in gene expression that contribute to maladaptive growth of the heart represent major challenges in the field.

Pathological cardiac hypertrophy is coupled to the activation of a fetal cardiac gene program, which results in the expression of fetal proteins involved in contractility,

metabolism, and calcium handling that are incompatible with sustained function of the adult myocardium (19). The myocyte enhancer factor-2 (MEF2) transcription factor activates many fetal cardiac genes and serves as a nuclear endpoint for stress signals in the adult myocardium (29). The transcriptional activity of MEF2 is tightly governed by its interaction with histone acetyltransferases (HATs) (39, 45) and histone deacetylases (HDACs) (9, 20, 21, 27, 28, 30, 40, 46-48, 50), which stimulate or suppress transcription, respectively, through their effects on histone acetylation and chromatin configurations. HATs acetylate the conserved amino-terminal tails of nucleosomal histones resulting in relaxation of chromatin structure and consequent transcriptional activation (13, 37). The gene-activating functions of HATs are counteracted by HDACs, which remove acetate groups from histone tails, promoting chromatin condensation and transcriptional repression (42).

Mammalian HDACs can be classified into multiple classes based on their structure and homology to three yeast HDACs (42). Class I HDACs (HDACs 1, 2, and 3) are expressed ubiquitously and are comprised simply of a catalytic domain. The class II HDACs (HDACs 4, 5, 7, and 9) are expressed at highest levels in heart, brain and skeletal muscle, and contain a C-terminal catalytic domain and an N-terminal extension that mediates interactions with other transcriptional repressors and activators.

Interaction of MEF2 with class II HDACs silences the expression of MEF2 target genes (20, 21, 27, 28, 30, 40, 46-48, 51). Calcium, calmodulin-dependent protein kinase (CaMK) and other kinases that are activated by stress signaling in the heart phosphorylate the amino-terminal extensions of class II HDACs, which results in their dissociation from MEF2 and export from the nucleus (27, 28, 46, 48). HATs are then enabled to interact with the

HDAC binding region of MEF2 and promote transcription of MEF2 target genes and cardiac hypertrophy (reviewed in 26 and 44).

Consistent with the notion that class II HDACs suppress pathological cardiac growth, at least in part by modulating MEF2 activity, mice lacking *HDAC9* display enhanced hypertrophy and super-activation of MEF2 in response to cardiac stress signals (46). To further explore the functions of class II HDACs in vivo, we generated mice lacking *HDAC5*. Like *HDAC9* mutant mice, these mice spontaneously develop cardiac hypertrophy with age, and display cardiomegaly in response to constitutive calcineurin activation or pressure overload due to aortic constriction. In contrast, mice lacking either *HDAC5* or *HDAC9* display a normal hypertrophic response to chronic β-adrenergic signaling. We also show that compound mutant mice lacking both *HDAC5* and *HDAC9* are prone to embryonic and early postnatal death from a spectrum of cardiac abnormalities including ventricular septal defects (VSDs) and thin-walled myocardium. These findings reveal redundant roles for HDACs 5 and 9 as counter-regulators of a specific subset of hypertrophic signaling pathways in the adult heart, as well as in normal cardiac growth and development during embryogenesis.

Materials and Methods

Generation and genotyping of HDAC5 mutant mice.

A 129s6/SvEvTAC mouse genomic BAC-library (BACPAC Resources, Oakland) was screened for the *HDAC5* gene employing a PCR amplification product corresponding to the coding region for the MEF2 binding domain of mouse HDAC5. Two independent *HDAC5* genomic clones were mapped by Southern blotting and partial sequencing. The *HDAC5* targeting construct was generated using the pN-Z-TK_2 vector, which contains a nuclear *lacZ* cassette and a neomycin-resistance gene (kindly provided by R. Palmiter). The ~1.5 kb 5' arm was generated by PCR. The ~6.5 kb 3' arm was subcloned from a BAC clone. The *lacZ* cDNA and neomycin-resistance cassette under control of the *PGK* promoter were fused in-frame with the 5' region of exon 3, placing the ß-galactosidase reporter gene under the control of the endogenous *HDAC5* promoter. The targeting vector was linearized and electroporated into mouse D3 embryonic stem (ES) cells. Correctly targeted ES cell clones were identified by Southern blotting using both 5´ and 3´probes. Of 361 clones tested, 17 were positive for correct recombination at the *HDAC5* locus. Two independent clones were injected into 3.5-day mouse C57BL/6 blastocytes, and the resulting chimeric males were bred to C57BL/6 females to achieve germline transmission of the mutant allele. Subsequent genotyping was performed by PCR with the following primers:

HD5gt 5', 5'-CAAGGCCTTGTGCATGCTGGGCTGG-3';

HD5gt3', 5'-CTGCTCCCGTAGCGCAGGGTCCATG-3';

LacZ, 3'-GCCCGTTTGAGGGGACGACGACAGTATCG-3'

Knockout and transgenic mice.

HDAC9 mutant mice (46) and mice bearing the α-myosin heavy chain (MHC)-calcineurin transgene were described previously (31).

Throacic aorta banding and isoproterenol administration.

Six-to-eight-week-old male mice underwent either a sham operation or were subjected to pressure overload induced by thoracic aorta banding (TAB) as described (12). For chronic isoproterenol administration, miniosmotic pumps (Alzet, Palo Alto, CA) containing isoproterenol or saline vehicle were inserted subcutaneously in the backs of ten-to-twelve-week-old male mice. The pumps delivered 28 ug/hour per 25 kg of body weight of isoproterenol in a 0.9% saline solution or saline solution alone. Mice were sacrificed 7 days later for assessment of cardiac hypertrophy.

Histology and β-Galactosidase Staining.

Adult tissues and embryos were fixed with paraformaldehyde. Embedding of tissues, histological sectioning, and staining with hematoxylin and eosin (H&E) were performed by standard procedures. Briefly, for β-Galactosidase staining, the embryos were dissected out free of yolk sac and amnion. After 2 washes in cold PBS, they were fixed with cold PBS containing 4% paraformaldehyde and 0.2% glutaraldehyde. Embryos younger than E9.5 were fixed for 30 minutes; E10.5-E13.5 embryos were fixed for 1-1.5 hrs. After fixation, the embryos were washed with cold PBS for 3 times, 10 minutes each. Then they were stained in the dark overnight at room temperature with staining solution. The staining solution

contains: 4 mM Ferrocyanide, 4 mM Ferricyanide, 2 mM $MgCl_2$, and 1 mg/ml X-gal in PBS. The X-gal stock is 40 mg/ml in Dimethylformamide. After staining, the embryos were washed with PBS for 3 times and post-fixed with the same fix solution overnight at 4 ^{o}C.

RNA analysis.

Total RNA was isolated with Trizol reagent (Invitrogen). Briefly, muscle tissues were homogenized in Trizol reagent. After a 5 minutes incubation at room temperature, 1/5 volume of chloroform was added and the mixture was vigorously shaken for 15 seconds. After centrifugation at 12,000 g for 5 minute at 4 ^{o}C, the upper phase was transferred to a new tube and 500 ul of isopropanol was added. After 10 minutes of incubation at room temperature, the mixture was centrifuged at 12,000 g for 10 minutes at 4 ^{o}C. After washed with 70% ethanol, the pellet was dissolved in TE buffer containing 0.1U/ul of RNase inhibitor.

After treatment with DNaseI, 1ug of RNA was used as a template for reverse transcription with random hexamer primers (Invitrogen). Briefly, 1 ug of RNA was mixed with 1x DNase buffer, 1 ul DNase (Invitrogen) and ddH_2O in a 10 ul reaction volume. After 15 minutes of incubation at room temperature, 1 ul of 25 mM EDTA was added and the DNase was inactivated by 10 minutes incubation at 65 ^{o}C. 150 ng of random primers and 2 ul of 5 mM dNTP mix were added to the mixture and incubated at 65 ^{o}C for 5 minutes. After a quick chill on ice, 4 ul of 5x first strand buffer, 2 ul of 0.1 mM DTT and 1 ul of RNase inhibitor and 1 ul of reverse transcriptase and ddH_2O were added to make a 20 ul reaction. After 10 minutes of incubation at room temperature, the reaction was incubated at 42 ^{o}C for

50 minutes to complete the reverse transcription. The final cDNA products were stored at -20 ^{o}C. All PCR products span intron regions of the genes. RT-PCR reactions were performed under conditions of linearity with respect to input RNA. The sequences of the primers used for RT-PCR are:

HDAC1 Forward: 5' – CTG AGC AAG GTC TGC AGC – 3'

Reverse: 5' – GGC ATC TTA GCA GAG CTC C – 3'

HDAC2 Forward: 5' – CCA AGG ACA ATA GTG GTG AG – 3'

Reverse: 5' – AAA TCA GCT CAG AAA GGC CA – 3'

HDAC3 Forward: 5' – TTT GAG TTC TGC TCG CGT TAC – 3'

Reverse: 5' – TAA ATC TCC ACA TCA CTT TCC – 3'

HDAC4 Forward: 5' – CAG ATG GAC TTT CTG GCC G – 3'

Reverse: 5' – CTT GAG CTG CTG CAG CTT C – 3'

HDAC5 Forward: 5' – GAA GCA CCT CAA GCA GCA GCA GG – 3'

Reverse: 5' – CAC TCT CTT TGC TCT TCT CCT TGT T – 3'

HDAC6 Forward: 5' – ACG CTG ACT ACA TTG CTG CT – 3'

Reverse: 5' – TCT CAA CTG ATC TCT CCA GG – 3'

HDAC7 Forward: 5' – CTC CAG CCA GAC ACA CCA GGC – 3'

Reverse: 5' – CTG CAA CAC CCC CAA CGC CTG – 3'

HDAC8 Forward: 5' – AAC ACG GCT CGA TGC TGG – 3'

Reverse: 5' – CCA GCT GCC ACT TGA TGC – 3'

HDAC9 Forward: 5' – TCA GAG GTT CCT ATG GGC CTG – 3'

Reverse: 5' - TGG AGA CGT TCC ACT GAG GG – 3'

RNA dot blot analysis was performed with 2 ug of total RNA and transcripts were quantified using a Storm 820 phosphoimager (Molecular Dynamics). The sequences of the probes used for dot blot are as following:

α-MHC: 5' – CGA ACG TTT ATG TTT ATT GTG GAT TGG CCA CAG CGA GGG TCT GCT GGA GAG G – 3'

β-MHC: 5' – GCT TTA TTC TGC TTC CAC CTA AAG GGC TGT TGC AAA GGC TCC AGG TCT GAG GGC TTC – 3'

ANF: 5' – AAT GTG ACC AAG CTG CGT GAC ACA CCA CAA GGG TCC AGG ATC TTT TGC GAT CTG CTC AAG – 3'

BNP: 5' – CAG CTT GAG ATA TGT GTC ACC TTC GAA TTT TGA GGT CTC TGC TGG ACC CGG AGG GTG CTG – 3'

α- Skeletal Actin: 5' – TGG AGC AAA ACA GAA TGG CTG GCT TTA ATG CTT CAA GTT TTC CAT TTC CTT TCC ACA GGG – 3'

GAPDH: 5' – GGA ACA TGT AGA CCA TGT AGT TGA GGT CAA TGA AG – 3'

Results

Generation of HDAC5 mutant mice

The mouse *HDAC5* gene encodes a protein of 1114 amino acids (Figure 2.1A). We introduced a loss of function mutation in the *HDAC5* gene by replacing coding exons 3-7 with a lacZ-neomycin-resistance cassette through homologous recombination. These exons, which encode residues 32-298 of HDAC5, include the region of the protein responsible for interaction with MEF2. Targeting of the *HDAC5* allele in ES cells was confirmed by Southern blot and PCR of genomic DNA. ES cells heterozygous for the *HDAC5* mutation were used to generate chimeric mice, which transmitted the mutation through the germline (Figure 2.1B). Breeding of $HDAC5^{+/-}$ mice in a mixed SvEv129/C57BL6 background yielded $HDAC5^{-/-}$ mice at predicted Mendelian ratios (data not shown). Homozygous mutants were viable and fertile and showed no abnormalities at early age (data not shown).

To confirm that the targeted mutation eliminated functional HDAC5, we sequenced the transcript from the mutant allele following RT-PCR. Sequencing showed that exon 2 was spliced to the 5' end of exon 3, which was linked to the lacZ-coding region (data not shown). Using primers representing exons within and surrounding the deleted region of the gene, we were unable to detect HDAC5 transcripts in RNA from hearts of homozygous mutant mice (Figure 2.1D). Thus, the mutant allele is expected to function as a null. We also examined the expression of HDACs 1, 2, 3, 4, 6, 7, 8 and 9 by RT-PCR in hearts and brains from wild-type and mutant mice. Transcripts encoding these HDACs were expressed at normal levels (Figure 2.1E), indicating that other HDACs were not up-regulated to compensate for the absence of HDAC5.

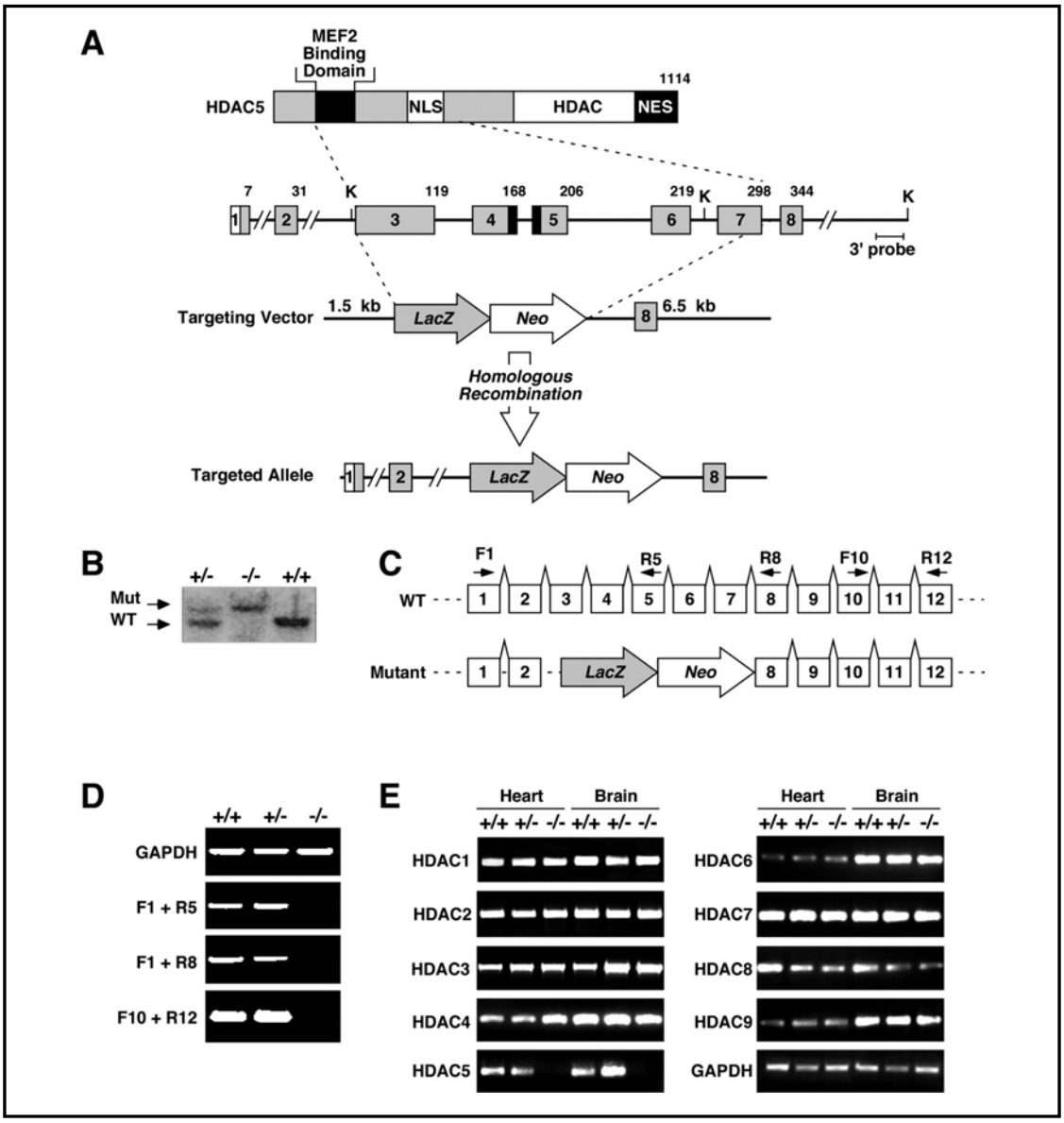

Fig. 2.1. Targeting the mouse HDAC5 gene. (A) A diagram of the HDAC5 protein is shown above a portion of the mouse HDAC5 locus encompassing coding exons 1-8. NES, nuclear export sequence; NLS, nuclear localization sequence. In the targeting vector, a nuclear lacZ reporter was inserted in-frame with exon 3. Homologous recombination resulted in deletion of exons 3-7, which encompass the MEF2 binding domain and NLS. (B) Southern blot analysis of genomic DNA from mice of the indicated genotypes. A 500-bp DNA fragment downstream of exon 7 was used as to probe Kpn I-digested tail DNA. Wild type (WT) and mutant (Mut) bands of 8.5 kb and 15.5 kb, respectively, are shown. (C and D) RNA from hearts of mice of the indicated genotypes was analyzed by RT-PCR. Positions of primers used for RT-PCR are shown (panel C). No functional HDAC5 mRNA was detected in homozygous mutants. GAPDH transcripts were measured as a control. (E) Transcripts for HDACs 1-9 were detected by RT-PCR using RNA isolated from hearts and brains of mice of the indicated genotypes.

Expression of lacZ from the targeted *HDAC5* allele

We stained embryos from HDAC5$^{+/-}$ intercrosses for lacZ expression in order to assess the tissue distribution of HDAC5 expression. As shown in Figure 2.2, β-galactosidase staining was detected in the looping heart tube at E9.5. At E10.5, expression in the heart increased further, and expression was also evident in the ventral region of the neural tube. β−galactosidase staining was also apparent in a variety of adult tissues, including heart, brain, skeletal muscle, kidney, lung, and liver (data not shown). The expression pattern of β−galactosidase during pre- and postnatal development reflected that of the endogenous *HDAC5* gene (data not shown).

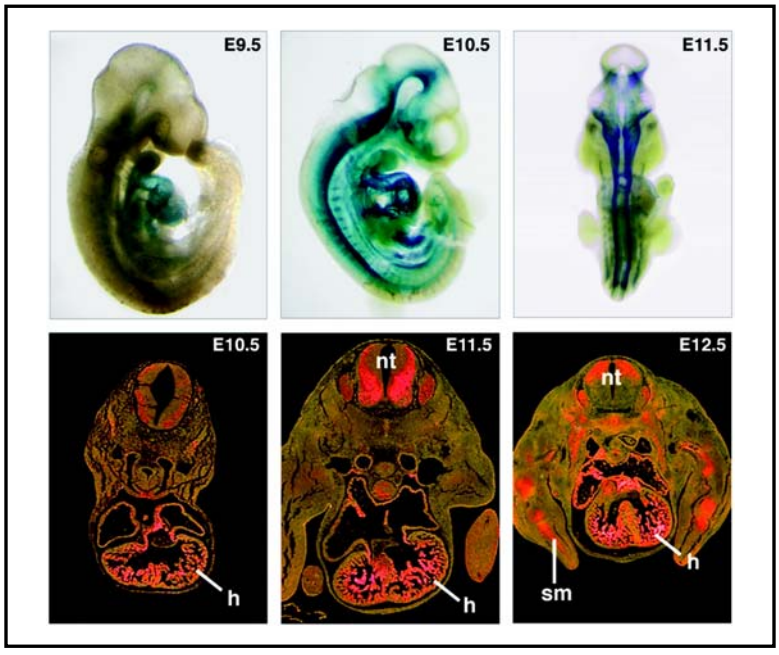

Fig. 2.2. Expression of lacZ from the targeted *HDAC5* allele. Embryos heterozygous for the targeted *HDAC5* allele were stained for lacZ expression on the indicated days of embryogenesis. Strong expression of lacZ was seen in the looping heart tube at E9.5 and in the heart and spinal cord at later stages. LacZ expression was also detected in the muscle forming regions of the limbs at E12.5. The lower panels show transverse sections through lacZ-stained embryos visualized in bright field with lacZ staining indicated in pink. h, heart; ht, neural tube; sm, skeletal muscle.

Enhanced hypertrophy of HDAC5 mutant mice in response to calcineurin activation

In light of the importance of HDAC9 in the control of cardiac growth (46), we focused on possible cardiac abnormalities in *HDAC5* null mice. At 6 weeks of age, the hearts of HDAC5 mutant mice were similar in size to those of wild-type littermates (Figure 2.3A). However, by eight months of age, *HDAC5* mutant hearts were 20% larger than normal ($p < 0.01$). Histological analysis indicated that age-dependent enlargement was due to hypertrophy (data not shown). Kaplan-Meyer survival curves showed no difference in survival of wild type and *HDAC5* mutant mice, indicating that the age-dependent hypertrophy in the homozygous mutants was not fatal.

To begin to investigate possible abnormalities in cardiac stress-responsiveness of HDAC5 mutant mice, we examined their response to constitutive calcineurin activation by intercrossing them with mice bearing an αMHC-calcineurin transgene that promotes cardiac hypertrophy (31). As shown in Figure 2.3 (panels B and C), hearts from HDAC5 null mice showed an exaggerated hypertrophic response to activated calcineurin and achieved a size of approximately three times normal by four weeks of age. Analysis of histological sections indicated that the increase in cardiac mass in the mutant was due to hypertrophy of cardiac myocytes (Figure 2.3B). We detected no evidence of cardiomyocyte hyperplasia in *HDAC5* mutant hearts. *HDAC5* mutant mice bearing the calcineurin transgene were highly prone to sudden death, and none of these mice survived beyond 8 weeks of age. Calcineurin transgenic mice in the wild-type background also die prematurely, but not until at least 12-16 weeks of age. Thus, the lack of HDAC5 resulted in enhanced sensitivity to the pathological consequences of cardiac calcineurin signaling.

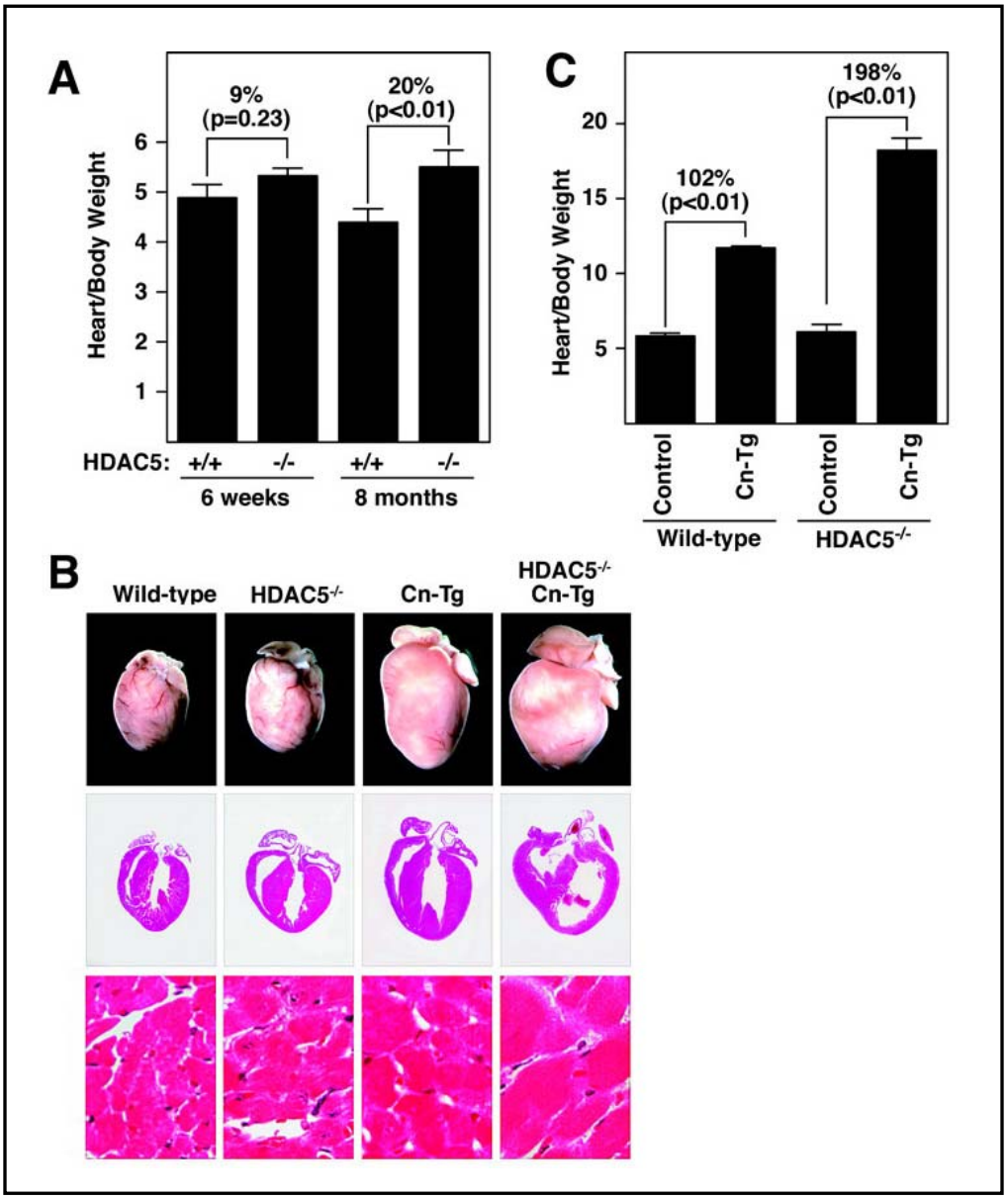

Fig. 2.3. Enhanced hypertrophy in HDAC5 mutant mice. (A) Hearts were dissected from mice of the indicated genotypes at six weeks and eight months of age and heart weight-to-body weight ratios were determined. Values represent the mean +/- standard deviation. Heart sizes of wild type (n = 4) and HDAC5 mutant (n = 4) mice were similar at six weeks, but by eight months, the mutant mice (n = 5) displayed enlarged hearts compared to wild type (n = 3). (B) HDAC5 mutant mice were bred with mice harboring the □-MHC-calcineurin transgene (Cn-Tg). Hearts from one-month-old mice of the indicated genotype were isolated (top images), sectioned, and stained with H&E (bottom images). (C) Heart weight/body weight ratios of mice of the indicated genotypes are shown. Wild type control (n = 6), Cn-Tg (n = 10), HDAC5-/- (n = 5), HDAC5-/-/Cn-Tg (n = 4).

The remarkable hypertrophy in *HDAC5* mutant mice was accompanied by a dramatic increase in expression of fetal cardiac genes. As shown in Figure 2. 4, expression of three representative fetal cardiac genes, *ANF*, *BNP*, and *β-MHC*, in response to calcineurin activation was enhanced in *HDAC5* null mice. The *ANF* and *β-MHC* genes were particularly sensitive to the *HDAC5* gene dosage and showed a heightened response to calcineurin even in $HDAC5^{+/-}$ mice.

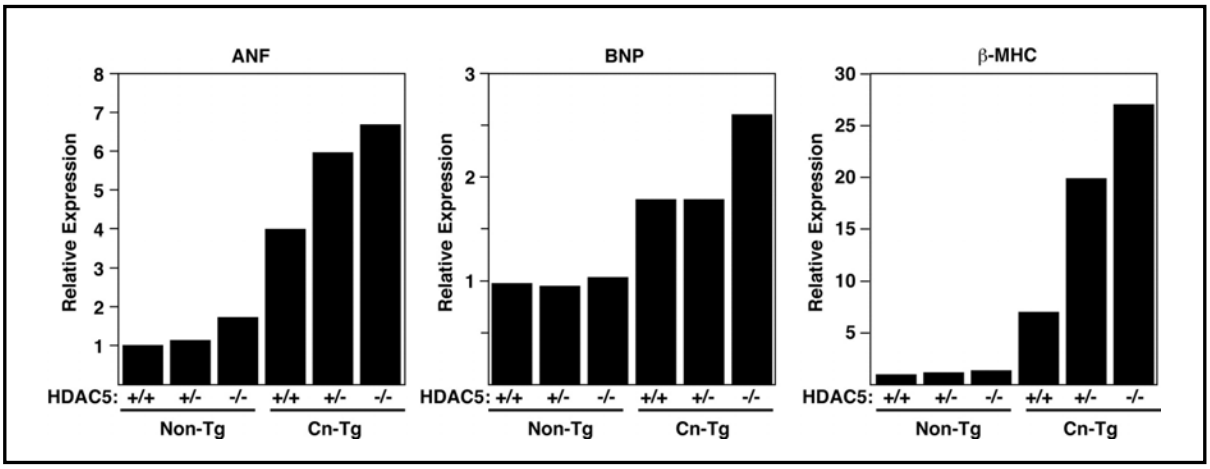

Fig. 2.4. Fetal gene expression in HDAC5 KO mice. RNA was isolated from hearts of mice with the indicated genotypes and expression of fetal cardiac genes was measured by dot blot analysis. Values are expressed as the level of expression of each transcript relative to that in hearts from wild type mice.

Enhanced hypertrophy in HDAC5 mutant mice in response to thoracic aortic banding

To further explore the role of HDAC5 in modulation of cardiac growth, we compared the responses of wild-type and *HDAC5* mutant mice to thoracic aortic banding (TAB), which causes hypertrophy due to increased afterload. Constriction of the thoracic aorta for 21 days in wild-type mice resulted in a 46% increase in heart weight/body weigh ratios (Figure 2.5A and B). *HDAC5* mutant mice showed an exaggerated hypertrophic response to TAB, and

increased in size by approximately 96% over the same time period. Thus, like their response to calcineurin activation, the hearts of HDAC5 null mice were sensitized to pressure overload.

Normal responsiveness of HDAC5 and HDAC9 mutant mice to chronic isoproterenol stimulation

We also investigated the potential role of HDAC5 in the hypertrophic growth response of the heart to β-adrenergic stimulation, using osmotic mini-pumps to deliver a chronic isoproterenol stimulus for 7 days. In contrast to the severe hypertrophy seen in response to calcineurin activation or TAB, wild-type and *HDAC5* mutant mice showed comparable responses to isoproterenol stimulation (Figure 2.5C). *HDAC9* mutant mice, which show an exaggerated response to calcineurin and pressure overload similar to that of *HDAC5* mutants (46), also displayed a normal response to isoproterenol infusion (Figure 2.5C). These findings suggest that HDAC5 and HDAC9 selectively act within the signaling pathways activated by calcineurin and pressure-overload, but not in the β-adrenergic signaling pathway.

Cardiac defects in HDAC5/9 double mutant mice

The similarity in responses of *HDAC5* and *HDAC9* mutant mice to cardiac stresses suggested that these HDACs played similar roles in the control of cardiac growth. To explore the extent of their functional overlap, we generated compound *HDAC5/9* mutant mice by interbreeding heterozygous mutants. Genotypes of offspring are shown in Table 1.

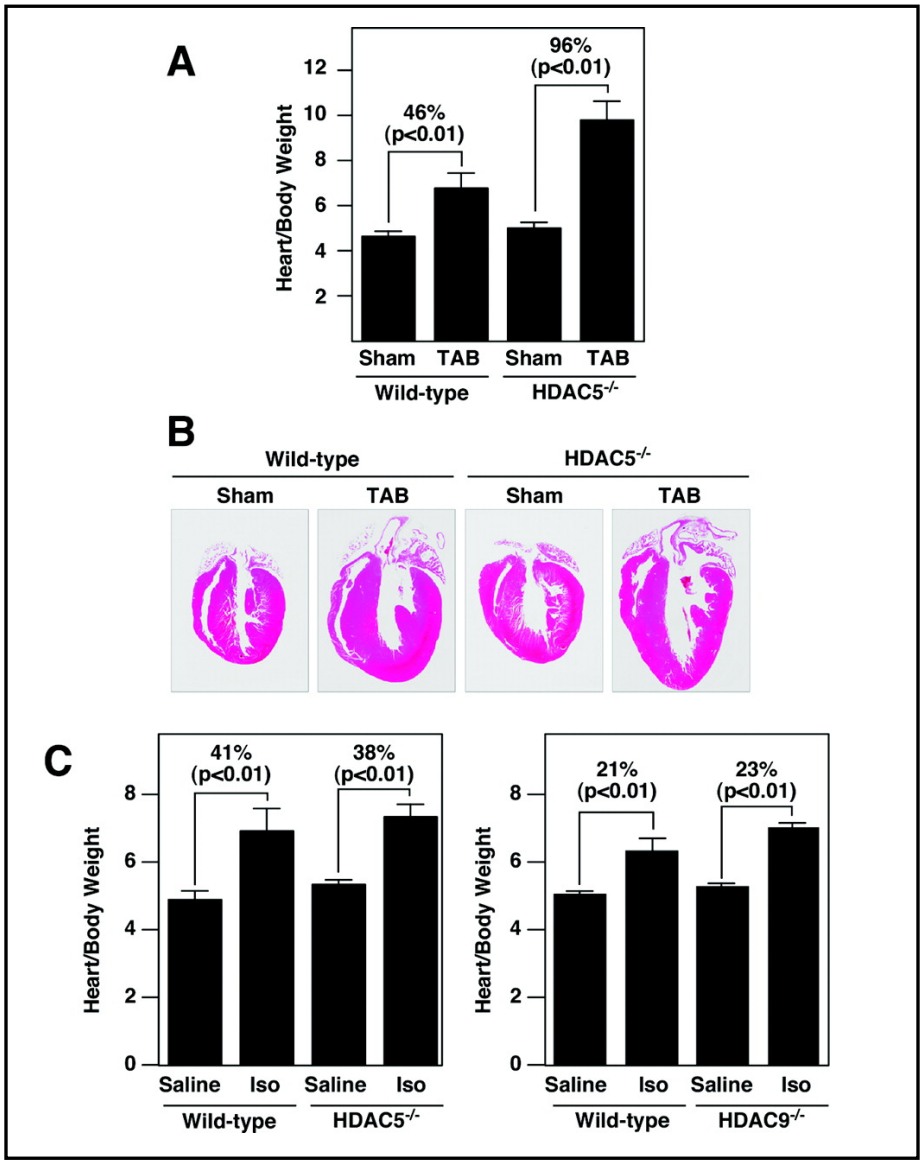

Fig. 2.5. Cardiac responses to thoracic aortic banding and chronic isoprotenernol administration. (A and B) Wild type and HDAC5 null mice at six weeks of age were subjected to thoracic aortic banding (TAB) for 21 days, at which time heart weight-to-body weight ratios were determined. (A). Values represent the mean +/- standard deviation. Wild type sham (n = 3), wild type TAB (n = 3), HDAC5-/-, sham (n = 4), HDAC5-/-/TAB (n = 4). (B) Hearts were sectioned and stained with H and E. (C) Wild type, HDAC5 and HDAC9 mutant mice at 8 weeks of age were infused with isoproterenol (Iso) or saline alone for 7 days, at which time heart weight-to-body weight ratios were determined. The hypertrophic responses of the HDAC5 and HDAC9 mutant mice was not statistically different from that of wild type mice. Left panel: wild type saline (n = 3), wild type Iso (n = 4), HDAC5-/-, sham (n = 4), HDAC5-/-, Iso (n = 6). Right panel: wild type saline (n = 4), wild type Iso (n = 4), HDAC9 -/-, sham (n = 4), HDAC 9 -/-, Iso (n = 4).

Mice homozygous for either null allele and heterozygous for the other were viable and showed mild cardiac hypertrophy (Figure 2.6A and data not shown). In contrast, *HDAC5/9* double null offspring in the mixed C57BL6/129 genetic background were underrepresented at postnatal day 7 and showed severe growth retardation (Figure 2.6A and Table 1). The few double mutants that survived to adulthood weighed about one-third that of wild-type littermates.

Table 2.1. Genotypes of offspring from HDAC5$^{+/-}$; HDAC9$^{+/-}$

	Genotype								
	1	2	3	4	5	6	7	8	9
HDAC5	+/+	+/+	+/+	+/-	+/-	+/-	-/-	-/-	-/-
HDAC9	+/+	+/-	-/-	+/+	+/-	-/-	+/+	+/-	-/-
Observed no.	15	39	15	32	77	33	18	33	3
Predicted %	6.25	12.5	6.25	12.5	25.0	12.5	6.25	12.5	6.25
Observed %	6	15	6	12	29	12	7	12	1

Genotypes were determined at postnatal day 7.

Analysis of internal organs of double null mice that survived to one month of age did not reveal obvious abnormalities at either the gross organ or histological levels. However, while the hearts were smaller than normal, reflecting the overall reduced size of the double mutant mice, as a fraction of overall body weight, the hearts from the double mutants were enlarged compared to wild type littermates (Figure 2.6B and C). Moreover, markers of cardiac hypertrophic were up-regulated in the hearts of adult double mutant mice (Figure 2.6D). One interpretation of these findings is that the lack of HDACs 5 and 9 imposes stress on the heart, which leads to an augmentation in cardiac growth. Another interpretation is that the age-dependent hypertrophy seen in mice homozygous for either the *HDAC5* or *HDAC9*

null alleles is accelerated in the absence of both genes. We also cannot rule out the possibility that the heart is less sensitive than the rest of the body to general growth retardation resulting from the lack of *HDAC5* and *HDAC9*.

The under-representation of *HDAC5/9* double mutant mice at postnatal day 7 suggested that this genotype caused embryonic or early perinatal lethality with variable penetrance. To establish the time of death of double mutants, we determined the genotypes of litters from timed matings. Double mutants were observed at predicted Mendelian frequencies until E15.5, at which time a subset of embryos displayed hemorrhages throughout the body (Figure 2.6E). Thereafter, there was a gradual decline in the frequency of viable double mutants. Embryos that displayed multifocal hemorrhages at E15.5 also had VSDs and thin ventricular walls (Figure 2.6F). Similar defects were seen in a subset of double mutant offspring that survived to birth (Figure 2.6E). Overall, 77% of double mutants had VSDs and about 20% had thin-walled myocardium. However, the 12% of double mutants that survived to adulthood showed no obvious cardiac malformations.

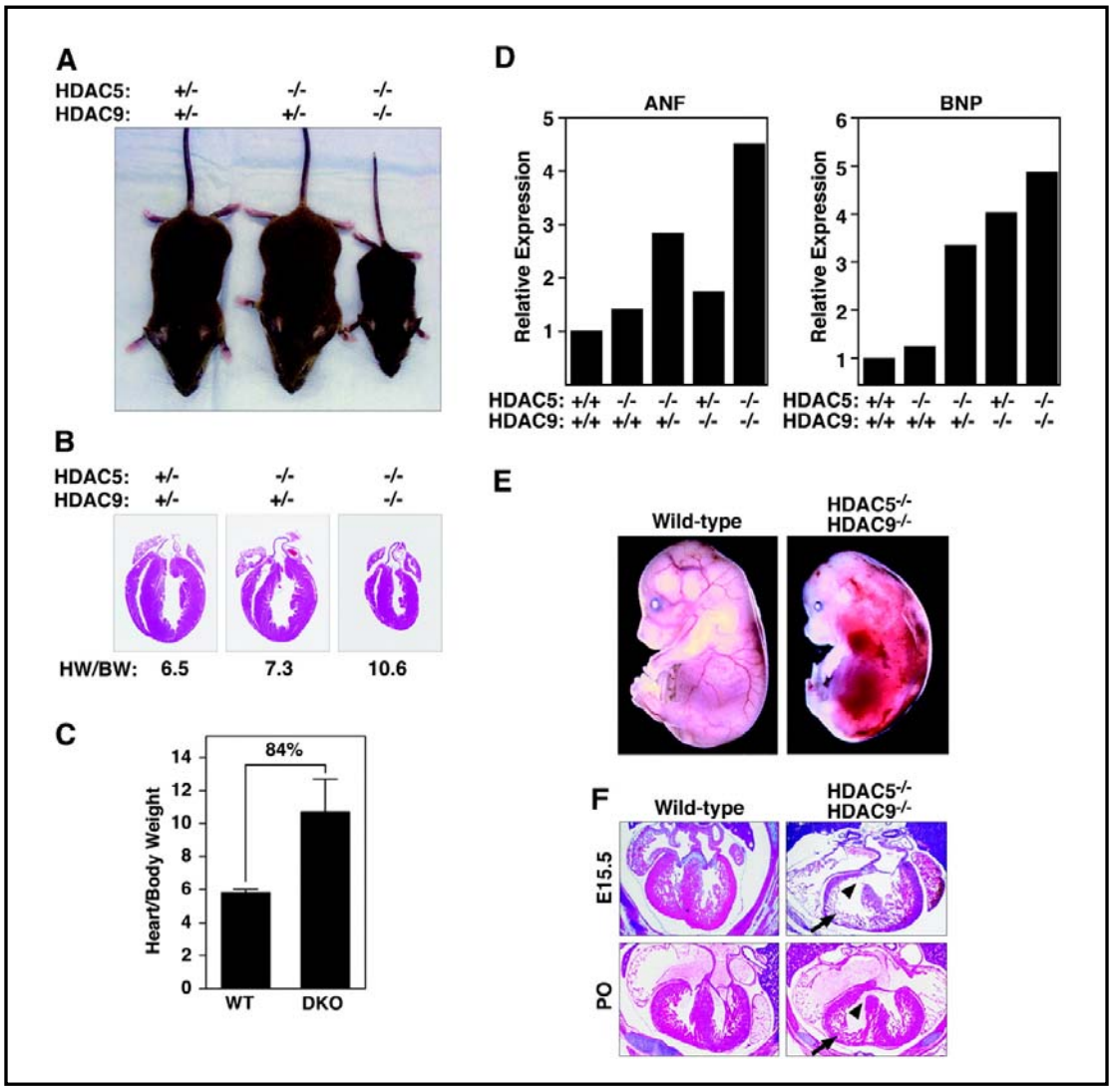

Fig. 2.6. Growth defect and cardiac abnormalities in HDAC5/9 double mutant mice. One-month-old mice of the indicated genotypes**.** (A) The HDAC5/9 double mutant animals are severely growth retarded. (B) Hearts were dissected from the wild type and HDAC5/9 double mutant mice shown in Panel A and heart weight-to-body weight ratios were determined. The double mutant mice have smaller hearts than wild type, but they are enlarged when compared to body weight. Heart weight/body weight (HW/BW) ratios are shown. (C) Hearts were dissected from wild type (n = 6) and HDAC5/9 double knockout (n = 5) mice (DKO) at six months of age and heart/body weight ratios were determined. Values represent the mean +/- standard deviation. (D) RNA was isolated from hearts of mice with the indicated genotypes and expression of fetal cardiac genes was measured by dot blot analysis. Values are expressed as the level of expression of each transcript relative to that in hearts from wild type mice. (E) Wild-type and HDAC5/9 double mutant embryos at E15.5. The double mutant shows multifocal hemorrhages. (F) staining of cardiac sections from wild type and HDAC5/9 double mutant mice at E15.5 (top panels) and at birth (P0, bottom panels). Note the VSD (arrowhead) and thin-walled myocardium (arrow) in the double mutant.

Discussion

The results of this study demonstrate that HDAC5 acts as an antagonist of a specific set of pathological signaling pathways leading to cardiac hypertrophy. In the absence of HDAC5, the heart becomes profoundly enlarged in response to calcineurin signaling and pressure overload. The cardiac phenotype of *HDAC5* mutant mice is remarkably similar to that of *HDAC9* mutant mice (46), strongly suggesting that these two HDACs play comparable roles in the control of cardiac growth. HDACs 5 and 9 also appear to play overlapping roles in development of the heart, as evidenced by cardiac malformations that occur in mice lacking both genes but not in mice lacking only one gene or the other.

Suppression of stress-dependent cardiac growth by HDACs 5 and 9

We showed previously that HDAC9 acts as a suppressor of cardiac hypertrophy and that hypertrophic signals inactivate HDAC9 and other class II HDACs through phosphorylation of their regulatory domains (46). Signal-dependent phosphorylation of class II HDACs creates binding sites for 14-3-3 chaperone proteins, which mediate their translocation from the nucleus to the cytoplasm and consequent de-repression of the hypertrophic gene program (9, 27, 28). Calcineurin activation and pressure overload both activate cardiac kinase(s) specific for the regulatory serine residues in class II HDACs (46), supporting a model in which these stress stimuli act, at least in part, by counter-acting the growth-inhibitory functions of class II HDACs (Figure 2.7). The notion that calcineurin and pressure overload promote hypertrophy through a common signaling pathway is compatible

with studies showing that calcineurin is activated in response to pressure overload and that calcineurin inhibition prevents hypertrophy in response to TAB (reviewed in 7).

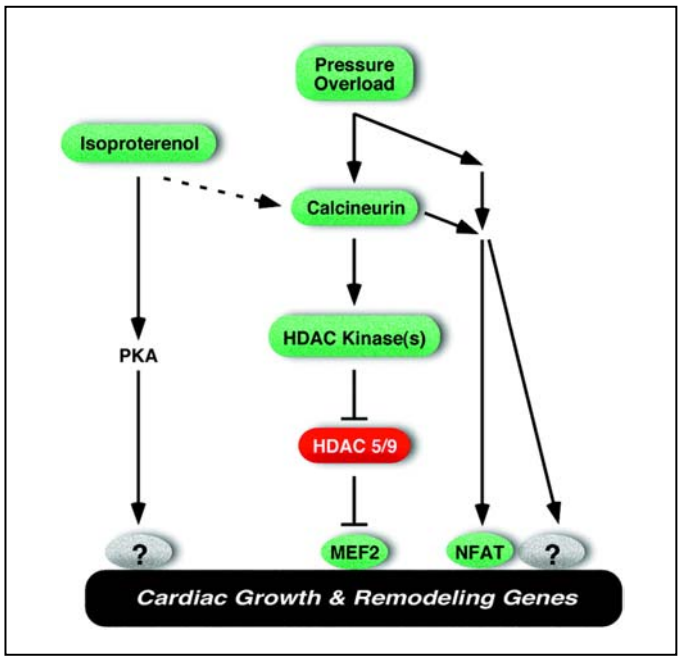

Fig. 2.7. A schematic of the roles of HDACs 5 and 9 as antagonists of hypertrophic signaling.

In contrast to their sensitized response to calcineurin and pressure overload, mice lacking *HDAC5* or *HDAC9* did not show a heightened hypertrophic response to chronic administration of isoproterenol. We suggest two possible explanations for this observation. 1) Isoproterenol could induce hypertrophy through a signaling pathway independent of class II HDACs. 2) Isoproterenol stimulation could super-activate HDAC kinase(s) leading to the complete nuclear export and inactivation of class II HDACs, such that genetic deletion of HDACs 5 and 9 would not further enhance the hypertrophic response to this stimulus. On the contrary, the enhanced sensitivity to calcineurin and pressure overload of mice lacking either *HDAC5* or *HDAC9* suggests that these stimuli inactivate only a fraction of the entire

pool of class II HDACs. Otherwise, if the entire pool of class II HDACs were inactivated in response to these stress stimuli, genetic deletion of HDACs would not be expected to increase the hypertrophic response.

Isoproterenol promotes hypertrophic growth via the β-adrenergic receptor, which stimulates the production of cAMP by adenylyl cyclase with consequent activation of protein kinase A (PKA) (34). PKA phosphorylates numerous proteins within the sarcomere and the sarcoplasmic reticulum that control cardiac contractility and calcium handling. The mechanisms whereby PKA signaling affect cardiac gene expression are less well understood, but the CREB transcription factor is one PKA target that has been shown to promote cardiac growth (5). Neither cAMP nor activated PKA promotes the phosphorylation or nuclear export of class II HDACs (27). However, several studies have implicated calcineurin signaling in the pathway whereby isoproterenol stimulates cardiac hypertrophy. Inhibition of calcineurin activity by elevation in the expression of the modulatory calcineurin interacting protein (MCIP) or other inhibitory proteins, for example, can suppress hypertrophy in response to isoproterenol administration (4, 38). We have also found that isoproterenol efficiently stimulates protein kinase D, which acts as an HDAC nuclear export kinase (R. Vega, E. Olson, and T. McKinsey, unpublished results). Thus, signals emanating from the β-adrenergic receptor may modulate HDAC function through multiple mechanisms (Figure 2.7). It should be noted in this regard that many forms of cardiac stress are accompanied by the activation of complex neurohumoral signals that act through interconnected signaling pathways (2, 8, 22, 34). Indeed, failing human hearts have been reported to demonstrate

elevated activities of calcium, calmodulin-dependent protein kinase (15), which phosphorylates HDACs (27, 28, 46), as well as PKA, which does not directly act on HDACs.

While we favor the notion that MEF2 is a critical direct target for class II HDACs in the signaling pathway for cardiac hypertrophy, modulation of HDAC activity can also influence the activity of other transcription factors involved in hypertrophy, such as GATA4 and NFAT, which interact with MEF2 (32, 45). Stress signals that inactivate class II HDACs also activate p300, which serves as a coactivator for the above transcription factors (10, 44). Thus, there are multiple points of crossover among the signaling pathways that govern cardiac growth in response to stress signaling.

Potential roles of class II HDACs in heart development

Whereas mice lacking either HDAC5 or HDAC9 display normal cardiac structure and function at birth, a high percentage of HDAC5/9 double mutants die during embryogenesis and the perinatal period from VSDs and thin ventricular walls, which typically arise from abnormalities in growth and maturation of cardiomyocytes. Both HDAC5 and HDAC9 are expressed in the developing myocardial chambers and interventricular septum during embryogenesis (49 and this study). Given the interaction between class II HDACs and MEF2, and the central role of MEF2 in the control of cardiomyocyte differentiation (27), the cardiac developmental defects in double mutants may result from super-activation of MEF2 with consequent precocious differentiation and cell cycle withdrawal of cardiomyocytes causing hypocellularity of the myocardium. In addition, class II HDACs participate in multi-protein repression complexes and modulate the activities of numerous transcription factors

involved in myocardial growth, such as the retinoic acid receptor, serum response factor, and myocardin (2, 6, 16, 43, and our unpublished results). The absence of HDACs 5 and 9 may therefore affect the activities of other cardiac transcriptional activators and repressors, thereby perturbing the precisely regulated programs of gene expression required for cardiac development.

It is interesting that genetic deletion of either HDAC5 or HDAC9 sensitizes the heart to pathological stress signals, yet developmental effects require loss of both genes. These differential effects suggest that different types or strengths of signals regulate these HDACs during development and adult heart disease. Alternatively, or in addition, the responsiveness of HDAC5 and HDAC9 to developmental and stress signals could be influenced by other proteins that differ between the embryonic and adult heart.

Control of cell growth and homeostasis by class II HDACs

Congenital and acquired cardiac disease phenotypes in humans and animal models frequently display variability in penetrance and expressivity, indicative of genetic modifiers. Whereas mice lacking as many as three of the four HDAC5/9 alleles are normal at early age, deletion of even one HDAC alleles augments the growth response of the heart to hypertrophic signals. The sensitized phenotypes of HDAC5 and 9 mutant mice demonstrate that heart size depends on class II HDAC gene dosage, and point to these HDACs 5 and 9 as modifiers of cardiac responses to pathological signals. It will be of interest to determine whether these HDACs modify cardiac disease phenotypes in humans.

The specific role of class II HDACs in a given cell type depends on the spectrum of available transcription factors with which they can interact. In addition to the influence of HDACs 5 and 9 on cardiac growth, we have recently found that HDAC4 regulates bone growth and development by repressing chondrocyte hypertrophy (R. Vega and E. Olson, unpublished results). In mice lacking HDAC4, chondrocytes undergo inappropriate hypertrophy, which leads to premature and ectopic mineralization of the cartilaginous skeleton due to activation of the Runx2 transcription factor. Thus, the first three class II HDACs to be analyzed through genetic loss of function in the mouse (HDACs 4, 5 and 9) all result in abnormal hypertrophic phenotypes, suggesting that class II HDACs act as general regulators of cellular hypertrophy during development and disease.

Class II HDACs have also been implicated in cell proliferation and oncogenesis (reviewed in 23). The *HDAC5* gene is located on human chromosome 17q21, which has frequently been associated with chromosomal alternations in human cancer (reviewed in 24). It will be interesting to determine whether mice lacking HDAC5 other class II HDACs display a predisposition to tumorigenesis.

The signal-responsiveness of class II HDACs provides a mechanism for linking extracellular signals with the genome during cellular transitions in development and disease and offers therapeutic opportunities for manipulating gene expression through the modulation of their regulatory kinases and phosphatases. Such approaches hold great promise for the modification of pathological cardiac growth and gene expression. Given the multiplicity of class I and II HDACs, there has also been interest in the identification of small molecular inhibitors that can selectively modulate the functions of individual HDAC isoforms. In this

regard, the recent finding that HDAC inhibitors block cardiac hypertrophy and thereby mimic the activity of class II HDACs (1, 11, 16) raises interesting questions about possible antagonistic roles of class I and II HDACs in cardiac growth (1, 11, 16). Analysis of the responsiveness of cells derived from HDAC knockout mice should facilitate the characterization of such inhibitors and their specific enzymatic targets.

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Chapter III

Histone Deacetylase 7 Maintains Vascular Integrity by Repression of MEF2-dependent Matrix Metalloproteinase Expression

Abstract

Development and homeostasis of the cardiovascular system require intimate interactions between endothelial and smooth muscle cells and the formation of a seamless circulatory network. We show that histone deacetylase 7 (HDAC7) is specifically expressed in the vascular endothelium during early embryogenesis where it maintains vascular integrity by repressing the expression of matrix metalloproteinase (MMP)-10, a secreted endoproteinase that degrades the extracellular matrix. Disruption of the *HDAC7* gene in mice results in embryonic lethality due to a failure in endothelial cell-cell adhesion and consequent dilatation and rupture of blood vessels. HDAC7 represses *MMP10* gene transcription by associating with MEF2, a direct activator of *MMP10* transcription and essential regulator of blood vessel development. These findings reveal an unexpected and specific role for HDAC7 in the maintenance of vascular integrity and have important implications for understanding the processes of angiogenesis and vascular remodeling during cardiovascular development and disease.

Introduction

Changes in histone acetylation alter chromatin structure and serve as a key mechanism for the control of gene expression (18). Histone acetyltransferases (HATs) stimulate gene transcription by acetylating nucleosomal histones which relaxes chromatin structure and facilitates recruitment of transcription factors to DNA target sequences. The stimulatory effect of HATs on gene expression is counteracted by histone deacetylases (HDACs), which remove acetyl groups from histones and promote chromatin condensation with consequent transcriptional repression (18).

Mammalian HDACs can be classified into different classes based on their structure, expression pattern and sequence homology to *Saccharomyces cerevisiae* HDACs (15). Class I HDACs (HDAC1, 2, 3, 8 and 11), which are ubiquitously expressed, are homologous to yeast RPD3 and simply consist of a catalytic domain (15). Class II HDACs (HDAC 4, 5, 7, 9) are homologous to yeast HDA1, and are highly expressed in heart, brain and skeletal muscle (14, 51). In addition to their carboxyl-terminal catalytic domain, class II HDACs contain an amino-terminal extension that interacts with other transcriptional cofactors and confers responsiveness to a variety of external signals (5, 30, 33, 59). Phosphorylation of the amino-termini of class II HDACs by calcium/calmodulin-dependent kinase (CaMK) and protein kinase D (PKD) creates docking sites for the 14-3-3 family of intracellular chaperone proteins, which promote shuttling of these HDACs from the nucleus to the cytoplasm, thereby de-repressing HDAC target genes and coupling external signals to the genome (16, 31, 33, 34, 49).

While the biochemical functions of HDACs have been extensively analyzed, their functions *in vivo* are only beginning to be elucidated. The functions of class II HDACs appear to be remarkably tissue-specific and dedicated to the control of tissue growth and development. HDAC5 and HDAC9 have been implicated in the repression of cardiomyocyte growth in the adult heart. Mutant mice lacking either of these HDACs are viable, but develop extremely enlarged hearts in response to pathological signals (6, 57). Conversely, signal-resistant mutants of HDAC5 and HDAC9 lacking the conserved phosphorylation sites, prevent cardiomyocyte growth (57). Consistent with these observations, HDAC5 and HDAC9 associate with and repress the activity of myocyte enhancer factor-2 (MEF2), a MADS-box transcription factor required for muscle development and growth (30-32, 34, 57-59).

HDAC4 is expressed in prehypertrophic chondrocytes of the developing skeleton and negatively regulates the activity of runt-related transcription factor-2 (Runx2), which is required for chondrocyte hypertrophy (50). Mice homozygous for an *HDAC4* mutation exhibit lethal ossification of endochondral cartilage due to precocious and ectopic hypertrophy of chondrocytes, whereas ectopic expression of HDAC4 in proliferating chondrocytes inhibits hypertrophic growth and differentiation. Thus, the repression of Runx2 activity by HDAC4 in the pre-hypertrophic zone of developing bones determines the timing and extent of endochondral bone formation (50).

In the present study, we generated mice lacking HDAC7, the last of the class II HDACs to be analyzed *in vivo*. Remarkably, *HDAC7* null mice die during mid-gestation from vascular dilatation and rupture caused by loss of adhesion between endothelial cells.

Consistent with this phenotype, HDAC7 is expressed specifically in the developing vascular endothelium where it represses the expression of matrix metalloproteinase-10 (MMP10), a secreted endoproteinase that degrades the extracellular matrix and perturbs vascular integrity. In *HDAC7* mutant embryos, MMP10 expression is dramatically up-regulated in the vascular endothelium and its antagonist tissue inhibitor of metalloproteinase-1 (TIMP1) is down-regulated. We show that HDAC7 is recruited to the promoter of the *MMP10* gene and represses its expression by association with MEF2, a direct activator of *MMP10* transcription. These findings reveal an unexpected and specific role for HDAC7 in the maintenance of vascular integrity and have important implications for understanding the mechanisms of angiogenesis and vascular remodeling during cardiovascular development and disease.

Materials and Methods

Generation of *HDAC7* Mutant Mice

The *HDAC7* targeting construct was generated by cloning a 2.9kb genomic fragment upstream of exon 2 and a 7.3kb fragment from exon 5 to exon 13 into the pN-Z-TK2 vector, which contains a promoterless nuclear *LacZ* (*nLacZ*) cassette and a *neomycin-resistance* gene (kindly provided by R. Palmiter). Following electroporation into ES cells, resulting clones were screened for correct homologous recombination by southern blot analysis. Two independent clones carrying a disrupted *HDAC7* allele were injected to E3.5 C57BL/6 mouse blastocysts to produce chimeric mice. Chimeras were mated with C57BL/6 females to obtain F1 mice carrying the targeted allele. Mice were generated in the isogenic 129/Sv background by breeding the chimeras with 129/Sv females. Genotyping of the mutant mice was performed using following primers:

HDAC7 SA3':5'- CCA GTG GAC GAG CAT TCT GGA GAA AGG C – 3'

LacZ 3'-2: 5' – GCC AGT TTG AGG GGA CGA CGA CAG TAT CG – 3'

HDAC7 SA5': 5'- GTT GCA GGG TCA GCA GCG CAG GCT CTG – 3'

RNA in situ Hybridization and Histology

Embryos and tissues used for histology were fixed in 4% paraformaldehyde, sectioned, and processed for hematoxylin and eosin (H&E) staining or in situ hybridization by using standard procedures (44). ^{35}S-labeled RNA probes were generated using Maxiscript kit (Amersham). Signals were pseudo-colored in red using Adobe Photoshop.

RT-PCR and Microarray Analysis

Total RNA was extracted from embryos or HUVECs using Trizol reagent (Invitrogen) and used as a template for reverse transcriptase with random hexamer primers (Invitrogen). Primer sequences for specific genes are available upon request. The details were described in Chapter II. Microarray analysis was performed using Human Genome U133 Plus 2.0 array (Affymetrix).

Whole Mount Staining and Immunohistochemistry

Immunostaining of embryos was performed as described previously (23). Briefly, embryos were fixed in 4% paraformaldehyde, bleached with 5% H_2O_2, blocked in 3% milk, incubated with primary antibodies and second antibody sequentially, and finally stained with the DAB kit (Vector labs). Antibodies used were anti-mouse CD31 (clone MEC13.3, Pharmingen), anti-SM α-actin (Clone 1A4, Sigma) and HRP-conjugated goat anti-rat IgG (Jackson ImmunoResearch). β-Galactosidase staining of embryos and tissues was performed as described (7). After staining, samples were sectioned and counter-stained with light eosin. Immunohistochemistry were described previously (45). Primary antibodies used were anti-SM α-actin, anti-MMP10 (clone IVC5, Chemicon) and anti-TIMP1 (polyclonal, Chemicon).

Electron Microscopy

Wild type and HDAC7 mutant embryos were fixed overnight in 2% glutaraldehyde in 0.1M cacodylate buffer at 4^{o}C, then postfixed, and dehydrated in an ethanol series. Samples

were then embedded in Spurr resin (Ted Pella, Inc., Redding, CA), stained with uranyl acetate and lead citrate and sectioned at 80 nm as described (22).

Cell Culture and Immunocytochemistry

HUVEC cells were purchased from ATCC. HAEC cells were obtained from Clonetics. Both cell lines were cultured in endothelial cell growth medium (Clonetics/Cambrex). For immunostaining, cells were plated on gelatin-coated glass coverslips and were fixed and stained as described (59). Briefly, the cells were fixed with 3.7% formaldehyde at room temperature for 5 minutes. After 3 washes with PBS, 5 minutes each, the cells were then permeated with blocking buffer (0.1% NP-40 and 3% BSA in PBS) for 15 minutes and incubated with primary antibody, which is diluted at 1:200 with the blocking buffer, for 45-60 minutes. After 3 washes with PBS containing 0.1% NP-40, 5 minutes each, the cells were incubated with secondary antibody diluted at 1:200 with blocking buffer for 45-60 minutes. After 3 washes again with PBS containing 0.1% NP-40, and a final wash with ddH2O, the coverslips were mounted with 15ul mounting medium containing DAPI from Vectashield. HDAC7 antibody was purchased from Santa Cruz.

RNAi and In Vitro Angiogenesis

SiRNA oligonucleotides were purchased from Ambion and used at a concentration of 40nM. HUVECs were transfected with Lipofactomine Plus reagent (Invitrogen). Four days after transfection, cells were harvested either for RNA analysis or in vitro angiogenesis. Matri-gel was purchased from Chemicon and the assays were performed as instructed by the

manufacturer. RT-PCR and in vitro angiogenesis were performed multiple times with comparable results. HDAC7-SiRNA sequence is: TCACTGACCTCGCCTTCAA. Primer sequences for RT-PCR are:

HDAC7 RT 5': 5' – CAG AGC ACT TCC CTC TGC GCA AGA CA – 3'

HDAC7 RT 3': 5' – GAG AAT GGG CTG CAG GCG AGA GGG C – 3'

MMP10 RT 5': 5' – GTC CTT CGA TGC CAT CAG CAC TCT GAG – 3'

MMP10 RT 3': 5' – CTA GGG AAG CCT TGC TCC ATG GAC TG – 3'

TIMP1 RT 5': 5' – GGA CAC CAG AAG TCA ACC AGA CCA CC – 3'

TIMP1 RT 3': 5' – CCG TCC ACA AGC AAT GAG TGC CAC TC – 3'

Reporter Assays

The MMP10-luciferase construct contained a DNA fragment extending from -886bp to +11bp of the mouse *MMP10* gene. The N-terminal flag-tagged HDAC7 and HDAC7 mutants were subcloned into pcDNA3.1 vector (Invitrogen). Amino acids 76-130 were deleted in the HDAC7-ΔMEF mutant. MEF2D and myc-tagged MEF2C were previously described (39). COS cells in 24-well plates were transfected with 150ng of reporter plasmids in the presence or absence of MEF2D or HDAC7. The reporter assays were performed as described (5).

Gel Mobility Shift Assay

Gel-mobility shift assays were performed as described (4). COS cells were lysed 48 hrs after transfection with a myc-tagged MEF2C expression plasmid or empty vector. The

DNA probe was labeled with ^{32}P-dCTP using a Klenow-fill in method and purified using a G25 DNA purification column from Roche. The 20ul binding reaction contained 1 ug of poly (dI-dC) (Sigma), 50,000 cpm of probe and 2ul of cell lysate. After incubation at room temperature for 15 minutes, the reactions were separated on a 5% PAGE gel containing 0.5 X TBE. The sequences of probes used are:

MCK MEF2 probe 5': 5'- GGG GAT CGC TCT AAA AAT AAC CCT GTC G -3'

MCK MEF2 probe 3': 5' – GGG CGA CAG GGT TAT TTT TAG AGC GAT C – 3'

MMP10 MEF2 wild-type probe 5': 5'- GGG CAT TTG GAT TAA AAA TAG CAC CCA TG – 3'

MMP10 MEF2 wild-type probe 3': 5' – GGG CAT GGG TGC TAT TTT TAA TCC AAA TG – 3'

MMP10 MEF2 mutant probe 5': 5' - GGG GTT CTT GTA GTC ATT TGG ATT AGG GGT AGC ACC CAT GTG AGT CAT GC – 3'

MMP10 MEF2 mutant probe 3': 5' – GGG GCA TGA CTC ACA TGG GTG CTA CCC CTA ATC CAA ATG ACT ACA AGA AC – 3'

Chromatin Immuno-Precipitation Assay

A ChIP kit was used for the assays according to manufacturer's instructions (Upstate Biotechnology). Basically, cells were crosslinked with 1% formaldehyde at 37 ^{o}C for 10 minutes. After washing with cold PBS twice, cells were scraped in 500 ul of PBS and centrifuged at 5000 g for 15 minutes at 4 ^{o}C. The pellets were then resuspended in 300 ul of SDS Lysis Buffer (1% SDS, 10 mM EDTA, 5 0 mM Tris-HCl, pH 8.1, and protease

inhibitors). The lysate was sonicated three times for 10 seconds each, to shear the DNA to between 200 bp to 1000 bp in length. After centrifugation at 14,000 rpm for 10 minutes in a benchtop eppendorf centrifuge, the supernatant was diluted 10 times with CHIP Dilution Buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCL, pH 8.1, 167 mM NaCl, and protease inhibitors). 1 ml of the diluted lysate was then cleared with 10 ug salmon sperm DNA and 20 ul Protein A-agrose beads (Santa Cruz) at 4 ^{o}C for 2 hrs. After a brief centrifugation to pellet the beads, the supernatant was incubated with 2 ug of specific antibodies over night at 4 ^{o}C. The next day, 10 ug salmon sperm DNA and 40 ul Protein A-agarose beads were added to the supernatant and incubated for 2 hrs. The beads were then washed sequentially with Low Salt Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8., 150 mM NaCl), High Salt Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8, 500 mM NaCl), and LiCl Wash Buffer (0.25 mM LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCL, pH 8.1) for 5 minutes each. The beads were then washed with TE for three times, and the precipitants were eluted twice with 250 ul of Elution Buffer (1% SDS, 0.1 M NaHCO3). 20 ul of 5M NaCl was added to each elute and incubated at 65 ^{o}C for 4 hrs to reverse crosslink. DNA fragments were then purified with QIAquick Spin column. Acetyl-histone3 antibody was purchased from Upstate Biotechnology and MEF2 antibody was purchased from Santa Cruz. Primer sequences are:

Human MMP10 5': 5' - CAA CTC CCA CAT TTA GAC CAC GAC TA – 3'

Human MMP10 3': 5' - CAG AGA CAA TGC TGA GAT CAC CAA CAG – 3'

Results

Generation of *HDAC7* mutant mice

To explore the functions of HDAC7 in vivo, we inactivated the mouse *HDAC7* gene by homologous recombination in ES cells and generated *HDAC7* mutant mice. The targeted mutation deleted exons 2 to 5, which encode residues 1-130 that encompass the MEF2-binding domain of the protein. The mutation also introduced a promoter-less nuclear β-galactosidase protein coding region in-frame with the initiation codon of the *HDAC7* gene and a neomycin-resistance cassette (Figure 3.1A). Targeting of *HDAC7* in ES cells was confirmed by Southern blot analysis (Figure 3.1B). ES cells heterozygous for *HDAC7* mutation were injected into E3.5 blastocysts to generate chimeric mice, which transmitted the mutant allele through the germ line.

Mice heterozygous for the *HDAC7* mutation were viable, fertile, and phenotypically normal. Heterozygote intercrosses in the isogenic SvEv129 background, as well as in the SvEv129/C57BL6 mixed background failed to yield any live *HDAC7* homozygous mutant pups, indicating that *HDAC7* was required for embryonic development. By timed matings of heterozygous mutant mice, we determined that the genotypes of embryos showed Mendelian ratios of inheritance up to E11.0, but no live homozygous mutants were obtained at later developmental time points (Table 3.1).

HDAC7 mutant mice were phenotypically undistinguishable from wild type embryos before E10.75. However, six hours later at E11.0, *HDAC7* mutant embryos died from widespread cardiovascular defects. This lethal phenotype was 100% penetrant. Homozygous

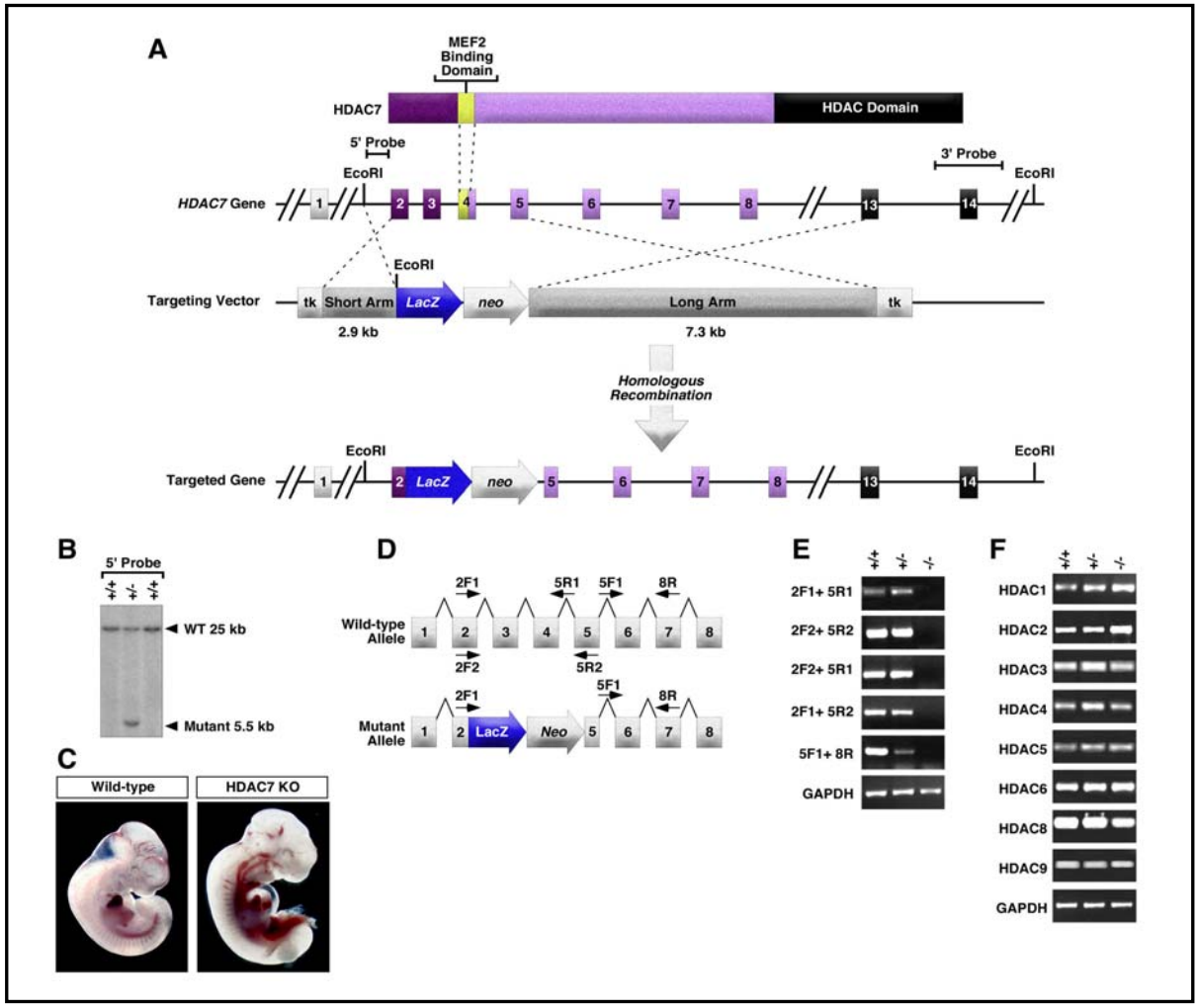

Fig. 3.1. Generation of HDAC7 mutant mice. (A) Strategy to generate HDAC7 mutant mice by homologous recombination. Exons 2-5, which encode the MEF2-binding domain, were replaced with a promoter-less nuclear lacZ cassette in frame with exon 2 and a neomycin-resistance cassette. (B) Southern blot analysis of genomic DNA from ES cells. DNA was digested with EcoRI; the positions of the wild type (25kb) and mutant (5.5kb) bands are indicated. Genotypes are shown on the top. (C) Wild type (1) and HDAC7 mutant (right) embryos at E11.0. The HDAC7 mutant showed dramatically enlarged dorsal aortae, pericardial effusion and hemorrhages. (D) Schematic of HDAC7 exons with positions of primers used for RT-PCR analysis. (E) Analysis of HDAC7 transcripts by RT-PCR. Genotypes are shown on the top and primer pairs at the right. (F) Transcripts for HDACs 1-6, 8 and 9 from embryos of indicated genotypes were analyzed by semi-quantitative RT-PCR. GAPDH transcripts were detected as a control.

mutant embryos showed pericardial effusion and enlarged dorsal aortae at the time of death, indicative of abnormalities in the circulation system (Figure 3.1C).

RT-PCR analysis of RNA from *HDAC7* mutant embryos at E10.5 using primers representing exon sequences within and surrounding the deleted region of the gene revealed that *HDAC7* transcripts were not present in homozygous mutants (Figure 3.1D and E) and suggested that the *HDAC7* mutant allele was a true null allele. To determine whether other HDACs might be up-regulated in the mutant embryos to compensate for the loss of HDAC7, we analyzed the expression of class I and II HDACs in wild type and mutant embryos by RT-PCR. Transcripts encoding other HDACs were expressed at normal levels in the mutant embryos (Figure 3.1F).

Table 3.1. Genotypes of Offspring from *HDAC7*$^{+/-}$ Intercross

	Offspring of Each Genotype		
Age	+/+	+/-	-/-
Before E11.0	21(28%)	37(50%)	16(22%)
E11.0-E12.5	10(30%)	16(47%)	8*(22%)
P10	45(38%)	72(62%)	0

* 5 out of 8 were dead at the time of dissection

Numbers of offsprings of each genotype and percent of total are shown.

Endothelial cell-specific expression of *HDAC7*

The embryonic expression pattern of *HDAC7* has not been previously defined. To begin to determine the cause of lethality in *HDAC7* mutant embryos, we analyzed *HDAC7*

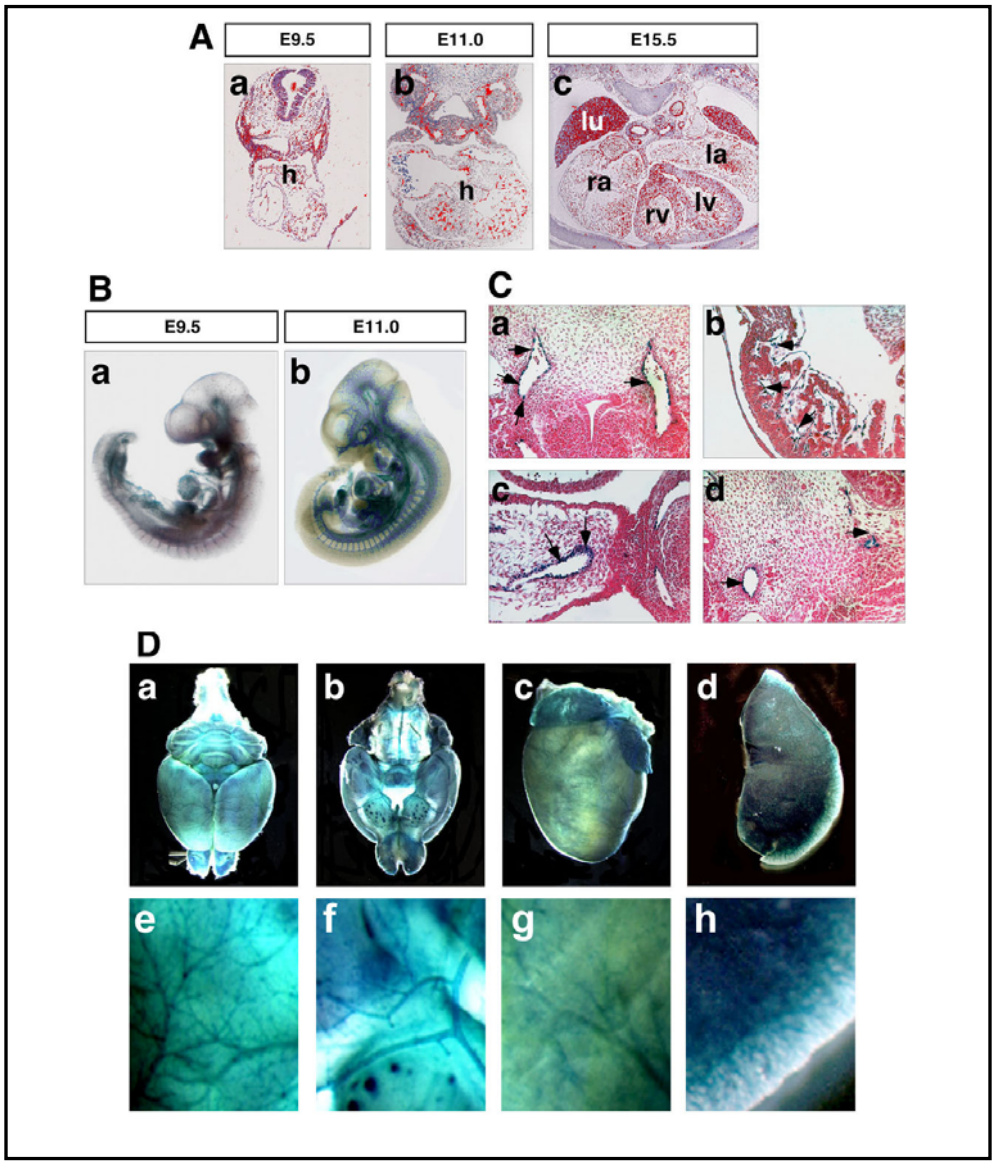

Fig. 3.2. Expression pattern of HDAC7 during mouse embryogenesis. (A) HDAC7 transcripts were detected by RNA in situ hybridization to transverse sections as follows: (a) staining of endothelial cells and surrounding mesenchyme cells at E9.5; (b) staining of vascular endothelium and endocardium at E11.0; (c) staining of heart, lung and major vessels at E15.5. h, heart; la, left atrium; lu, lung; lv, left ventricle; ra, right atrium; rv, right ventricle. (B) Expression of LacZ from targeted HDAC7 allele. (a) E9.5, (b) E11.0. (C) Light eosin staining of histological sections of HDAC7+/- embryos at E11.0 prestained for LacZ. Arrows point to endothelial cells stained for beta-galactosidase expression. (a) communication between dorsal aorta and branchial arch arteries; (b) endocardium; (c) outflow track; (d) small vessels in brain. (D) Expression of LacZ from targeted HDAC7 allele in adult tissues. (a-b) brain, (c) adult, (d) lung. Notice the high level expression in the blood vessels on the high magnification view (e-f).

expression by in situ hybridization. *HDAC7* transcripts were first detected at E9.5 in endothelial cells within the heart and blood vessels and in mesenchyme (Figure 3.2Aa). At E11.0, *HDAC7* expression was restricted to endothelial cells lining the vessels and the cardiac chambers (Figure 3.2Ab). At later stages, expression of *HDAC7* was also prominent in the myocardial layer of the heart, while expression in the endothelium was maintained. At E15.5, high expression of *HDAC7* in lung tissue was also observed (Figure 3.2Ac).

Mouse embryos harboring the *HDAC7* mutation expressed lacZ in a pattern corresponding to that of the endogenous *HDAC7* gene. Cardiovascular specific expression of lacZ was observed at E9.5 (Figure 3.2Ba). At E11.0, lacZ was predominantly expressed in the heart and vessels. Low expression in neural tube was also observed (Figure 3.2Bb). Serial sections of E11.0 embryos indicated that lacZ expression was restricted to vascular and cardiac endothelial cells (Figure 3.2Ca-d). LacZ expression was also observed in skeletal muscle from E12.5 to adulthood (data not shown). LacZ staining of adult tissues from heterozygous mutant mice showed that *HDAC7* was highly expressed in brain, lung, heart, and vessels (Figure 3.2D), which is consistent with the pattern of the HDAC7 transcript detected by northern blot (19). HDAC7 expression was undetectable in the yolk sac vasculature, which indicates differential regulation of embryonic and extraembryonic vessels (data not shown).

Cardiovascular abnormalities in *HDAC7* mutant mice

Transverse sections of mutant embryos at E11.0 showed that the dorsal aortae and cardinal veins were severely enlarged and frequently ruptured (Figure 3.3A). Although the

gross morphology of the hearts in mutant embryos appeared normal with completed rightward looping and normal chamber formation, the myocardial walls of the ventricular chambers were abnormally thin and the atria were dilated (Figure 3.3A). Less smooth muscle cells surrounding dorsal aortae were observed in the mutant embryos compared to wild type littermates (Figure 3.3A).

Possible endothelial abnormalities of *HDAC7* mutant embryos were further examined by electron microscopy. Whereas tight junctions between adjacent endothelial cells were observed in wild type embryos, such junctions were missing in mutant embryos prior to death (Figure 3.3B). Instead, endothelial cells appeared to extend long processes that failed to establish tight endothelial cell-cell interactions, which are essential for maintaining vascular integrity.

Normal differentiation and patterning of endothelial and smooth muscle cells in *HDAC7* mutant embryos

In light of the specific expression of *HDAC7* in embryonic endothelial cells by E11.0 and the obvious morphological abnormalities in this cell population as revealed by electron microscopy, we examined for possible defects in endothelial cell differentiation in *HDAC7* mutant embryos. A panel of molecular markers important for vasculogenesis, including *VEGF*, *flk1*, *flt1*, *tie1*, *tie2*, *Flt4, ET-1, ETA, Erb3, Neuregulin, ARNT, Hif1a, Hrt1, Hrt2, Ang-1, Ang-2, endoglin-1, Smad5, LKLF, eNOS, PDGFb, hb-EGF, MEF2C*, was analyzed either by RT-PCR or RNA *in situ* hybridization, but no changes in the expression levels of these genes were observed (data not shown). Among these genes, *tie1*, *tie2*, *ang-1* and *ang-2*

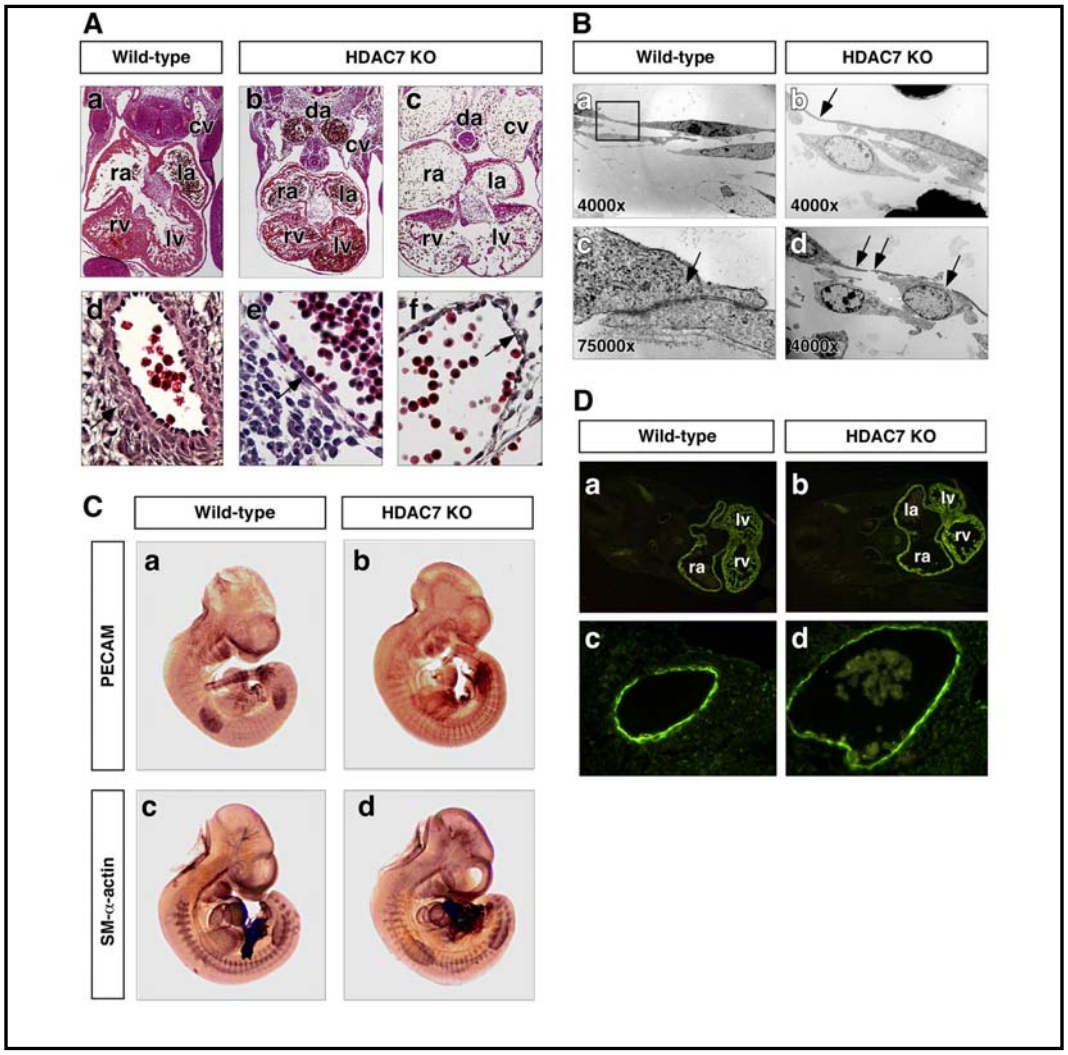

Fig. 3.3. Cardiovascular defects in HDAC7 mutant mice. (A) Histological analysis of HDAC7 mutant mice. (a-c) Low magnifications of H&E staining of transverse sections from E11.0 embryos of indicated genotypes. Note dilated vessels in HDAC7 mutants. (d-f) High magnification of dorsal aortae. Note that there are less supporting cells in mutant embryos (arrows). cv, cardinal vein; da, dorsal aorta; la, left atrium; lv, left ventricle; ra, right atrium; rv, right ventricle. (B) Electron microscopy analysis of HDAC7 mutant mice. Endothelial cell-cell adhesions were absent in mutant sections. Box in panel a is enlarged in c. Arrow in panel c points to tight junction in wild type embryo. Arrows in panels b and d point to abnormal endothelial cell extension and lack of tight junction in the mutant embryo. (C) Whole mount PECAM staining of wild type (a) and mutant (b) embryos at E10.5. The vascular plexus is well formed in mutant mice. (c and d) Smooth muscle □-actin staining of embryos at E11.0. Staining of the dorsal aorta in mutant embryos (arrows) is less prominent than in wild type embryos. (D) Immunohistochemistry against SM α-actin. (a and b) Transverse sections of wild type and mutant embryos were stained for SM α-actin (green). (c and d) High magnification of dorsal aorta shows dilatation and reduced staining in mutant embryos.

are key molecules involved in recruitment of smooth muscle cells by endothelial cells (11, 29, 41, 47).

To visualize the primative vasculature plexus, we stained E10.5 embryos with an antibody against PECAM-1, an endothelial cell-specific marker. As shown in Figure 3.3Ca-b, differentiated endothelial cells were properly positioned in the mutant embryos. The complexity and patterning of the cranial vasculature, the intersomitic vasculature, and the dorsal aortae as revealed by PECAM-1 staining were also similar in wild type and *HDAC7* mutant embryos. Transverse sections showed the presence of endothelial cells in the dorsal aortae and cardinal veins in both wild type and *HDAC7* mutant embryos, although the vessels in the mutant embryos were larger than normal. Similarly, PECAM staining was detected in the endocardium of the hearts of wild type and mutant embryos (data not shown). These results indicated that endothelial cell differentiation and patterning were unaffected by disruption of the *HDAC7* gene.

Recruitment of smooth muscle cells by arterial endothelial cells provides contractility and mechanical support for the developing arteries. To investigate whether the vascular abnormalities in *HDAC7* mutant embryos were caused by a defect in recruitment or differentiation of smooth muscle cells, we performed whole mount antibody staining for smooth muscle α-actin, a smooth muscle cell-specific marker. Normal patterning of smooth muscle cells was observed in the mutant embryos, suggesting that vascular endothelial cells were capable of recruiting smooth muscle cells (Figure 3.3Cc-d). Immunostaining of embryo sections for SM α-actin revealed the presence of smooth muscle cells in the dorsal aorta,

indicating that the dorsal aorta in the mutant embryos correctly adopted the arterial fate during development (Figure 3.3Da-b), which was further confirmed by immunostaining against Ephrin B, an arterial specific endothelial cell marker (data not shown). However, fewer SM α-actin positive cells were observed in mutant compared to wild-type embryos (arrowheads in Figure 3.3Dc-d), suggesting a possible abnormality in recruitment of smooth muscle cells by endothelial cells in the absence of HDAC7. TUNEL staining showed no difference in apoptosis in endothelial and smooth muscle cell populations between wild type and mutant embryos (data not shown). We conclude that the absence of HDAC7 results in a specific abnormality in endothelial cell-cell adhesion and recruitment of smooth muscle cells with consequent vascular rupture and embryonic death.

Altered gene expression by knockdown of *HDAC7* in endothelial cells *in vitro*

In an effort to identify potential HDAC7 target genes in endothelial cells that might reveal the mechanism by which HDAC7 controls vascular integrity, we used RNAi to suppress HDAC7 translation in Human Umbilical Vein Endothelial Cells (HUVECs). In HUVECs transfected with siRNA specific for HDAC7 mRNA, HDAC7 transcripts were down-regulated at least 3-fold compared to controls (Figure 3.4A). Down-regulation of HDAC7 protein was confirmed by immunostaining using an antibody against HDAC7 (Figure 3.4B).

When cultured on matri-gel, HUVECs spontaneously organize themselves into a primative vascular network. In the presence of HDAC7 siRNA, HUVECs failed to adhere to each other and instead remained as single cell or clumps of cells (Figure 3.4Cb). In contrast,

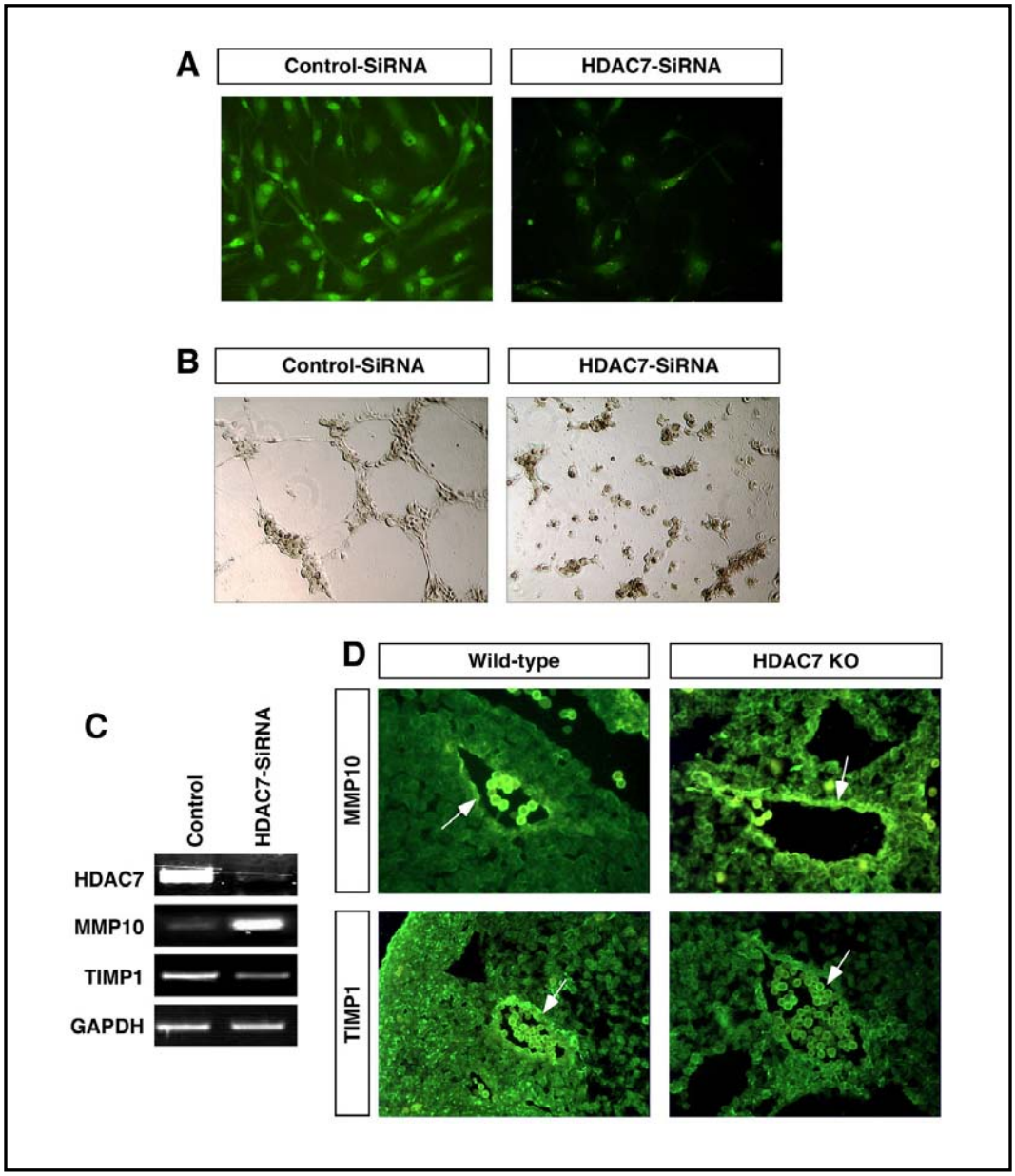

Fig. 3.4. Identification of HDAC7 target genes in endothelial cells. (A) Immunocytochemistry against HDAC7 in HUVECs. HDAC7 protein level was significantly down-regulated by SiRNA transfection. **(**B) In vitro angiogenesis assay. HUVECs failed to form vascular network after HDAC7 knockdown. **(**C) RT-PCR analysis to confirm microarray data. RT-PCR was performed to confirm the efficiency of HDAC7 knockdown. At least 70% of HDAC7 transcripts were eliminated by SiRNA transfection. **(**D)Immunohistochemistry against MMP10 and TIMP1. Note that MMP10 is up-regulated (arrows) and TIMP1 is down-regulated (arrows) in the endothelium of branchial arch arteries of HDAC7 mutant embryos.

GFP siRNA, as a control, had no effect on the ability of HUVECs to become organized into an endothelial lattice (Figure 3.4Ca).

Microarray analysis to compare the gene expression profiles of HUVECs treated with HDAC7 siRNA and controls revealed that numerous genes encoding extracellular matrix and adhesion proteins were dysregulated in the presence of HDAC7 siRNA (Table 3.2). Of particular interest were the genes encoding matrix metalloproteinase 10 (MMP10), also referred to as stromelysin-2, which was up-regulated by 6.5 fold, and its inhibitor tissue inhibitor of metalloproteinase 1 (TIMP1), which was down-regulated by 8.6 fold. MMP10 is a secreted proteinase that degrades the extracellular matrix, and TIMP1 inhibits MMP10 activity (42, 53). Thus, the up-regulation of MMP10 and concomitant down-regulation of TIMP1 would be predicted to disrupt cell-cell adhesion and result in leakage and dilatation of blood vessels as seen in *HDAC7* mutant embryos. The changes in expression of these genes in HUVECs treated with HDAC7 siRNA were validated by RT-PCR (Figure 3.4Ca).

We further examined the expression of MMP10 and TIMP1 in *HDAC7* mutant embryos by immunohistochemistry. Consistent with their expression in cultured endothelial cells, MMP10 was dramatically up-regulated in the perivascular region, while TIMP1 was down-regulated in vascular endothelial cells in mutant embryos (Figure 3.4D).

MMP10 is a direct target of MEF2 and HDAC7

Given the fact that HDAC7 is a transcriptional repressor and *MMP10* is up-regulated in *HDAC7* mutant embryos, we searched for transcription factors that control expression of *MMP10* in endothelial cells. Previous reports indicate that two AP-1 sites and two PEA3

Table 3.2. Genes regulated by HDAC7 in HUVECs

Fold change	Genes
48.5	calcineurin-binding protein calsarcin-1
7	growth arrest-specific 1 (GAS1)
6.5	matrix metalloproteinase 10 (stromelysin 2) (MMP10)
5.2	histone deacetylase 9
4.6	retinol dehydrogenase homolog isoform-1 (RDH)
4	sprouty (Drosophila) homolog 1 (antagonist of FGF signaling)
-22.6	DBL proto-oncogene splicing variant 1 (Ras GEF)
-13.9	5-hydroxytryptamine (serotonin) receptor 2B (HTR2B)
-11.3	retinol-binding protein 4, interstitial (RBP4)
-8.6	tissue inhibitor of metalloproteinase 1 (TIMP1)
-7	claudin 14 (CLDN14)
-6.5	hairy and enhancer of split (Drosophila) homolog 2 (HES2)
-5.7	angiopoietin-related protein (ANGPTL2)
-5.3	fibroblast growth factor 1 (acidic) (FGF1)
-4	retinoic acid receptor, gamma (RARγ)

Representative genes that are dysregulated in HUVECs upon HDAC7 knockdown are listed.

sites in the *MMP10* promoter are responsible for constitutive expression and induction by inflammation (2). By searching for evolutionarily conserved sequences in the upstream region of the *MMP10* gene, we identified a potential MEF2 site in the promoter (Figure 3.5A).

To confirm this site was a bona fide binding site for MEF2, we performed DNA binding assays using extracts from COS cells transfected with a Myc-MEF2C expression plasmid. The MEF2-consensus sequence from the *MMP10* control region bound MEF2C comparably to the canonical MEF2 site from the *muscle creatine kinase* (*MCK*) enhancer (13) (Figure 3.5B). This DNA-protein complex was super-shifted by anti-Myc antibody, and was abolished in the presence of an excess of the unlabeled cognate DNA sequence or the MCK MEF2 site as a competitor, whereas a mutant sequence failed to compete for MEF2C binding (Figure 3.5B). Similarly, the DNA-protein complex between MEF2 and the MCK MEF2 site was abolished by an excess of unlabeled MCK MEF2 sequence or MMP10 MEF2 sequence but not by the mutant sequence (Figure 3.5B).

A DNA fragment containing this site conferred MEF2 responsiveness to a luciferase reporter in transfected COS cells (Figure 3.5C), and mutation of the MEF2 site completely abolished responsiveness to MEF2. Moreover, HDAC7 prevented activation of the *MMP10* promoter by MEF2. A mutant of HDAC7 lacking the MEF2-binding domain (HDAC7-ΔMEF) failed to inhibit the *MMP10* promoter, whereas a mutant lacking the signal-responsive serines (HDAC7-S/A), which functions as a "super-repressor", showed greater repressive activity against MEF2 (Figure 3.5D).

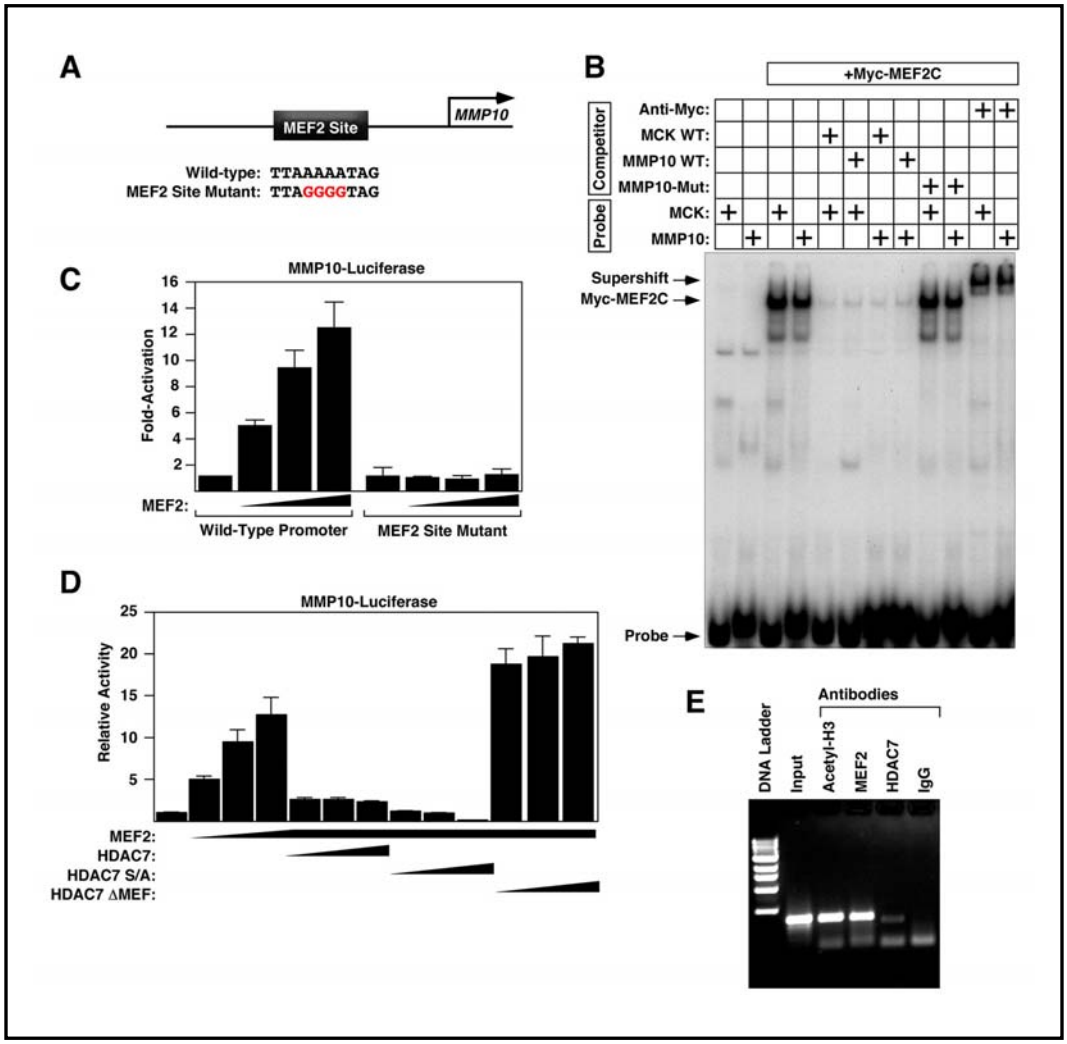

Fig. 3.5. MMP10 expression is controlled by MEF2 and HDAC7. (A) The sequence of the mouse MMP10 gene containing the potential MEF2 site and the TATA box is shown. (B) Gel mobility shift assays were performed with a labeled MMP10 probe or MCK probe and extracts from COS cells overexpressing Myc-MEF2C. Unlabeled MMP10 probe and MCK probe were indistinguishable in their ability to compete with labeled probes to form DNA-protein complex. The DNA-protein complex was supershifted in presence of anti-myc antibody. (C) Responsiveness of MMP10 gene promoter to MEF2. COS-1 cells were transfected with pGL3-Luc reporter containing the region from -886bp to +11bp of MMP10 gene with indicated amounts of MEF2D. Mutation of the predicted MEF2 site completely abolished the responsiveness of MMP10 promoter to MEF2D. (D) Activation of MMP10 promoter by MEF2 is inhibited wild-type HDAC7 and signal-resistant HDAC7 mutant (HDAC7-S/A), but not by an HDAC7 mutant lacking the MEF2-binding domain (HDAC7-ΔMEF). (E) ChIP assays were performed with chromatin prepared from HAECs. Chromatin was immunoprecipitated with mouse IgG or antibodies against acetylated histone H3, MEF2 or HDAC7, and precipitated genomic DNA was analyzed by PCR using primers for the MMP10 promoter, which contains a MEF2 binding site. PCR amplification was performed prior to immunoprecipitation for the input control.

To further validate the association of MEF2C with the *MMP10* promoter, we performed chromatin immunoprecipitation (ChIP) assays. As shown in Figure 3. 5E, MEF2, which is expressed in and required for endothelial cell differentiation (24, 25), was detected on the *MMP10* promoter in the context of native chromatin in Human Aortic Endothelial Cells (HAECs). We conclude that MEF2 binds directly to the regulatory region responsible for expression of *MMP10* in the endothelial cells and that HDAC7 is recruited by MEF2 to the *MMP10* gene where it represses *MMP10* expression.

Discussion

The results of this study reveal a key role of HDAC7 in maintenance of vascular integrity. Disruption of the *HDAC7* gene, which is expressed specifically in endothelial cells, results in embryonic lethality by E11.0 due to cardiovascular defects that include dilated and ruptured blood vessels, dilated atria, and thin-walled myocardium. Endothelial cells of *HDAC7* mutant embryos appear normal during initial differentiation and patterning, but display cell-cell adhesion defects. In addition, the expression levels of two proteins involved in regulating the interaction of endothelial cells with the extracellular matrix, MMP10 and TIMP1, are perturbed in endothelial cells of *HDAC7* mutant embryos. Promoter analysis revealed that *MMP10* is a direct target of MEF2, a key transcription factor required for endothelial cell differentiation. We conclude that HDAC7 regulates vascular permeability by modulating MMP activity and expression through inhibiting MEF2 activity, as schematized in Figure 3.6.

Regulation of vascular integrity by HDAC7

Formation of the vasculature involves two sequential steps, vasculogenesis, the initial formation of primary vessels, and angiogenesis, a process of sprouting or splitting of blood vessels and remodeling of vascular beds (40). During both processes, endothelial cells recruit supporting cells, smooth muscle cells in major arteries and pericytes in microvessels, to provide contractility and mechanical support. The extracellular matrix surrounding endothelial cells and tight junctions between adjacent endothelial cells protect the vasculature from invasion.

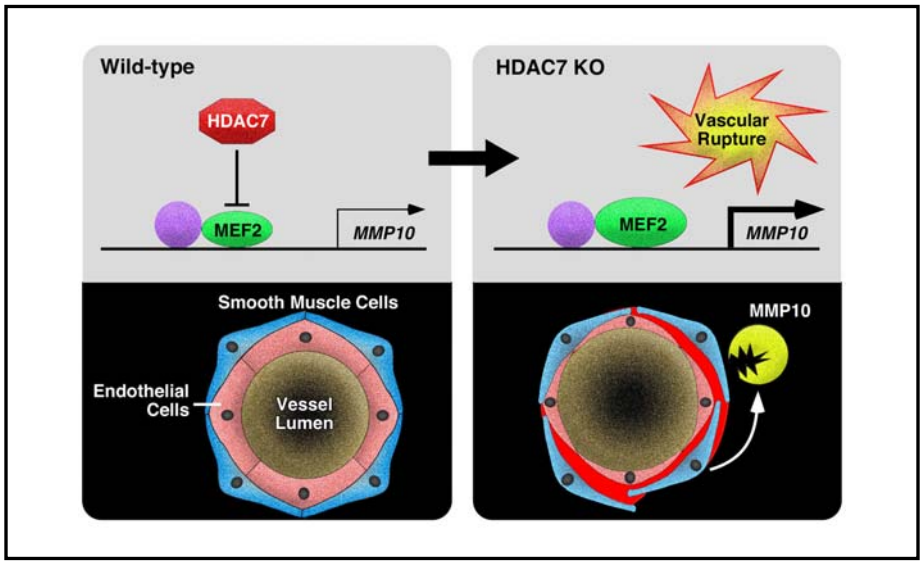

Fig. 3.6. A model for modulation of MMPs by HDAC7 through inhibition of MEF2 activity. MMP10 expression is controlled by MEF2, which is antagonized by HDAC7 in endothelial cells. In the absence of HDAC7, MMP10 is over-expressed. Combined with down-regulation of TIMP1, extracellular matrix is greatly disrupted, which leads to rupture of blood vessels.

The primary defect in *HDAC7* mutant embryos is the loss of vascular integrity with consequent embryonic demise. Formation of the primary vascular plexus – vasculogenesis, which begins at E7.5 in mice, is unperturbed in *HDAC7* mutant embryos. Recruitment of smooth muscle cells takes place at around E9.0, and by E9.5, when *HDAC7* expression is first detectable. In the vascular endothelium, the recruitment of the first layer of smooth muscle cells is essentially complete, which explains why smooth muscle α-actin staining is detected in *HDAC7* mutant embryos. However, by E11.0, the ability of endothelial cells in *HDAC7* mutant embryos to recruit supporting cells is compromised, as evidenced by the reduction of surrounding cells in the mutants, which is associated with partial loss of endothelial cell-cell adhesion. These findings demonstrate that the major embryonic role of HDAC7 is to maintain vascular integrity. Confirmation of the specific function of HDAC7

in the developing vascular endothelium was provided by the creation of a conditional *HDAC7* mutant allele. Endothelial cell-specific deletion of *HDAC7* results in the same phenotype as global deletion of the gene.

The endothelium, which forms the seamless inner lining of all blood vessels, is essential for maintenance of vascular integrity. Vascular leakage causes circulatory collapse and contributes to the pathogenesis of numerous, usually life-threatening, diseases, such as atherosclerosis and aneurysms. Vascular integrity is maintained by tight junctions between adjacent endothelial cells. During angiogenesis, MMPs degrade components of the extracellular matrix including the junctional complex and participate in the remodeling of vascular basement membranes and extracellular matrix, which facilitates detachment of supporting cells from endothelium and permits endothelial cells to migrate into surrounding tissues (42, 43, 48, 54).

TIMPs, which represent a family of endogenous MMP inhibitors, are highly expressed in endothelial cells to provide native protection from breakdown of the endothelium (17). The up-regulation of MMP10 was accompanied by down-regulated of its inhibitor TIMP1, which would be expected to further exacerbate the abnormalities in vascular integrity.

Regulation of *MMP10* by MEF2 and HDAC7 during endothelial development

MEF2C is highly expressed in the developing vascular endothelium (24), and MEF2C knockout mice die at E9.5 from vascular abnormalities that have been attributed in part to a failure of the endothelium (24, 25). Recent studies have shown that MEF2 serves as a

platform for the mutually exclusive recruitment of class II HDACs or the histone acetyltransferase p300, which occupy a docking site within the MEF2 DNA binding domain (55, 56). Class II HDACs, including HDAC7, contain a short peptide sequence near their N-termini that tethers them to MEF2, resulting in repression of MEF2 target genes, such as *MMP10*. A variety of signaling pathways involving G-protein-coupled receptors and calcium-dependent protein kinases lead to the phosphorylation of a set of serine residues in the N-terminal regulatory domains of class II HDACs (5, 30, 31, 38, 49). When phosphorylated, these sites bind 14-3-3 chaperone proteins, which escort class II HDACs from the nucleus to the cytoplasm, freeing MEF2 from the repressive influence of class II HDACs and allowing p300 to dock on MEF2 and stimulate MEF2-dependent transcription (16, 31). Our results show that HDAC7 potently represses the *MMP10* promoter, whereas a mutant form of HDAC7 lacking the MEF2 binding domain is unable to repress transcription.

It has also been shown that class I HDACs are involved in regulation of extracellular matrix proteins including MMPs through unknown mechanisms (52). In addition, histone deacetylase inhibitors regulate MMP activity by inducing the expression of RECK, a repressor of MMPs (1, 3, 27).

Unique functions of class II HDACs

HDAC7 is the last of the four mammalian class II HDACs to be analyzed by gene disruption in the mouse. The different class II HDACs play unique and highly specific roles in the control of cell growth and homeostasis. The role of HDAC7 in maintenance of vascular integrity is distinct from the roles of other class II HDACs. HDACs 4, 5 and 9 share

common function in regulating cellular hypertrophy. Mice lacking HDAC4 display chondrocyte hypertrophy during skeletogenesis and consequently postnatal lethality (Vega et al., 2004b); HDAC5 or HDAC9 mutant mice are viable but show enhanced cardiac growth in response to stress signals inducing cardiac hypertrophy (Zhang et al., 2002; Chang et al., 2004b).

Since HDAC7 regulates *Nur77* transcription and TCR-mediated apoptosis during thymus development (12), we examined *HDAC7* mutant embryos by TUNEL assays (data not shown). However, we do not see any increase in apoptosis compared to wild type embryos, which indicates that HDAC7 may play different roles in different organs. Given the importance of MEF2 in skeletal muscle and cardiac development and growth, it would also be very interesting to investigate the function of HDAC7 in these tissues. The availability of the HDAC7^{flox} allele will allow this analysis in the future.

Implications

The realization that HDAC7 regulates MMP10 expression has interesting implications for a variety of human disorders. The balance between MMP and TIMP activity is critical for maintaining the integrity of the cardiovascular system. The up-regulation of MMP10 and concomitant down-regulation of TIMP1 expression observed in HDAC7 mutant embryos is reminiscent of the change in expression of these two families of antagonistic proteins in a variety of cardiovascular disorders. Following myocardial infarction, for example, MMP9 expression is up-regulated, while TIMP1 expression is down-regulated (8-10). Heart failure and ventricular dilatation is associated with similar changes in expression

of MMPs and TIMPs (46). This imbalance between these proteins has been implicated in pathological remodeling of the myocardium in these disease settings, and has pointed to the potential usefulness of MMP inhibitors in restoration of cardiac function in these disorders (10, 21). The mechanisms responsible for the dysregulation of MMP and TIMP expression in these disorders have not been defined.

Aortic aneurysms, which are characterized by destructive remodeling of the vascular extracellular matrix and consequent rupture of the vessel wall, account for an estimated 15,000 deaths per year in the United States. MMP10 and TIMP1 have been shown to be associated with aortic aneurysm and are likely to contribute to disease pathogenesis (36). Thus, strategies to suppress MMP10 expression and maintain TIMP expression by maintaining HDAC7 activity would be predicted to have therapeutic value.

Tumor angiogenesis is also an essential step in metastasis that requires formation of a stable. endothelial plexus. MMP inhibitors as well as HDAC inhibitors have been used to prevent metastasis and treat cancer (1, 20, 26-28, 35, 37). Based on the results of this study, we predict that inhibition of HDAC7 activity in tumors and consequent up-regulation of MMPs would also have anti-angiogenic consequences and eventually lead to regression of tumors due to disruption of vascular supply. Hence, these findings point to HDAC7 as a potential drug target for vascular-related disorders including heart failure, aortic aneurysm and tumor.

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Chapter IV

Signal-dependent Regulation of Class II Histone Deacetylases

Abstract

Class II histone deacetylases (HDACs) repress transcription by associating with a variety of transcription factors and corepressors. Phosphorylation of a set of conserved serine residues in the N-terminal extensions of class II HDACs creates binding sites for 14-3-3 chaperone proteins, which trigger nuclear export of these HDACs, thereby derepressing specific target genes in a signal-dependent manner. We show here that class II HDACs are substrates for a stress-responsive kinase specific for conserved serines that regulate MEF2-HDAC interactions. To identify intracellular signaling pathways that control phosphorylation of the class II HDAC, HDAC5, we designed a eukaryotic cDNA expression screen in which a GAL4-dependent luciferase reporter was expressed with the DNA binding domain of GAL4 fused to the N-terminal extension of HDAC5 and the VP16 transcription activation domain fused to 14-3-3. Transfection of COS cells with cDNA expression libraries results in activation of luciferase expression by cDNAs encoding HDAC5 kinases or modulators of such kinases that enable phosphorylated GAL4-HDAC5 to recruit 14-3-3-VP16 with consequent reconstitution of a functional transcriptional complex. Our results reveal a remarkable variety of signaling pathways that converge on the signal-responsive phosphorylation sites in HDAC5, thereby enabling HDAC5 to connect extracellular signals to the genome.

Introduction

Changes in histone acetylation represent a key mechanism for the modulation of gene transcription (22). Acetylation of nucleosomal histones by histone acetyltransferases enhances transcription by relaxing the condensed structure of the nucleosome, whereas deacetylation of histones by histone deacetylases (HDACs) reverses this process and promotes chromatin condensation and transcriptional repression. The recruitment of histone acetyltransferases and HDACs by specific transcription factors enables these chromatin-modifying enzymes to regulate specific sets of target genes.

Three different classes of HDACs can be distinguished by their structures, expression patterns, and catalytic mechanisms (15). The four class II HDACs (HDAC4, HDAC5, HDAC7 and HDAC9) contain a bipartite structure with an N-terminal extension of approximately 600 amino acids followed by a catalytic domain (49). The N-terminal regions of class II HDACs interact with a variety of transcription factors and corepressors and contain a set of conserved phosphorylation sites that control their distribution between the nucleus and the cytoplasm (16, 31-33, 47, 52, 53). The signal-responsive serines in the N-terminal regions of class II HDACs are phosphorylated by calcium, calmodulin-dependent protein kinase (CaMK) and protein kinase D (PKD) (10, 32, 33, 41, 47, 52, 53). When phosphorylated, these sites recruit the chaperone protein 14-3-3, which masks the nuclear localization sequence (NLS) in class II HDACs with consequent export of the HDAC/14-3-3 complex from the nucleus to the cytoplasm and derepression of specific genes (16, 23, 28, 33, 35, 50). Thus, phosphorylation of class II HDACs provides a mechanism for coupling external signals to the genome.

Class II HDACs have been implicated in a variety of developmental and signal-dependent processes. HDAC5 and HDAC9 suppress hypertrophic growth of cardiomyocytes, such that knockout mice lacking these HDACs develop grossly enlarged hearts in response to cardiac stress (6, 52). HDAC9 has also been shown to repress the responsiveness of skeletal muscle genes to motor innervation (36). The actions of these HDACs in cardiac and skeletal myocytes correlate with their ability to associate with and repress the activity of myocyte enhancer factor-2 (MEF2). HDAC4 acts as a repressor of chondrocyte hypertrophy via its association with the Runx2 transcription factor, a master regulator of chondrocyte hypertrophy; chondrocytes from HDAC4 knockout mice undergo precocious and ectopic hypertrophy resulting in lethal ossification of endochondral cartilage (48). HDAC7 has been implicated in negative selection and apoptosis of T cells as a result of its association with the orphan nuclear receptor Nur77 (10, 11).

Here, we show that diverse hypertrophic signals *in vivo* lead to the activation of a cardiac HDAC kinase that phosphorylates the signal-responsive sites in class II HDACs and MITR. In an effort to identify kinases and other signaling molecules capable of regulating the phosphorylation of HDAC5, we designed a eukaryotic cDNA expression screen for cDNAs whose products could induce the interaction of GAL4-HDAC5 and 14-3-3-VP16 chimeric proteins and consequent activation of a GAL4-dependent luciferase reporter in transfected COS cells. A collection of regulators of HDAC5 phosphorylation was uncovered in this screen. Our results reveal a remarkable number of signaling pathways that culminate with the phosphorylation of HDAC5 and suggest that HDAC5 integrates diverse signaling pathways

and transduces their effects to “downstream” target genes as a result of its signal-dependent nuclear export.

Materials and Methods

HDAC Kinase Assays

Adult mouse hearts were homogenized in 1 ml of lysis buffer (PBS containing 1 mM EDTA, 0.1% Triton X-100, 1 mM PMSF, and protease inhibitor cocktail (Roche)). After brief sonication, lysates were clarified by ultra-centrifugation, and protein concentration was determined.

GST-HDAC substrates contained amino acids 208-310 of HDAC4, 218-328 of HDAC5, or 180-283 of HDAC9 fused to glutathione S-transferase (GST). GST-fusion proteins were also made with serine-to-alanine mutations at positions 246, 259 and 218 in HDACs 4, 5, and 9, respectively. GST-HDAC protein (1 □g) was conjugated to glutathione-agarose beads. GST-HDAC-bound beads were washed with PBS and subsequently incubated with heart protein lysate (100 μg) in lysis buffer for 2 h at 4°C. Beads were washed twice with the same buffer and equilibrated with kinase reaction buffer (25 mM Hepes [pH7.6], 10 mM $MgCl_2$, 0.1 mM $CaCl_2$). Beads were then resuspended in kinase reaction buffer (30 μl) containing 12.5 μM ATP and 5 μCi γ-^{32}P-ATP and reactions were allowed to proceed for 30 min at room temperature. Reactions were then boiled, and phosphoproteins were resolved by SDS-PAGE, visualized by autoradiography, and quantified using a phosphorimager.

Constructs and cDNA expression library

HDAC5 derivatives in which residues 2-664 were fused to the DNA-binding domain of GAL4 (GAL4BD-HDAC5) and 14-3-3 fused to the VP16 transcription activation domain have been described (47). Human fetal heart and mouse embryonic day 10.5 cDNA

expression libraries were purchased from Invitrogen. cDNA pools were prepared using PerfectPrep Plasmid 96 Vac Direct Bind kit (Eppendorf). Each cDNA pool used in the screening contained 50-100 single cDNA clones.

Transient transfections and luciferase reporter assays

In each well of a 24-well plate, $5x10^4$ COS cells were cultured in DMEM with 10% fetal bovine serum. Cells were transfected with 250ng of cDNA expression library together with 100ng of UAS-Luciferase reporter plasmid, 50ng each of expression plasmids encoding GAL4BD-HDAC5 and VP16-14-3-3 using 1.4 ul of Fugene 6 reagent (Roche Molecular Biochemicals). Transfection efficiency was normalized by cotransfection of 10ng of pCMV-LacZ. At 48 h post-transfection, the cells were harvested in 150ul passive lysate buffer (Promega) and 20ul of cell lysate was used for luciferase or β-galactosidase assays. For sib-selection, positive pools of cDNA expression clones were transformed into *E.coli* DH5α competent cells and plated on LB agar dishes. For each positive clone, 96 single clones were picked from the dish and grown up in LB liquid media. Twelve single clones were combined as subpools and plasmids were prepared and transfected for the reporter assay as described above. Single clones from the positive subpools were prepared and tested for their ability to promote HDAC5 phosphorylation.

Immunocytochemistry

COS cells plated on glass coverslips were fixed and stained as described (53). Fluorescent images were collected on a Leica fluorescent microscope and were processed

with Adobe Photoshop. Anti-FLAG (Sigma) and fluorescein-conjugated anti-mouse IgG (Vector Labs) were used at 1:200 dilution.

Results

Activation of a cardiac HDAC kinase by hypertrophic stimuli

Class II HDACs and MITR possess a common structure with two conserved CaMK phosphorylation sites flanking a nuclear localization sequence (NLS) near their N-termini (Figure 4.1A and B). To determine whether a kinase specific for these phosphorylation sites might be activated in the heart in response to hypertrophic stimuli, we developed an *in vitro* kinase assay using portions of HDAC4, HDAC5 and HDAC9/MITR encompassing the conserved CaMK sites fused to GST as substrates (Figure 4.1C). Phosphorylation of GST-HDAC proteins was readily detected in this assay (Figure 4.1C, WT). GST-HDAC fusion proteins in which the CaMK sites (Ser-246 in HDAC4, Ser-259 in HDAC5 and Ser-218 in HDAC9/MITR) were mutated to alanines (Figure 4.1C, Mut) were not phosphorylated by cardiac extracts, demonstrating that the kinase activity is specific for the CaMK sites.

Constriction of the thoracic aorta in mice creates a pressure gradient in excess of 50 mm Hg and results in approximately a 50% increase in cardiac mass within 21 days (19). Analysis of cardiac extracts from sham-operated and thoracic aortic-banded (TAB) mice showed that pressure-overload increased HDAC kinase activity 2.1- and 1.7-fold, using GST-HDAC9 and GST-HDAC5 substrates, respectively ($P < 0.05$) (Figure 4.1D). Hypertrophic hearts from transgenic mice expressing activated calcineurin (37) also showed pronounced elevation of HDAC kinase activity (Figure 4.1E). Cardiac hypertrophy due to expression of activated forms of CaMK and the MAP kinase MEK5 in the heart also resulted in stimulation of HDAC kinase activity (data not shown).

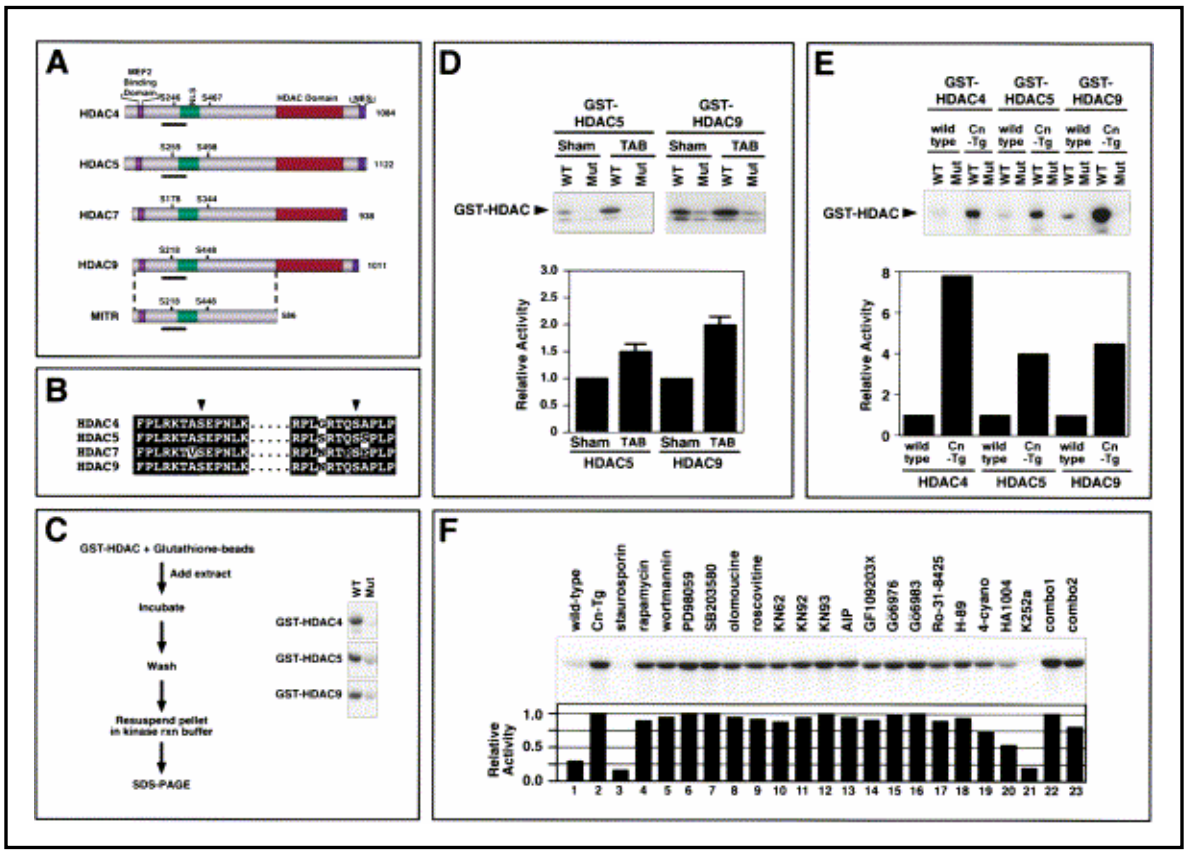

Fig. 4.1. Elevated HDAC kinase activity in hypertrophic hearts. (A) Schematic diagram of class II HDACs. Class II HDACs have a bipartite structure, with a C-terminal catalytic (HDAC) domain and an N-terminal extension with a MEF2-binding domain. Conserved phosphorylation sites flank the nuclear localization sequence (NLS) and a nuclear export sequence (NES) is near the C-terminus. MITR is a splice variant of HDAC9 that lacks an HDAC domain. (B) Amino acid homologies surrounding the regulatory serines (arrowheads) in the class II HDACs. (C) Schematic diagram of HDAC kinase assay. Kinase assays were performed with GST-HDAC substrates. The region of each HDAC that was used as substrate is underlined in panel A. A kinase from heart lysates phosphorylates the wild-type (WT) substrates, but not mutant (Mut) substrates containing alanine in place of the regulatory serine residue. (D,E) HDAC kinase assays were performed with heart extracts from thoracic aorta-banded (TAB) or sham-operated mice (D) or from wild-type (wt) or MHC-calcineurin transgenic (Cn-Tg) mice. Values represent the average of at least three independent assays. (F) HDAC kinase assays were performed with heart extracts derived from wild-type (lane 1) and Cn-Tg (lanes 2-23) mice in the absence (lanes 1 and 2) or presence (lanes 3-23) of diverse inhibitors (upper panel). Kinase activity relative to that in extracts from Cn-Tg mice (lane 2) was determined (lower panel). Inhibitors were added to reactions at concentrations 20 times the IC50. Combo1 contained AIP and Go6983 and combo2 contained HA1004 and Go6983. Inhibitor experiments were performed at least three times with comparable results. A representative experiment is shown. For D-F, enzyme activity was quantified as described in Experimental Procedures.

To further characterize the HDAC kinase, we tested its sensitivity to a panel of inhibitors (Figure 4.1F and data not shown). The HDAC kinase was inhibited by staurosporin and K252a, which are general serine/threonine kinase inhibitors. The kinase was also partially inhibited by HA1004, which is a broad inhibitor known to inhibit CaMK, PKA, and PKG. However, the kinase was not inhibited by compounds that inhibit CaMK (KN62, KN93 or AIP), PKA (H-89 or 4-cyano-3-methylisoquinoline), PKC (GF109203X, Gö6976, Gö6983 or Ro-31-8425), MEK (PD98059), p38 (SB203580), cdk (olomoucine and roscovitine) or PI-3-kinase (rapamycin and wortmannin). The kinase was also not inhibited by inhibitors of PKG, Raf or MLCK (data not shown). Combinations of the above inhibitors also failed to inhibit the kinase. Paradoxically, inhibitor combinations that contained HA1004 were less effective than HA1004 alone. We suspect this is due to counteracting effects of one inhibitor on the action of another.

These results demonstrate that diverse hypertrophic cues stimulate the activity of a kinase (or kinases) specific for the phosphorylation sites that inactivate class II HDACs and suggest that the kinase measured in this assay does not correspond to kinases previously implicated in hypertrophic signaling.

A cDNA expression screen for activators of HDAC5 phosphorylation

In an effort to identify protein kinases capable of phosphorylating the 14-3-3 consensus sites in HDAC5, we designed a eukaryotic expression screen as schematized in Figure 4.2A. In this screen, the N-terminal extension of HDAC5 was fused to the GAL4 DNA binding domain and 14-3-3 was fused to the VP16 transcription activation domain. In

transfected COS cells, HDAC5 is not phosphorylated, so GAL4-HDAC5 cannot interact with 14-3-3-VP16, and a GAL4-dependent luciferase reporter (UAS-luciferase) cannot be activated. Expression plasmids encoding these fusion proteins, together with UAS-luciferase, were transfected into COS cells along with pools of ~100 cDNA clones each from a fetal human heart or a 10.5 day mouse embryo cDNA expression library. Any cDNA pool containing a kinase capable of phosphorylating the 14-3-3 binding site in HDAC5 or an upstream activator of an endogenous kinase in COS cells for this site will result in the creation of a phospho-14-3-3 recognition motif, which will recruit 14-3-3-VP16 to activate the reporter. Individual clones were isolated from positive pools by sib-selection. Results obtained from a typical screen of cDNA pools in a 96-well plate and the luciferase expression in response to an activating pool containing a cDNA encoding the endothelin-1 (ET1) receptor type A (ETA) are shown in Figure 4.2B.

We screened ~2,000 individual cDNA pools, representing ~200,000 individual cDNA clones, and identified 36 cDNAs capable of activating the luciferase reporter. As shown in Table 4. 1, the screen resulted in the identification of known and novel HDAC kinases as well as cell surface receptors and other effector molecules. The specificity of activation of UAS-luciferase by individual clones was tested by mutating serine-259 and -498 of HDAC5 to alanine, which prevents 14-3-3 binding. All clones listed in Table 4.1 showed a dramatic reduction in their ability to activate UAS-luciferase when either 14-3-3 site was mutated and a complete loss of activity when both 14-3-3 sites were mutated. (Figure 4.3 and data not shown). To assess the relative potencies of activating cDNAs, we performed parallel transfection assays with an expression plasmid encoding CaMKI, which is known to

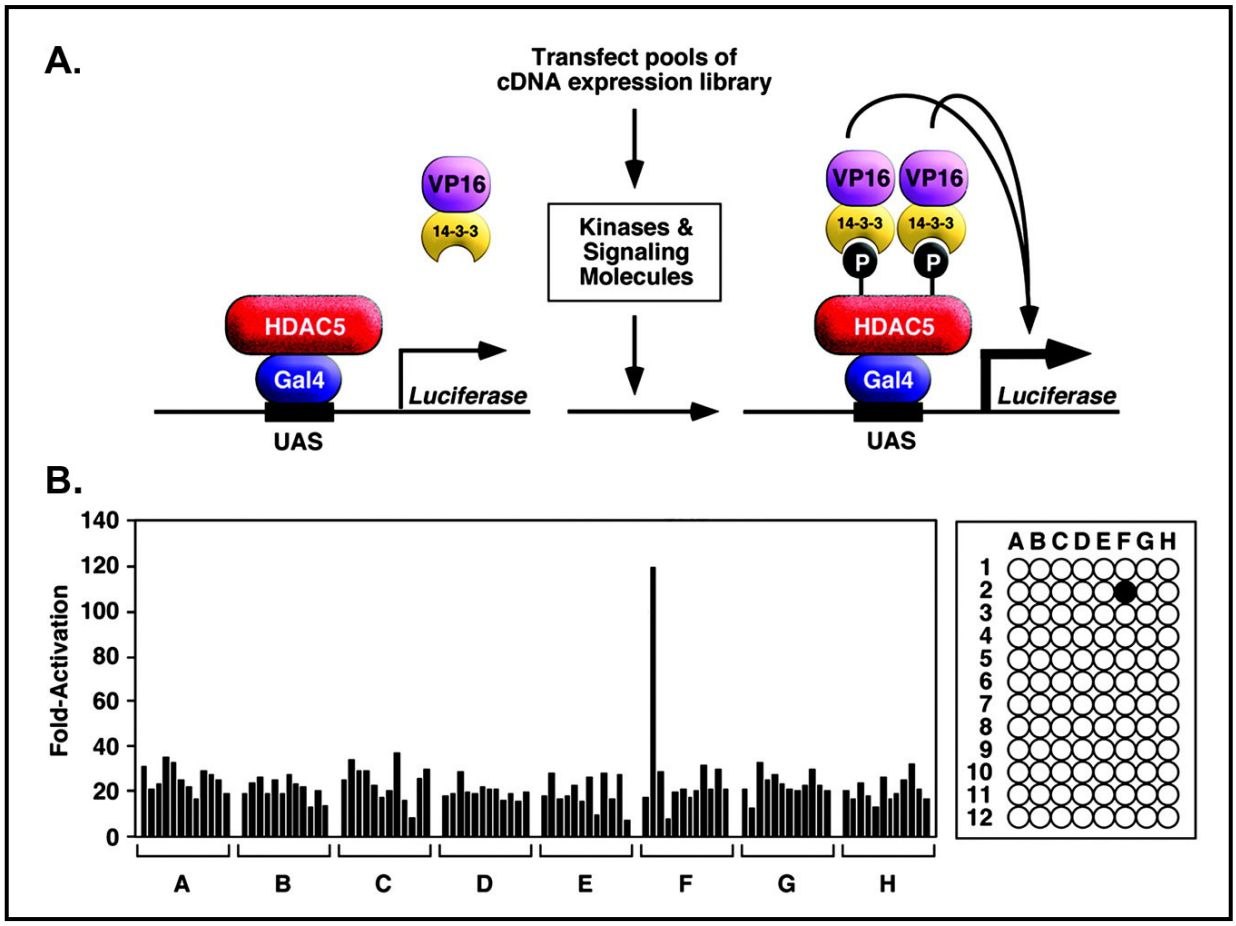

Fig. 4.2. Schematic diagram of the cDNA expression screening strategy. (A) The cDNA expression screen. The N-terminal extension of HDAC5 was fused to the DNA binding domain of GAL4 and 14-3-3 was fused to the activation domain of VP16. A luciferase reporter controlled by the GAL4 DNA binding site, referred to as the upstream activating sequence (UAS), is expressed at a basal level in control COS cells. Transfection of COS cells with pools of cDNAs results in activation of UAS-luciferase expression by pools containing kinases or activators of kinases that phosphorylate the 14-3-3 binding sites in HDAC5, resulting in recruitment of 14-3-3-VP16 and reconstitution of a transcriptional complex. (B) Results from a transfection assay in a representative 96-well plate are shown. Each well received a pool of ~50-100 cDNAs as described in panel B. The UAS-luciferase plasmid was specifically activated in well F2. Sib-selection from this pool identified endothelin-1 receptor A as the activating cDNA.

Table 4.1. Positive Clones from the Screen

Activators	# of clones	cDNA insert
Kinases:		
PKD2	1	aa118 to the end
PKC□	1	aa 237 to the end
Mark2	3	full length
Receptors:		
Endothelin receptor type A	5	all full length
EDG3	1	full length
EDG4	3	all full length
EDG7	1	full length
5HT2B	2	aa39 to the end and full length
ErbB-2	1	aa487 to the end
Regulators of Rho Signaling:		
RhoA	1	full length
Rho C	1	full length
Rho GEF1	1	aa383 to the end
Rho GEF5	1	aa1002 to the end
Transcriptional Regulators:		
PIAS4	1	full length
COUP-TFII	2	both full length
Ebfaz	1	aa6 to the end
Others:		
Cyclin A2	1	full length
Chloride channel intracellular protein 1	1	full length
PNAS-2	1	full length
SENP2	3	aa349 to the end and full length
Putative MAPK/NfkB a.p.	1	full length
Novel	3	unknown

Proteins encoded by cDNA clones isolated in the expression screen described in Figure 4. 2A are shown. The number of clones of each type that were isolated and the amino acids (aa) encoded by the clones are shown.

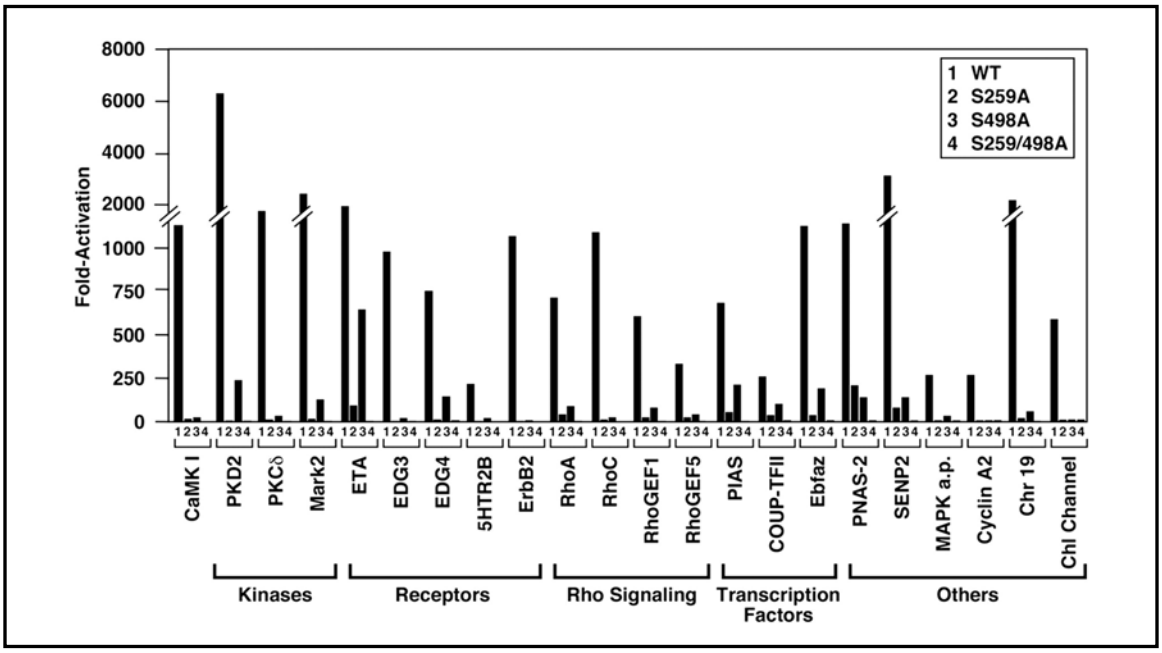

Fig. 4.3. Activation of UAS-luciferase by expression of cDNAs that promote the association of GAL4-HDAC5 and 14-3-3-VP16. COS cells were transfected with UAS-luciferase and expression plasmids encoding GAL4 fused to the wild type HDAC5 N-terminal extension or mutants of this region in which serine 259 and/or 498 were mutated to alanines, as indicated, along with 14-3-3-VP16 and expression plasmids for individual activating cDNAs. Mutation of single serines severely impaired activation of UAS-luciferase and mutation of both serines abolished activation.

phosphorylate serines 259 and 498 of HDAC5 (32). Activating cDNAs evoked increases in expression of UAS-luciferase that were generally of the same magnitude as seen with CaMKI. Characteristics of different activating clones are described below.

Kinases

As expected, we identified multiple protein kinases in the screen, including clones encoding PKCδ and PKD2, both of which have been shown to stimulate phosphorylation and nuclear export of HDAC5 (47). In addition, three independent cDNAs encoding microtubule-

associating regulatory kinase (Mark2) were isolated. Mark2 phosphorylates the sequence KxGS, which is similar to the signal-responsive sites in class II HDACs (20).

To examine whether Mark2 might act as a direct class II HDAC kinase, we performed an in vitro kinase assay using extracts from COS cells transfected with the Mark2 expression plasmid and GST-HDAC5 or GST-HDAC4 fusion proteins as substrates. As shown in Figure 4.4, extracts from Mark2-expressing cells efficiently supported the phosphorylation of these class II HDACs. Mutation of the signal-responsive serines in the GST-HDAC substrates abolished phosphorylation. We conclude that Mark2 can function as an HDAC kinase.

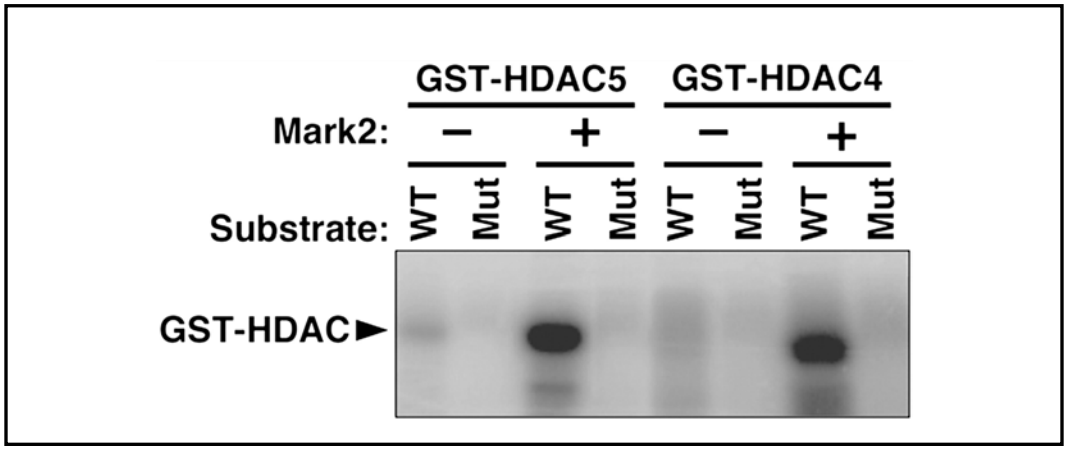

Fig. 4.4. Phosphorylation of HDACs 4 and 5 by extracts from Mark2 transfected cells. COS cells were transiently transfected with an empty pcDNA3 expression plasmid (-) or a pcDNA3-Mark2 expression plasmid (+). Cell extracts were prepared and used for in vitro kinase assays with GST-HDAC fusion proteins and [γ-^{32}P]-ATP. WT refers to GST fusion proteins with the wild type amino acid sequence and Mut refers to fusion proteins in which the signal-responsive serines were mutated to alanines.

Cell Surface Receptors

A number of cell surface receptors were identified in the expression screen (Table 4. 1 and Figure 4. 3). Although these receptors require ligand binding for signaling, we presume

that their over-expression leads to signaling in the absence of ligand or that ligand present in culture medium activates the receptors. Consistent with prior studies demonstrating that ET-1 can promote nuclear export of HDAC5 in cardiomyocytes (47), we isolated five independent clones encoding the ET-1 receptor type A (ETA). We also identified three clones encoding the cell surface receptors for lysophosphatidic acid (LPA), referred to as EDG4 and EDG7 , which belong to a family of G-protein coupled receptors referred to as EDG (endothelial differentiation genes) receptors (40). Another clone encoded the EDG3 receptor, which binds sphingosine-1-phosphate (S1P) (40). These receptors activate calcium currents and couple to Rho and PKC-dependent signaling pathways (40, 57).

In addition, two cDNAs encoding the serotonin receptor, 5-hydroxytryptamine receptor-2B (5HTR2B), were found in the screen. The ability of the 5HT2RB to promote HDAC5 phosphorylation confirms our prior studies in which we showed that the 4-amino-pyridine derivative, pyridine activator of myocyte hypertrophy (PAMH), which promotes cardiomyocyte hypertrophy, acts through this receptor to stimulate HDAC5 phosphorylation (5). The 5HTR2B triggers intracellular calcium release and PKC activation (8), which is likely to account, at least in part, for the ability of the over-expressed receptor to induce HDAC5 phosphorylation.

Unexpectedly, a clone encoding the tyrosine kinase receptor ErbB-2, which is localized to the plasma membrane, was isolated in the screen. Since ErbB protein kinases are specific for tyrosine phosphorylation (25), the ability of ErbB-2 to activate the UAS-luciferase reporter by inducing the interaction of GAL4-HDAC5 and 14-3-3-VP16

apparently reflects the activation of an endogenous HDAC kinase in COS cells that responds to ErbB-2 signaling.

Regulators of Rho Signaling.

Multiple regulators of Rho signaling, including RhoA, RhoC and the Rho guanyl nucleotide exchange factors (GEFs) 1 and 5, were isolated in the screen (Table 4. 1 and Figure 4. 3). RhoGEFs activate Rho by stimulating the exchange of GDP for GTP, with consequent stimulation of Rho-dependent signaling pathways. RhoA activates two groups of kinases, protein kinase N/PKC-related kinases and Rho kinases (44), raising the possibility that these kinases may phosphorylate HDAC5.

Transcriptional Regulators.

Several transcriptional regulators, including protein inhibitor of activated STAT 4 (PIAS4), COUP-TFII and Early B-cell factor-associated zinc finger protein (Ebfaz), were as effective as protein kinases and signaling molecules in activation of the UAS-luciferase reporter (Table 4. 1 and Figure 4. 3). PIAS proteins function as transcriptional coregulators in various cellular pathways and have been shown to act as E3-like ligases involved in sumoylation (3). Since PIAS can interact with HDAC1 (14) and HDAC1 undergoes SUMO modification (9), it could potentially regulate HDAC5 phosphorylation by recruiting HDAC kinases to the complex, or its sumoylation of HDAC5 could make HDAC kinases more accessible to HDAC5.

The orphan nuclear receptor COUP-TFII functions as a transcriptional repressor (1), and is involved in many developmental processes including skeletal muscle differentiation, cardiogenesis and angiogenesis (20, 27, 43, 56). Ebfaz binds to and inhibits the activity of EBF, a basic helix-loop-helix transcription factor required for B-cell lineage commitment and development of the olfactory epithelium (51). Because these cDNAs also required serines 249 and 498 for activation, it seems likely that these transcriptional repressors stimulate phosphorylation of these sites through an indirect mechanism.

Clones that Activate HDAC5 Phosphorylation Through Unknown Mechanisms.

Finally, there were multiple cDNAs that act through mechanisms that would not be expected to affect phosphorylation of HDAC5, including cyclin A2, chloride intercellular channel 1 (CLIC1), the SUMO1/sentrin/SMT3-specific protease 2 (SENP2) and the putative mitogen-activated protein kinase/NF□B activating protein (MAPK a.p.) (Table 4. 1 and Figure 4. 3).

Cyclin A2 induces the activity of cyclin-dependent kinase 1 (Cdk1) during the G1/S transition and activates Cdk2 during G2/M transition (38). Since HDAC5 phosphorylation sites differ from the Cdk consensus site, and previous studies have shown that Cdk inhibitors failed to inhibit HDAC kinase activity (52), we believe that cyclin A2 stimulates HDAC5 phosphorylation by an indirect mechanism.

Chloride channels are frequently involved in calcium fluctuation and some are activated by calcium. Membrane depolarization caused by chloride conductance or chloride

movement also contributes to Ca^{2+} release from intracellular stores (25). Thus, it is tempting that CLIC1 regulates HDAC kinase(s) via an effect on calcium signaling.

SENP2 reverses the process of sumoylation by removing SUMO from modified proteins (54). Recently, it has been proposed that HDACs repress MEF2 activity by potentiating MEF2 sumoylation, and that SENP3, a closely related family member, removes SUMO from MEF2 and activates MEF2 (13). Our results suggest that SENP may also activate MEF2 through promoting phosphorylation of HDAC5. It is interesting that two proteins we identified in this screen, PIAS4 and SENP2, are involved in the same pathway and counter-act each other. These findings raise the possibility that sumoylation of HDAC5 is coordinated with phosphorylation. Prior studies have also shown that SENP2 interacts with Axin, a regulator of the Wnt signaling pathway, and therefore has been implicated as a possible regulator of β-catenin degradation (39). Thus, it is conceivable that SENP2 regulates HDAC5 phosphorylation via an effect on Wnt signaling.

The putative MAPK a.p. was originally identified in a similar expression screen for proteins that can activate MAPK and/or NFκB pathways (30). Since kinases in MAPK pathways do not phosphorylate the 14-3-3 sites in HDAC5, we presume that MAPK a.p. may induce HDAC5 phosphorylation either by regulating the NF□B pathway or by other unknown mechanisms.

We also identified three cDNAs encoding novel proteins with unknown function. Clone BC007457 encodes an SNF7 domain containing protein, clone AK001192 encodes a predicted protein with weak similarity to Splicing factor ARGININE/SERINE-RICH 4, and clone BC006701 encodes a novel open reading frame on mouse chromosome 19.

Differential responses of class II HDACs to activators of HDAC5 phosphorylation

We tested the effects of activating cDNAs on nuclear export of HDACs 5, 4 and 7. In contrast to HDAC5, which is completely nuclear in COS cells, HDACs 4 and 7 are distributed between the nucleus and the cytoplasm. CaMKI, which we used as a positive control for HDAC5 export, was the most potent export kinase, stimulating ~100% of cells to translocate HDAC5 from the nucleus to the cytoplasm. PKD2 was about half as potent as CaMK in the nuclear export assay (Figure 4. 5). Surprisingly, the other activating cDNAs were relatively weak inducers of HDAC5 nuclear export and caused HDAC5 to redistribute to the cytoplasm in only about 5% of cells. HDAC4 was present in the cytoplasm of about 50% of control COS cells. All of the activating cDNAs stimulated HDAC4 translocation to the cytoplasm, with CaMKI and PKD being most effective. HDAC7 was present in the cytoplasm of ~20% of COS cells. CaMK and PKD caused a complete relocalization of HDAC7 to the cytoplasm, whereas other activators stimulated HDAC7 nuclear export to intermediate levels.

These findings suggest that HDACs 4, 5, and 7 are all able to respond to the different types of activators identified in the screen, but the stimulus required for nuclear export of HDACs 4 and 7 is less stringent than for HDAC5. Because none of the activators shown in Table 4. 1 were able to stimulate expression of the UAS-luciferase reporter if serines 259 and 498 in HDAC5 were mutated to alanines, it is reasonable to conclude that all of these activators enhance the phosphorylation of HDAC5 at one or both of these sites with consequent recruitment of 14-3-3-VP16. Thus, it is puzzling that several of the activating

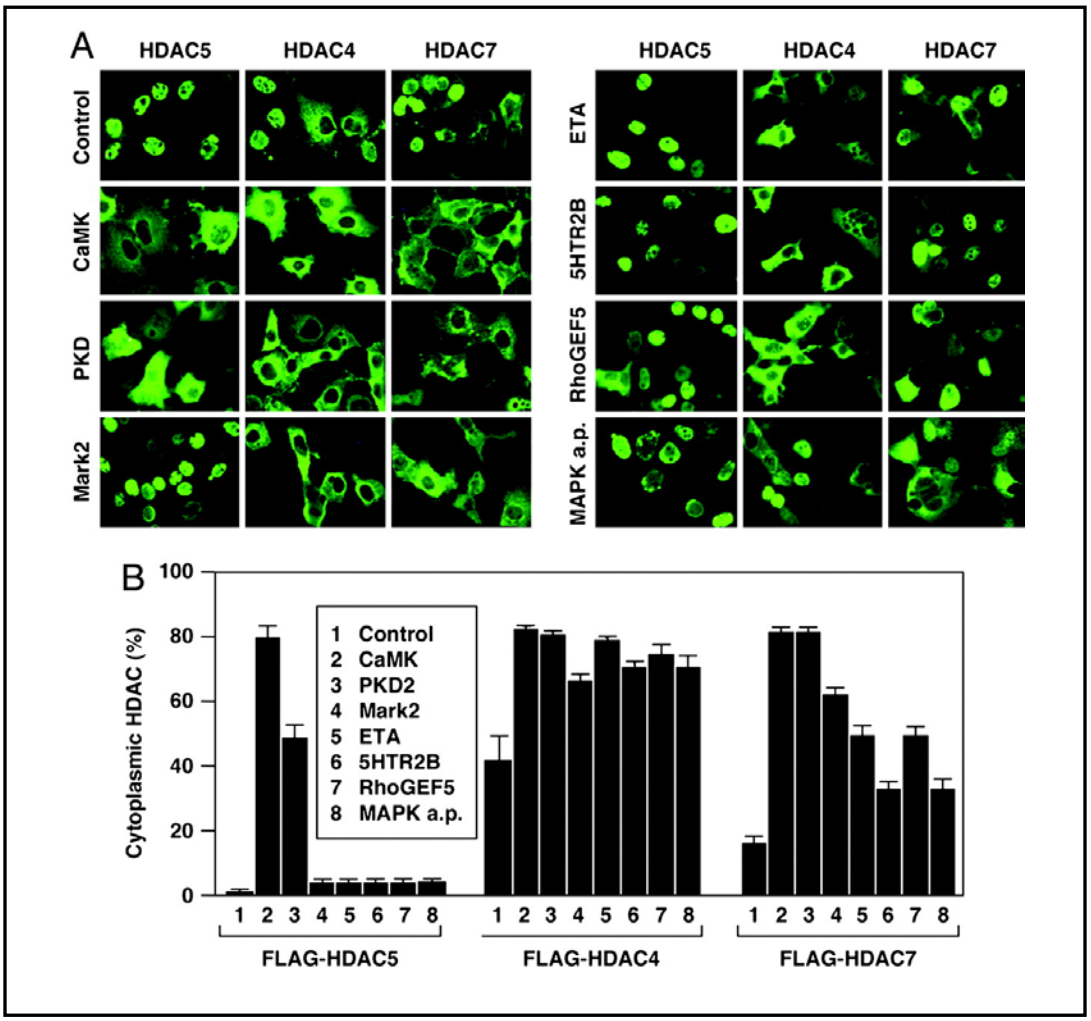

Fig. 4.5. Effects of activating cDNAs on nuclear/cytoplasmic distribution of class II HDACs. (A) COS cells were transiently transfected with expression plasmids encoding FLAG-tagged HDACs, as shown in each column, and activators of HDAC5 phosphorylation, as shown in each row. The subcellular distribution of HDACs was determined by immunostaining as described in Materials and Methods. Representative fields are shown. (B) The percentage of cells containing FLAG-tagged HDACs in the cytoplasm was determined by counting at least 100 transfected cells.

cDNAs have only minimal effects on nuclear export of HDAC5. One interpretation of this result is that nuclear export requires steps in addition to phosphorylation of the 14-3-3 docking sites in HDAC5. Export kinases such as CaMKI and PKD must be effective in triggering all the steps required for export, whereas other stimuli might activate only a subset of such steps.

Discussion

Stress signals stimulate adult cardiomyocytes to undergo hypertrophy, which is associated with the activation of a fetal cardiac gene program that results in maladaptive changes in cardiac contractility and calcium handling. The results of this study show that stress signals stimulate an HDAC kinase that phosphorylates conserved regulatory serine residues in class II HDACs. A eukaryotic expression screen to identify HDAC5 kinases and their effectors revealed known and unexpected regulators of HDAC5 phosphorylation. The remarkable number of signaling molecules that induce HDAC5 phosphorylation suggests that the signal-responsive serines in HDAC5 (and in other class II HDACs) serve as a point of convergence of diverse signaling cascades that culminate in the nucleus to control specific programs of gene expression (figure 4.6).

Activation of an HDAC kinase by hypertrophic signals

CaMK activity is elevated in failing human hearts (24) and constitutively activated CaM kinases can induce cardiac hypertrophy *in vivo* and *in vitro* (42, 55). The ability of CaMK signaling to phosphorylate the conserved regulatory sites in MITR and class II HDACs in transfected cells (32, 34, 53) suggested that CaMK might be involved in the transduction of hypertrophic stimuli through HDACs *in vivo*. Indeed, our results demonstrate the existence of a protein kinase activity in cardiac extracts that phosphorylates the CaMK sites in MITR and class II HDACs and point to this enzyme as an effector in hypertrophic signaling pathways. However, the kinase detected in this assay does not appear to correspond to a typical CaMK, based on its insensitivity to all of the CaMK inhibitors we tested (KN62, KN93, and AIP), as well as its failure to bind a calmodulin affinity column and its activity in the presence of EGTA (data not shown). The kinase is also not recognized by an anti-

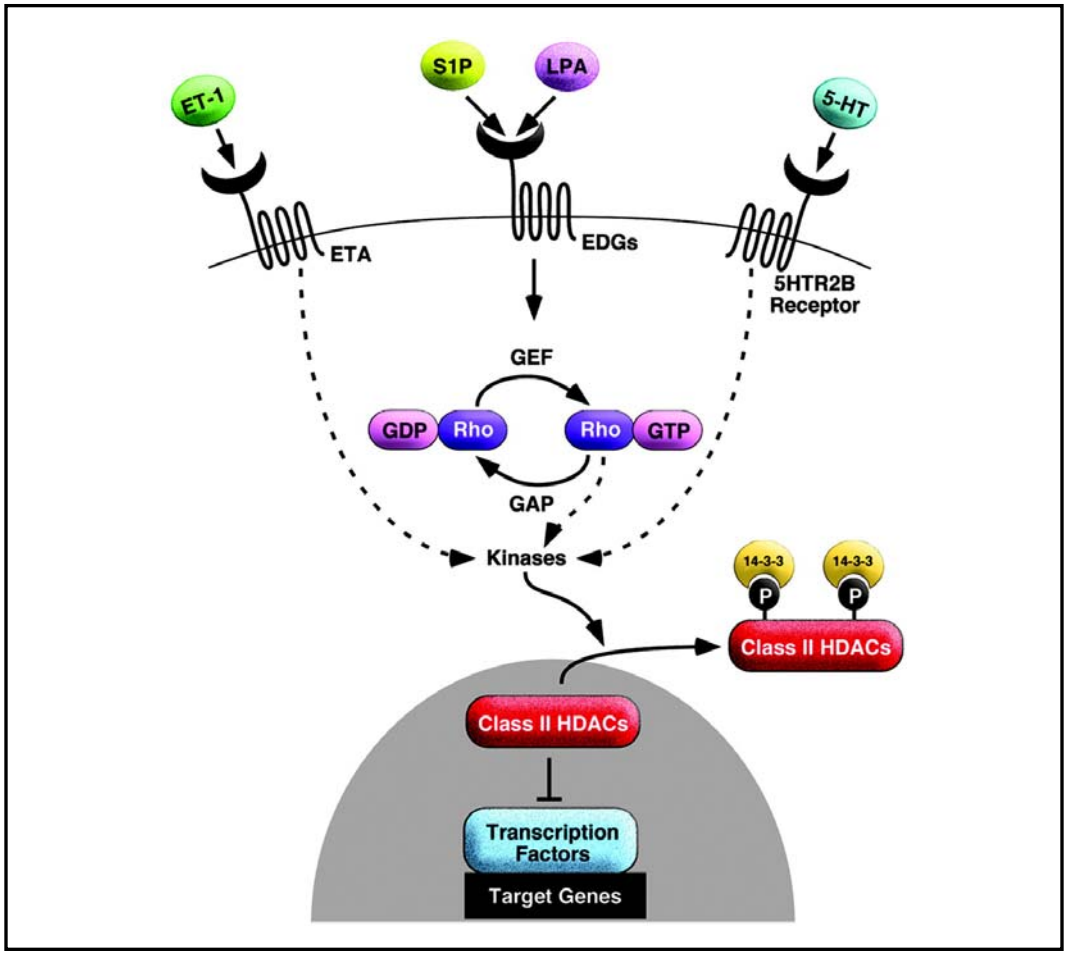

Fig. 4.6. Signaling pathways directed at serines 249 and 498 of HDAC5. The ability of class II HDACs to inhibit activity of transcription factors is abolished by phosphorylation at signal-responsive serines, which creates 14-3-3 docking sites and results in nuclear export of the complex. A variety of inducers of cardiac hypertrophy, including ET1, S1P, LPA, serotonin (5-HT) and RhoA signaling, stimulate HDAC kinase activity and consequently derepress HDAC targeted transcription factors. We propose that class II HDACs serve as a nodal point that translates extracellular and intracellular signals to the genome and controls gene expression.

CaMKIV antibody. Although the kinase was partially inhibited by HA1004, which is known to inhibit PKA, PKG, and CaMK, this inhibitor may also inhibit other untested or unknown kinases. Since other inhibitors for PKA, PKG, and CaMK do not inhibit the HDAC kinase, it does not appear that these kinases singly or in combination can account for the activity. Nevertheless, it is possible that the kinase is a CaMK-like kinase, since purified CaMK can phosphorylate the same sites in HDACs. The identity of this stress-responsive kinase remains to be determined.

The stimulation of HDAC kinase activity by stress signals *in vivo*, coupled with the ability of signal-resistant HDAC mutants to block hypertrophy of primary cardiomyocytes in the presence of diverse agonists *in vitro*, suggests that this kinase integrates multiple hypertrophic signaling pathways. Of course, activation of HDAC kinase activity by different hypertrophic stimuli need not be direct and could involve secondary pathways or autocrine signaling loops.

Phosphorylation of HDAC5 by Mark2

Mark2 was the only unknown HDAC kinase identified from the screen. Mark2, which is expressed in adult human heart, brain and skeletal muscle, phosphorylates microtubule-associated proteins and triggers microtubule disruption (12). *In vitro* kinase assays demonstrated that Mark2 could phosphorylate class II HDACs directly at the signal-responsive sites. *Mark2* null mice show growth retardation and immune system dysfunction (21). It will be interesting to determine if any of the actions of Mark2 are mediated by HDAC phosphorylation and whether Mark2 signaling may influence HDAC functions during cardiac development and growth.

Regulation of HDAC5 phosphorylation by EDG signaling

The EDG receptors EDG3, EDG4 and EDG7 were potent inducers of HDAC5 phosphorylation. These receptors, which bind LPA and S1P, respectively, have been implicated in cardiac hypertrophy (18, 45) and vascular development (2, 26, 29), but the downstream signaling pathways that link these receptors to the genome have not been identified. Our results suggest that EDG signaling stimulates cardiomyocyte growth, at least in part, by promoting the phosphorylation of class II HDACs.

Regulation of HDAC5 phosphorylation by Rho signaling

Activation of Rho signaling by RhoA, RhoC or RhoGEFs -1 or -5 induced HDAC5 phosphorylation. Rho signaling controls a variety of downstream kinases that are likely to couple Rho activation to HDAC5 phosphorylation. RhoA has been implicated in the signaling pathways whereby ET-1 and phenylephrine induce cardiac hypertrophy (7), but the downstream effectors of RhoA that promote hypertrophy are not fully understood. The ability of RhoA and RhoGEFs to induce the association of GAL4-HDAC5 and 14-3-3-VP16 suggests that these effectors control a protein kinase pathway leading to phosphorylation of the signal-responsive serines in HDAC5. Given the importance of Rho signaling for differentiation and morphogenesis of cardiac, skeletal and smooth muscle cells (7, 17, 46), and the role of MEF2 in these processes (4), it is likely that class II HDACs act as intermediaries in these pathways.

Transcriptional regulators of HDAC5 phosphorylation

It is curious that we identified several transcriptional regulators that induced the interaction of HDAC5 and 14-3-3 in a manner dependent on the signal-responsive serines in HDAC5. While we have not directly examined the effects of these activators on HDAC5 phosphorylation, the finding that serine-to-alanine mutations in HDAC5 abolish their stimulatory effects strongly suggests that they act via an HDAC kinase. We currently favor the possibility that these activators induce the expression of one or more HDAC kinases or suppress the expression of phosphatases that act on these sites.

Selective responsiveness of class II HDACs to different signaling pathways

It is remarkable that such a broad range of signaling pathways and transcriptional regulators modulate phosphorylation of the signal-responsive serines in HDAC5. These findings point to HDAC5 as a nodal point in intracellular signaling pathways that integrates diverse upstream signals and transmits them to the transcriptional machinery. While we have focused primarily on the regulation of HDAC5 phosphorylation, our results indicate that other class II HDACs display unique responses to the various signaling molecules we have identified. Such specificity allows different sets of target genes to be modulated by these signaling pathways depending on which class II HDACs are expressed in a particular cell type. Additional specificity of action can be achieved by the interaction of class II HDACs with different transcriptional activators and corepressors that may be cell type specific. Modification of this expression screen to employ different class II HDACs and cDNA expression libraries from different cell types should allow the identification of a large spectrum of molecules regulating a particular HDAC in tissues of interest.

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Chapter V

Identification of Genes Regulated by Histone Deacetylase 5 and MEF2 during Skeletal Muscle Differentiation

Abstract

Skeletal muscle differentiation is controlled by interactions between myogenic basic helix-loop-helix and myocyte enhancer factor 2 (MEF2) transcription factors. Association of MEF2 and class IIa histone deacetylases (HDACs) - HDAC4, 5, 7 and 9 - results in repression of MEF2 activity and inhibition of myogenesis. We show that ectopic expression of HDAC5 in C2C12 myocytes blocks myogenic differentiation, whereas an HDAC5 mutant lacking the MEF2-binding domain is ineffective as a repressor of myogenesis, indicating that HDAC5 inhibits myogenic differentiation mainly through its ability to repress MEF2 functions. In an effort to identify genes in the muscle differentiation pathway that are regulated by the HDAC5/MEF2 complex, we compared the gene expression profiles of control C2C12 myocytes infected with empty virus and myocytes infected with retroviruses encoding HDAC5 or an HDAC5 mutant lacking the MEF2-interacting domain. Using high-density cDNA microarray analysis, we identified 519 genes, among 16,297 genes surveyed, that are regulated by the HDAC5/MEF2 complex either in proliferating myoblasts or in differentiated myotubes. These data provide a comprehensive molecular description of the genes under the influence of HDAC/MEF2 partnership during myogenesis, as well as genes regulated by HDAC5 independent of its association with MEF2. Furthermore, we validated our premise by analysis of the regulatory region of one novel gene of these 519 genes, whose expression was proven to be dependent on MEF2. Functional analysis of these novel genes would surely provide insights in our understanding about muscle differentiation.

Introduction

The process of muscle differentiation is coupled to withdrawal of proliferating myoblasts from the cell cycle, fusion to form multinucleated myotubes, and transcriptional activation of an array of muscle-specific genes whose products are required for specialized contractile and metabolic functions of the differentiated myofiber. Members of the MyoD family of basic helix-loop-helix (bHLH) transcription factors, -MyoD, myogenin, Myf5 and MRF4 - are sufficient to induce the skeletal muscle differentiation program in non-muscle cells, and are essential for muscle development *in vivo* (25, 35).

The ability of myogenic bHLH proteins to promote muscle gene expression is dependent on their interactions with members of myocyte enhancer factor-2 (MEF2) family of transcription factors (24). The four mammalian MEF2 proteins, MEF2A, -B, -C and –D, and the single MEF2 dMEF2 in Drosophila share high homology in an N-terminal MADS (MCM1, Agamous, Deficiens, Serum response factor) box domain, which mediates DNA binding and dimerization. An adjacent MEF2-specific domain is important for cofactor association and dimerization. The C-terminal region of MEF2 factors, which are relatively divergent in primary sequence among the family members, function as a transcription activation domain (TAD). MEF2 proteins bind to a conserved A/T-rich sequence present in the regulatory regions of a variety of muscle and growth factor-inducible genes (26). The essential role of MEF2 in muscle development has been demonstrated by loss-of-function mutations of the single *Mef2* gene in *Drosophila*, which result in defects in a block to differentiation of all muscle cell types (3, 14), and by ectopic expression of dominant-

negative MEF2 protein in cultured mammalian cells, which prevents myoblast differntiation (27).

The transcriptional activity of MEF2 is modulated by its association with histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs activate transcription by acetylating necleosomal histones, resulting in relaxation of mucleosome structure and accessibility of transcriptional complexes to their target genes. Conversely, HDACs counteract the activity of HATs by deacetylating histones with consequent chromatin condensation. HDACs are categorized into multiple classes based on their size, sequence homology to yeast HDACs and protein-protein interactions (11). Class I HDACs, HDAC1, -2, -3, -8 and –11, which are expressed ubiquitously, are homologous to yRPD3 and contain only a catalytic HDAC domain. Class II HDACs are homologous to yHDA1, and are highly expressed in the heart, brain and skeletal muscles. Based on sequence homology and domain organization, class II HDACs are divided into two subclasses, IIa (HDAC4, -5, -7 and -9 and a splicing variant MITR), which are the focus of this paper, and IIb (HDAC6 and HDAC10) (8, 9). In addition to their C-terminal catalytic domain, class IIa HDACs have N-terminal extensions that mediate their interaction with MEF2 and repression of MEF2 target genes and myogenesis (16, 17, 21, 23).

Consistent with its inhibitory function during muscle differentiation, HDAC5 is predominantly localized to the nucleus of proliferating C2C12 myoblasts, and is translocated to the cytoplasm during differentiation (20). Calcium/calmodulin-dependent kinase (CaMK) stimulates myogenesis by phosphorylating HDACs at two conserved serine residues, which correspond to serine residues 259 and 498 in HDAC5. The phosphorylated serines serve as

docking sites for the intracellular chaperone protein 14-3-3. Consequently, the phospho-HDAC: 14-3-3 complex is exported to the cytoplasm with the assistance of nuclear exportin protein Crm1, resulting in the disruption of the MEF2: HDAC complex and derepression of MEF2-dependent genes (17, 20, 21).

To further investigate the function of HDAC5 during skeletal muscle differentiation and identify the target genes in the muscle differentiation pathway for the MEF2:HDAC5 complex, we used high-density microarrays to compare the gene expression profiles of control C2C12 muscle cells and C2C12 cells over-expressing either HDAC5 or an HDAC5 mutant defective in MEF2 binding. We show that of 16,267 genes surveyed, 519 genes (3.2%) showed greater than 2 fold changes in expression in myoblasts or myotubes due to the association of HDAC5 with MEF2. These data provide a comprehensive molecular description of the function of HDAC5 and the significance of its interaction with MEF2 during myogenesis.

Materials and Methods

DNA Constructs and Retrovirus Generation

FLAG-tagged derivatives of HDAC5 and a mutant form lacking MEF2-binding domain HDAC5-□MEF have been described (22, 23). For retrovirus production, cDNAs were cloned into pMINV vector, and the resultant constructs and empty pMINV vector (as a control) were transfected into 293T cells using Fugene 6 (Roche). Tongue-1-Luc was constructed by cloning 1kb upstream transcriptional start codon into pGL3-Luc vector.

Cell Culture

C2C12 mouse myoblasts (American Type Culture Collection, Manssasw, VA) were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and penicillin-streptomycin. Cells were grown to 85% confluency and induced to differentiate by replacing the growth medium with the differentiation medium (DMEM with 2% horse serum).

Retrovirus Infection

C2C12 myoblasts were grown to 35% confluency and infected with recombinant retrovirus for 4 hours in the presence of 8ug/ml hexadimethrine bromide. Twenty-four hours after infection, the infected cells were selected with 8mg/ml Genectin (Invitrogen) for 5 days. The infected cell populations were maintained in growth medium with 3mg/ml Genectin thereafter.

Western Blot

The infected cells were harvested in phosphate-buffered saline (PBS) containing 0.1% Triton X-100, 1 mM EDTA, 1mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Roche). After brief sonication and removal of cellular debris by centrifugation, the proteins in the supernatant were separated by SDS-PAGE, transferred to polyvinylidene fluoride membranes, and immunoblotted with a monoclonal anti-flag antibody (Santa Cruz; 9E10). Proteins were visualized with a chemiluminescence system (Santa Cruz).

RNA Isolation and Analysis

Total RNA was extracted from cultured cells with Trizol reagent (Invitrogen) according to the manufacturer's instructions. For RT-PCR, total RNA was used as a template for reverse transcriptase and random hexamer primers were used (Invitrogen). For the microarray analysis, the National Institute on Aging (NIA) mouse 16K cDNA chip was used as described (34). The slides were scanned with a GenePix 4000A scanner (Axon Instruments) and analyzed using the GENEPIX PRO 3.0 (Axon Instruments). The raw data was normalized using a total intensity normalization method and the spots that have median intensities less than the mean plus three times standard deviation of the background Cy3 or Cy5 intensity were eliminated (34). We further processed the data by removing any gene element that had not shown a change of greater than 2-fold at any time point or had shown significant change in HDAC5ΔMEF2 infected cells. These processes allowed us to focus analysis in genes that were specifically affected by the HDAC5/MEF2 interaction. For clustering analysis, we converted the Cy5/Cy3 ratio to a log ration (base 2), analysed with

GeneCluster 2.0 available at www-genome.wi.mit.edu and generated a 3x3 self-organization map (32).

Immunocytochemistry

Cells were grown on glass coverslips, fixed in 10% formalin, and stained in PBS containing 3% bovine serum albumin (BSA) and 0.1% Nonidet P-40. Primary antibodies recognized flag epitope and skeletal actinin (Sigma). Primary and secondary antibodies were used at a dilution of 1:200. Coverslips were mounted on slides using Vectorshield (Vector Laboratories).

Results

Inhibition of myoblast differentiation by HDAC5 requires the MEF2 binding domain

Previously, we showed that HDAC5 could block MyoD mediated conversion of 10T1/2 fibroblasts to muscle cells (17), which we attributed to the repressive influence of HDAC5 on MEF2. In an effort to identify genes in the muscle differentiation pathway that were regulated by the association of HDAC5 with MEF2, we infected C2C12 myoblasts with retroviral expression vectors encoding wild-type HDAC5 or an HDAC5 mutant (HDAC5-ΔMEF) lacking the MEF2-binding domain (schematized in Figure 5.1B). The retrovirally-expressed HDAC5 proteins contained a FLAG tag to permit their detection and were expressed at comparable levels (Figures 5.1C and D).

Cells expressing wild-type HDAC5 exhibited a normal morphology and growth rate, but when transferred to differentiation medium were unable to form myotubes (Figure 5.1D). During differentiation of C2C12 myoblasts, endogenous HDAC5 is translocated from the nucleus to the cytoplasm, which is likely to trigger the differentiation process (20). In contrast, the exogenous HDAC5 protein was never exported from the nucleus and the cytoplasm even after exposing the cells to differentiation medium for four days (Figure 5.1D), likely because the over-expressed protein overwhelms the endogenous kinase-dependent nuclear export mechanism.

To monitor myoblast differentiation, we examined the expression of skeletal actinin, a sarcomeric protein specific for differentiated muscle cells, by immunocytochemistry. After 2 or 4 days in differentiation medium, very few myocytes overexpressing HDAC5 were stained for skeletal actinin compared to the empty virus-infected controls, indicating

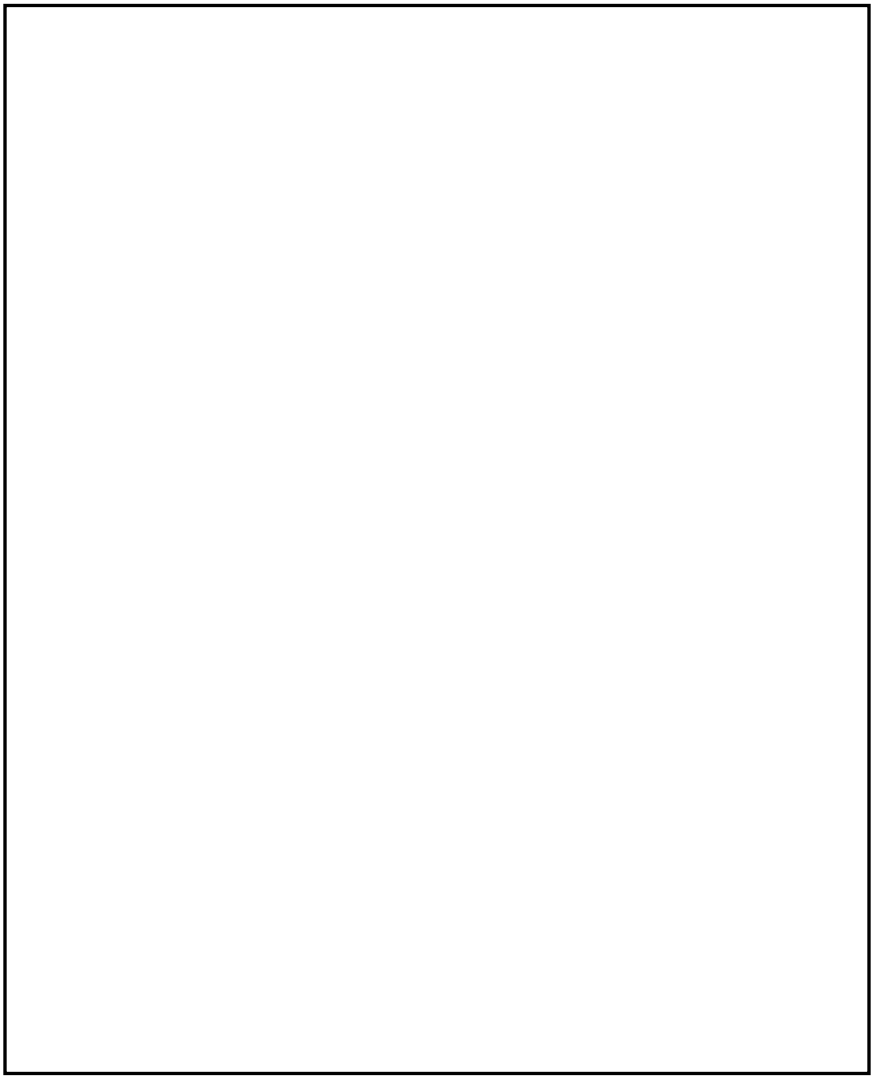

Fig 5.1. Expression of wild type and mutant HDAC5 in C2C12 cells. (A) Schematic diagrams of HDAC5 proteins. The HDAC5-ΔMEF mutant contains an internal deletion that removes the MEF2 binding domain. NES, muclear export sequence; NLS, nuclear localization sequence. **(**B) Experimental strategy for identification of target genes of HDAC5/MEF2. (C) C2C12 cells were infected with retrovirus encoding wild type HDAC5 or HDAC5-ΔMEF mutant proteins with FLAG epitope tags and proteins were detected by western blot using anti-FLAG antibody. A control retrovirus lacking a cDNA insert was included as a negative control and extracts from cells transfected with pcDNA-HDAC5 as a positive control. Wild type and mutant HDAC5 proteins were expressed at comparable levels. (D) C2C12 cells were infected with wild type HDAC5 and HDAC5-ΔMEF2 expressing retroviruses. Expression of exogenous HDAC5 proteins was detected by immunostaining with anti-FLAG antibody in myoblasts maintained in growth medium or following transferring to differentiation medium for 2 and 4 days, as indicated. Myocytes at indicated stages were also stained with anti-α-actinin, a sarcomeric protein expressed only in differentiated muscle cells. Overexpression of wild-type HDAC5 but not HDAC5-ΔMEF inhibited myoblast differentiation.

deficiency of differentiation (Figure 5.1D). In contrast, skeletal actinin-positive myotubes were observed throughout the cultures expressing the HDAC5-ΔMEF mutant (Figure 5.1D).

Transcriptional Profile of Myocytes Over-expressing HDAC5

To identify downstream target genes regulated by the association of HDAC5 with MEF2, we performed microarray analysis with myocytes infected with wild-type HDAC5 or HDAC5-ΔMEF and compared the expression of each of 16,267 genes in the arrays with myocytes infected with empty retrovirus as an infection control. Our premise was that the genes that are regulated by wild type HDAC5, but not by HDAC5-ΔMEF would be potential targets of the association of HDAC5 with MEF2.

Table 5.1. Schematic diagram for microarray analysis.

	Experiment Samples	Control Samples
HD-d0	Day 0 myocytes with wild-type HDAC5	Day 0 myocytes with empty virus
HD-d1	Day 1 myocytes with wild-type HDAC5	Day 1 myocytes with empty virus
HD-d2	Day 2 myocytes with wild-type HDAC5	Day 2 myocytes with empty virus
mHD-d0	Day 0 myocytes with HDAC5- ΔMEF2	Day 0 myocytes with empty virus
mHD-d1	Day 1 myocytes with HDAC5- ΔMEF2	Day 1 myocytes with empty virus
mHD-d2	Day 2 myocytes with HDAC5- ΔMEF2	Day 2 myocytes with empty virus

Of 16,267 genes on the chip, 960 (5.9%) showed more than a 2-fold change in expression in one or more microarrays performed (Table 5.1). The temporal expression patterns of these genes (Figure 5.2A) revealed the importance of HDAC5 during both myoblast proliferation and differentiation. Genes up- and down-regulated in cells over-expressing HDAC5 are represented with positive and negative bars, respectively. To distinguish genes regulated by the HDAC5/MEF2 complex from those regulated by HDAC5

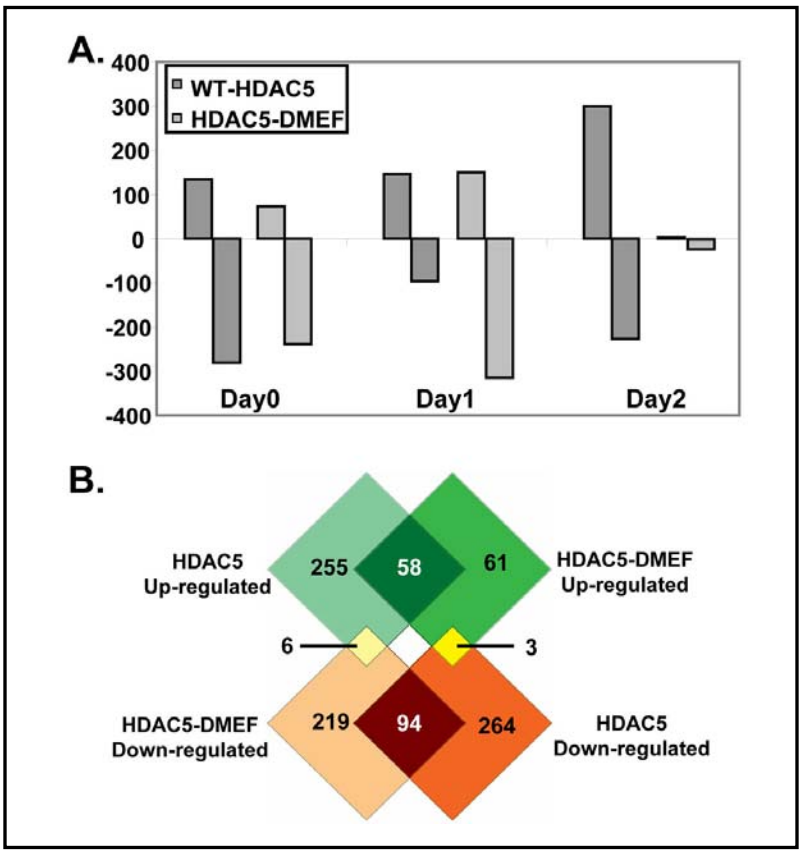

Fig. 5.2. Transcriptional profile of HDAC5 and HDAC5-ΔMEF over-expressing myocytes. Microarray analyses were performed to compare gene expression profile of myocytes infected with HDAC5 or HDAC5-ΔMEF with myocytes infected with empty retrovirus, as a control. (A) The numbers of genes up- or down-regulated in HDAC5 or HDAC5-ΔMEF over-expressing myocytes at different differentiation stages are shown. (B) Venn-diagram illustrating the number of genes regulated by HDAC5 or HDAC5-ΔMEF. Numbers of genes that are uniquely regulated are shown in the outer squares. Overlapping genes that are regulated by both HDAC5 and HDAC5-ΔMEF are denoted in the intersections. Notably, 255 genes were up-regulated and 264 genes were down-regulated only in wild-type HDAC5 over-expressing myocytes but not in HDAC5-ΔMEF overexpressing myocytes.

in a MEF2-independent manner, we generated a Venn diagram (Figure 5.2B). Altogether, 152 genes (58 up-regulated plus 94 down-regulated) were affected in cells infected with wild-type HDAC5 and HDAC5-ΔMEF, indicating that these genes are regulated by HDAC5 activity independent of MEF2 association. Genes affected by ectopic expression of HDAC5-ΔMEF, but not wild-type HDAC5 (61 up-regulated and 219 down-regulated), are likely to represent transcriptional targets of HDAC5 independent of MEF2. More importantly, 519 genes (255 up-regulated and 264 down-regulated) showed changes in myocytes infected with HDAC5 and no significant changes in cells infected with HDAC5-ΔMEF at any stage. The genes in this group that were down-regulated specifically by HDAC5 are likely targets of the HDAC5/MEF2 complex. It is intriguing that such a large group of genes was up-regulated by HDAC5, which acts as a transcriptional repressor. We speculate that these genes are regulated indirectly by HDAC5, possibly by repressing the expression of repressors.

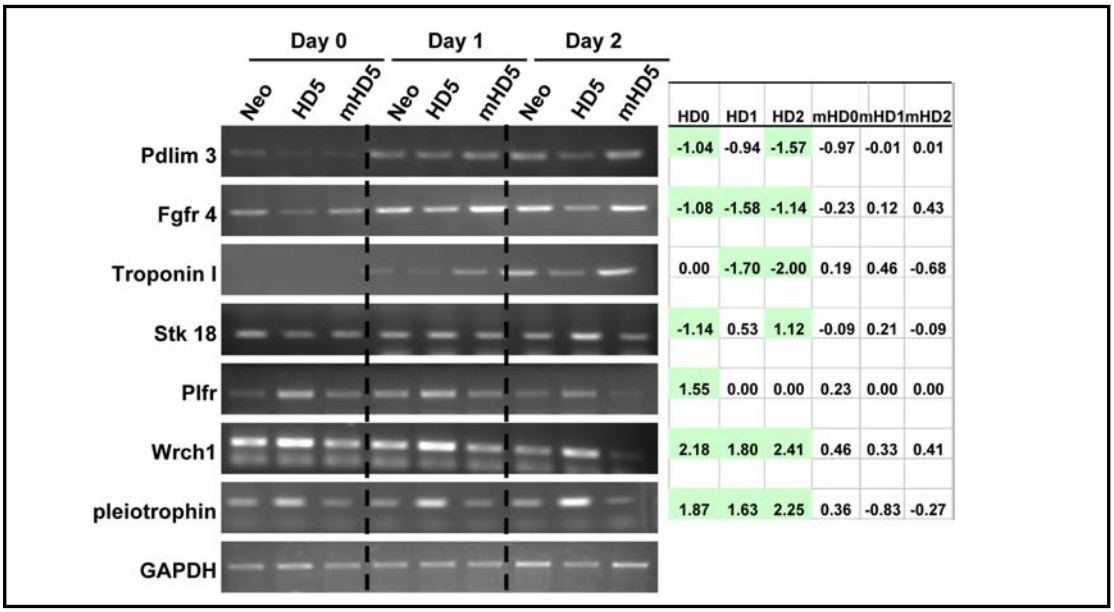

Fig. 5.3. RT-PCR analyses were performed to confirm the microarray data. The expression profiles of these genes are shown in the right panel.

To confirm the microarray data, we selected several genes randomly and performed RT-PCR with gene specific primers. As shown in Figure 5.3, the muscle structure gene troponin I, which is only expressed in differentiated myocytes, was dramatically down-regulated in the myotubes expressing wild type HDAC5 (lanes 5 and 8), while there was only a subtle change in the myotubes expressing HDAC5-ΔMEF (lanes 6 and 9). Other genes we tested were also consistant with the microarray data (Figure 5.3).

Genes regulated by the HDAC5/MEF2 complex

We focused our analysis on the 519 genes (Figure 5.4A) regulated by the association of HDAC5 with MEF2. Based on the similarity of the temporal expression patterns, these 519 genes were clustered into 9 groups designated as C0-C8 (Figure 5.4B and C). Clusters c0, c3, c6 and c7 showed increased expression patterns in cells infected with wild type HDAC5. In contrast, clusters c1, c2, c5 and c8 showed decreased expression pattern in cells expressing HDAC5. The timing of the stimulatory or inhibitory effects of HDAC5 expression varies among these clusters.

Based on the annotated function ascribed to each gene, we classified these 519 genes into different functional groups, including genes involved in amino acid metabolism, apoptosis, carbohydrate metabolism, defense, cell division and growth, adhesion and motility, morphogenesis and development, energy metabolism, gene regulation, lipid metabolism, muscle development and function, neuronal process, protein modification and metabolism, signaling, transport and genes with unknown functions (Figure 5.4D), indicating

that HDAC5/MEF2 is involved in regulation of a variety of aspects of cell growth and differentiation.

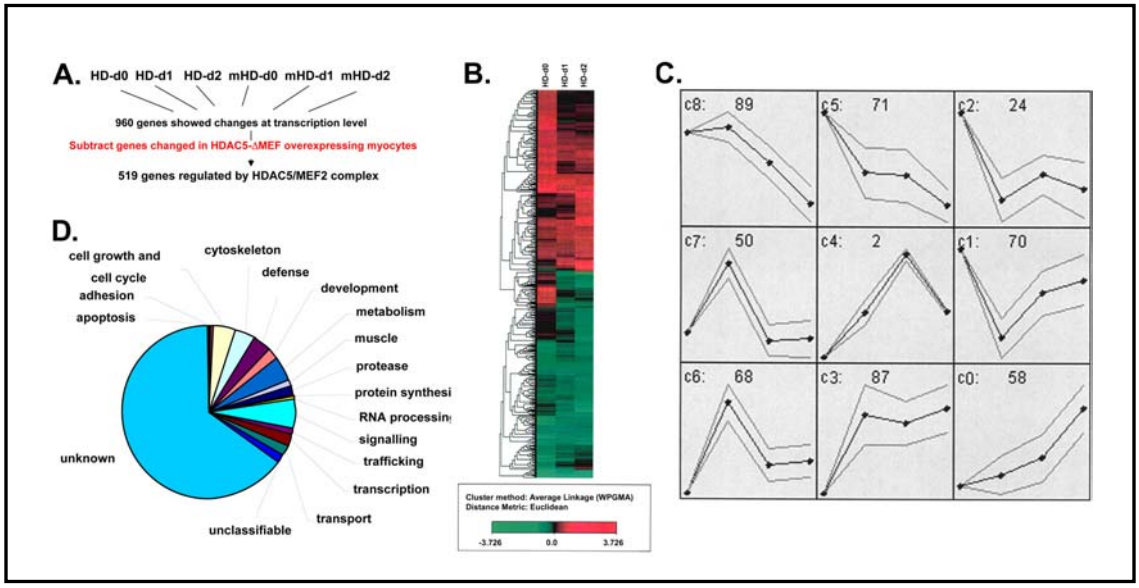

Fig. 5.4. Genes that are regulated uniquely by HDAC5. (A) Experimental scheme to identify HDAC5/MEF2 target genes. (B) Genes are clustered according to their temporal expression pattern. (C) Self-Organization Map (SOM) was used to analyze the data according to their temporal expression pattern. Basically, genes in clusters c1, c2, c5 and c8 were down-regulated upon HDAC5 overexpression, while genes in clusters c0, c3, c4, c6 and c7 were up-regulated. (D) Genes are categorized into different groups according to their functional annotation. Genes with unknown functions represent either novel genes or genes that are not functionally well-studied.

Cloning and characterization of tongue-1

To validate our approach for identifying target genes of the HDAC:MEF2 complex, we cloned a novel gene from these 519 genes, which we named as tongue-1 because of its high expression in the tongue muscles. Tongue-1 expression was down-regulated in myocytes expressing wild type HDAC5 but was unchanged in myocytes overexpressing HDAC5-ΔMEF. Based on our premise, tongue-1 would serve as an example of a gene regulated by the HDAC5:MEF2 complex.

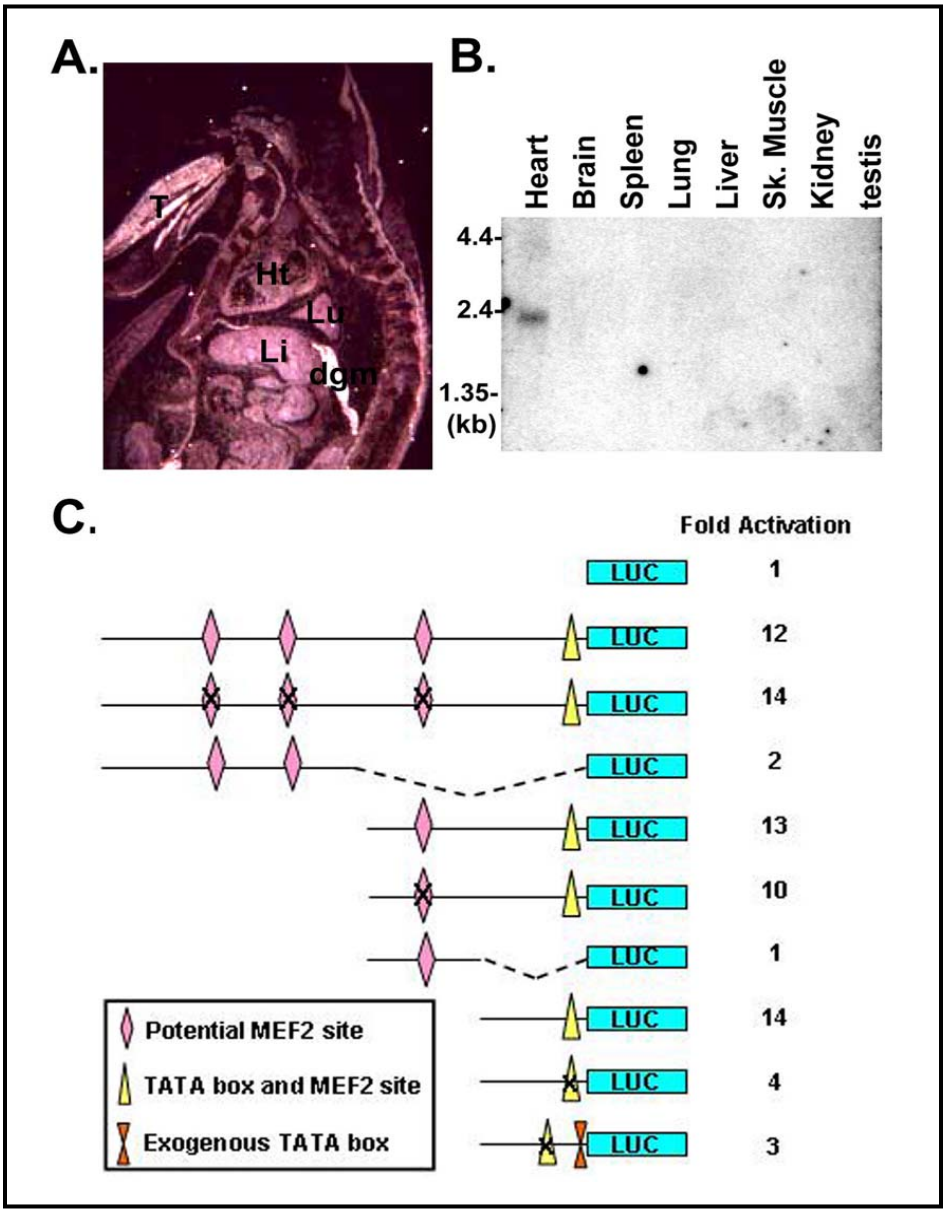

Fig. 5.5. Tongue-1, a novel muscle-specific gene, is a direct target of MEF2. (A) RNA in situ hybridization was performed using radioactively labeled tongue-1 anti-sense probe with sagittal section from E15.5 embryo. Tongue-1 is highly expressed in tongue and diaphragm. (B) Detection of tongue-1 mRNA by northern blot of adult tissues. Tongue-1 is highly and specifically expressed in heart. (C) Responsiveness of Tongue-1 promoter to MEF2. 1 kb promoter region upsteam of transcription start site of tongue-1 was cloned into pGL3-Luc vector. COS cells were transfected with Tongue-1-Luc and MEF2D, and luciferase assays were performed two days after transfection. Mutation of the most proximal MEF2 site, which also functions TATA box, abolished the responsiveness to MEF2D with or without exogenous TATA box, while mutation of the other three distal MEF2 sites failed to do so.

The expression pattern of tongue-1 in mice was examined by RNA *in situ* hybridization (Figure 5.5A). At embryonic day 15.5 (E15.5), tongue-1 is highly expressed in tongue and diaphragm muscles. Adult tissue northern blots indicate that tongue-1 expression is restricted to the heart (Figure 5.5B). Furthermore, the transcript of tongue-1 is down-regulated 9 fold in the muscles of MyoD-/- mice (2). These data are consistent with the notion that tongue-1 was isolated from C2C12 muscle cell line and was a potential target of MEF2.

To test if tongue-1 is a real target of HDAC:MEF2 complex, 1kb promoter region of tongue-1 was cloned into pGL3-Luc vector. As determined by in vitro luciferase transfection assay, this 1kb region was responsive to the MEF2 transcription factors (Figure 5.5C). Mutation of the three distal potential MEF2 sites did not affect the responsiveness to MEF2, while mutation of the most proximal MEF2 site, which may also function as TATA box, abolished the responsiveness to MEF2 with or without exogenous TATA box (Figure 5.5C). These data confirmed that Tongue-1 is a direct target of MEF2, as indicated by the microarray analysis.

Cell Cycle and muscle-specific genes affected by HDAC/MEF2 interaction

One noticeable feature of the expression patterns is the presence of two groups of genes, in clusters c0 and c8, with enhanced and reduced expression, respectively, only in HDAC5 expressing myocytes at a late stage of myogenic differentiation (Figure 5.4C). Of all the genes with known functions in cell cycle control, more than half, 23 out of 44, were found in cluster c0. Many of these genes are known to function directly in promoting cell cycle progression, such as MAD2, cyclin A2, cyclin B2, cdc2, cdc20, activator of S phase

kinase, E2F1 and p107, and in DNA replication, such as DNA replication factor and replicase 1 containing protein (12, 30). On the other hand, many well-known muscle-specific genes, such as cystein-rich protein 3, cardiac troponin T2, skeletal muscle troponin I slow, cardiac/skeletal muscle troponin C, fast myosin heavy chain, muscle creatine kinase, caridac alpha actin, skeletal muscle ryanodine receptor 1 and muscle glycogen phosphorylase, are clustered in c8 with a reduced expression at late stage of myogenic differentiation. We also found that cyclin-dependent kinase inhibitor $p21^{cip}$, which inhibits cell cycle progression (15), is down-regulated by HDAC5. These data suggest that HDAC5 may inhibit myogenesis by both suppressing the expression of muscle genes and preventing myoblasts from exiting cell cycle (Figure 5.6).

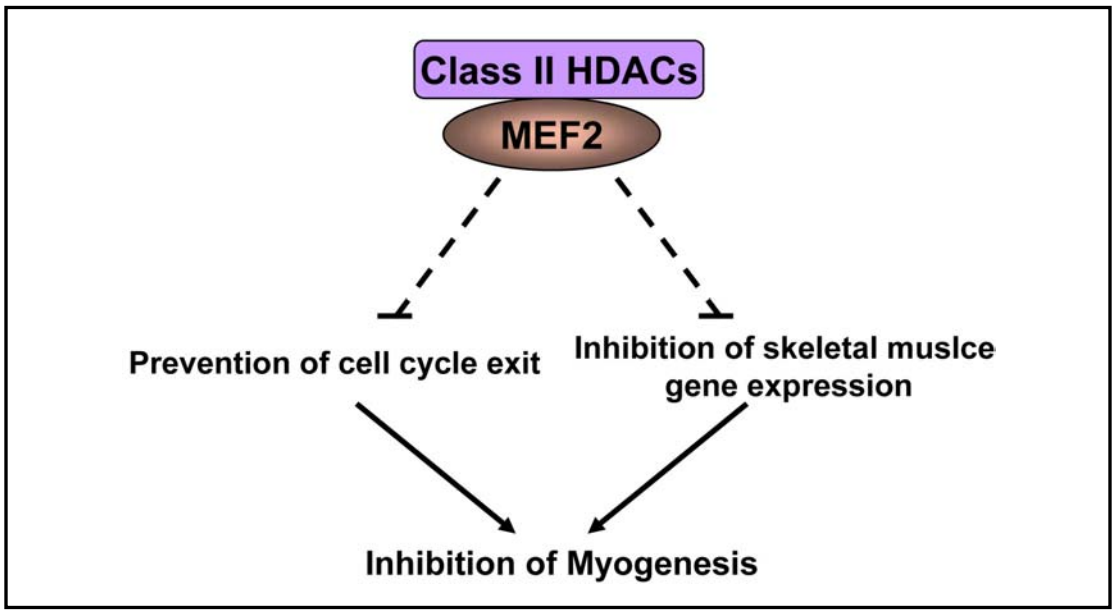

Fig. 5.6. A model for the control of skeletal muscle differentiation by HDAC5/MEF2. HDAC5 inhibits myogenesis by both repressing the expression of muscle genes and preventing myoblast from exiting cell cycle.

Genes that are regulated by HDAC5/MEF2 interaction in proliferative myogenic cells

It is interesting to note that MyoD was down-regulated by about 2-fold in myoblasts expressing HDAC5, but not in myocytes at later stages expressing HDAC5. This finding indicates that the inhibitory role of HDAC5 on MyoD transcription is specific to committed proliferating myogenic cells.

A subset of genes, grouped in c1 and c2 clusters, showed a similar expression pattern to that of *MyoD* in response to HDAC5 expression. Many of these genes are known to function in signaling and myogenic differentiation. Of particular interest are those genes related to Ca^{2+}- (calcineurin A beta) (5), hypoxia- (hypoxia inducible factor 1, alpha subunit) (7), Notch- (jagged 1) (31), Wnt- (dishevelled 2) (33) and BMP-dependent (LIM hemeobox protein 9) signaling (1), all of which have been demonstrated to play essential roles in development and cell communication. Contrary to the reduced expression pattern, a group of genes (in c7) showed induced expression only in proliferative myoblasts infected with HDAC5.

Genes that are generally inhibited or stimulated by HDAC5/MEF2 interaction

An expected expression pattern of HDAC5/MEF2 target genes that are independent of differentiation would be general repression or induction by expression of HDAC5, but not HDAC5-ΔMEF2, in both proliferating and differentiated myocytes. Here, we observed such expression patterns for a subset of genes as potential targets of HDAC5/MEF2 interaction, in c3, c5 and c6 clusters. In c3 cluster, there are 8 redundant genes of alpha 4 glutathione S-transferase with identical expression pattern, providing evidence of high fidelity of the

analysis. Genes in cluster c6 showed a similar expression pattern to that of c3 with more a profound change. There is an array of genes including the Wnt1 responsive cdc42 homolog (also called Wrch-1), with potential functions in cellular signaling, which have not been previously implicated in myogenic differentiation.

Discussion

Class II HDACs such as HDAC5 inhibit myogenesis, at least in part, by repressing the activity of MEF2, an essential myogenic cofactor for MyoD (16, 20). The results of this study demonstrate that deletion of the MEF2-binding domain in HDAC5 eliminates its ability to inhibit myogenesis and provide a genome-wide molecular description of the target genes of the HDAC5/MEF2 complex in the muscle differentiation pathway.

Repression of MEF2 activity by Class IIa HDACs

The roles of class IIa HDACs as signal-dependent repressors for cardiac hypertrophy have been well established (4, 36). Class IIa HDACs have also been shown to inhibit MyoD-mediated conversion of 10T1/2 fibroblasts into skeletal myocytes (16, 17). Endogenous HDAC5 is localized to the nucleus of proliferating myoblasts and is exported to the cytoplasm upon phosphorylation by HDAC kinase(s), which results in derepression of MEF2 transcriptional activity (16, 20-22). In this study, we found that HDAC5, when over-expressed, is localized in the nucleus even when myocytes are subjected to differentiation conditions, suggesting the regulated subcellular translocation is disrupted by HDAC5 overexpression. When induced to differentiate, myoblasts overexpressing HDAC5 are unable to fuse into myotubes. Persistent nuclear expression of HDAC5 presumably represses transcription factors, such as MEF2, resulting in inhibition of myogenic differentiation.

The results of this study show that a 17-amino acid deletion that removes the MEF2-binding domain disrupts the ability of HDAC5 to inhibit myogenesis, without affecting the localization of the protein to the nucleus. These findings provide strong support for the

conclusion that MEF2 is the key target for HDAC5, and presumably other class IIa HDACs, in the muscle differentiation pathway. Although MEF2 is not necessarily the only transcription factor that is functionally regulated during myoblast differentiation, the interaction of MEF2 with HDAC5 or other class IIa HDACs is likely to be a key regulatory event in the control of myogenic differentiation. It is plausible that HDACs are recruited to MyoD or other cofactors by MEF2 and repress their activity. We also cannot rule out the possibility that other unknown transcriptional effectors critical for myogenesis bind the same region of HDAC5 as MEF2 and are regulated by HDAC5. Nevertheless, the results presented here pinpoint the MEF2 binding domain of HDAC5 as a critical effector of the muscle differentiation pathway.

Target genes of the HDAC/MEF2 complex

Among 960 genes that are affected by overexpression of HDAC5, 441 were also affected by overexpression of HDAC5-ΔMEF2 mutant. These 441 genes are evidently affected by HDAC5 activity independent of the interaction with MEF2 since this mutant can not interact with MEF2. Since the cells overexpressiong HDAC5-ΔMEF2 are capable of normal myogenic differentiation upon serum withdrawal, we conclude that altered mRNA expression of these 441 genes is not sufficient to block myogenic differentiation.

The 519 genes that are affected by HDAC/MEF2 interaction were classified into different functional groups, including genes involved in adhesion, apoptosis, cell cycle and growth, communication, cytoskeleton, defense, differentiation, metabolism, motility, RNA processing, signal transduction, and transport and gene elements with unknown functions. These findings indicate that HDAC5 regulates many different, if not all, aspects cellular

functions either directly or indirectly. Half of these gene elements, 244 out of 519 (47.0%), are ESTs of unknown functions. Functional characterization of these genes will be very insightful for understanding the function of HDAC5 in the process of skeletal muscle development.

The self-organizing map (SOM) (32) was used to assemble and analyze the data. This procedure groups genes based on their common expression patterns. Several groups of genes (clusters c0, c3, c6 and c7) showed increased expression patterns in cells infected with HDAC5. We propose two explanations for the responsiveness of these genes to HDAC5. The straightforward one is that the expression of the corresponding genes is stimulated by HDAC5 overexpression. Alternatively, the expression of these genes, which is normally down-regulated during differentiation, is unusually maintained due to the defect in differentiation caused by HDAC5 expression. In contrast, clusters c1, c2, c5 and c8 showed decreased expression patterns in cells expressing HDAC5, consistent with the notion that these genes are directly repressed by HDAC5 or its normal induction during myogenic differentiation is blocked indirectly by the defect in differentiation. The timing of the stimulatory or inhibitory function of HDAC5 expression varies among these clusters, suggesting that HDAC5 may have different functions on myocytes at different differentiation stages.

Even though we did not observe any morphological changes in myoblasts overexpressing HDAC5, we did find that the expression of a number of genes (clusters c1, c2, c3, c5, c6 and c7) was changed in these myoblasts from the microarray analysis. This indicates that in addition to its inhibitory role in myogenic differentiation, HDAC5 may also

regulate cell growth and proliferation in myoblasts. Most of the genes in these clusters are involved in fundamental cellular processes including metabolism, protein trafficking, cytoskeleton, cell growth and signaling, which reflects HDAC5 as a general regulator in proliferating myoblasts.

Genes in cluster c0 showed enhanced expression in HDAC5 expressing myocytes at late stages of myogenic differentiation (Figure 5.4C), suggesting that these genes are either specifically induced or prevented from being down-regulated in differentiated myotubes due to HDAC5 expression. There is a significant enrichment in this cluster of genes related to cell cycle control and cell growth with functions in control of cell cycle progression and DNA replication. Since HDAC5 is a transcriptional inhibitor, it is mostly likely that the enhanced expression of these cell proliferative genes in cluster c0 is due to the inability of cells to exit from cell cycle and differentiate, but not stimulatory effects of HDAC5. On the other hand, we found that many muscle-specific genes (in cluster c8) showed reduced expression at late stages of myogenic differentiation. This indicates that HDAC5 directly or indirectly blocks the expression of these muscle-specific genes, providing molecular evidence that ectopic expression of HDAC5 in myocytes efficiently blocks myogenic differentiation.

As a master regulator of skeletal muscle differentiation, MyoD interacts with MEF2 and forms a feedback loop to further enhance MyoD expression and myogenesis (13). The inhibition of MEF2 and MyoD by HDAC5 may contribute to the overall effect of repression of myoblast differentiation. In this study, MyoD is down-regulated about 2-fold in the myoblasts, but not changed in myocytes overexpressing HDAC5-ΔMEF. This indicates that the inhibitory role of HDAC5 on MyoD transcription is mediated by MEF2 in proliferating

myogenic cells. The genes, grouped in c1 and c2 clusters, showed a similar expression pattern as that of MyoD in response to HDAC5 overexpression. Many of these genes are known to function in signaling pathways important to myogenic differentiation, suggesting that HDAC5 represses the expression of these signaling molecules to potentate its inhibitory effect on myogenic differentiation, an integrated process involved in multiple signaling pathways. Contrary to the reduced expression pattern, a group of genes (in c7) showing an induced expression only in proliferative myoblasts infected with HDAC5. The expression changes of genes in this cluster are probably due to cell autonomous response to the stress imposed by overexpression of HDAC5.

Roles of class I and class III HDACs in myogenesis

Recent studies have also implicated class I and class III HDACs in myogenesis. The class I HDAC, HDAC1, interacts with MyoD and inhibits MyoD-dependent transcription in undifferentiated myoblasts(18, 19), while in differentiating myocytes, retinoblastoma protein (pRb) disrupts the MyoD-HDAC1 complex by associating with HDAC1 directly to promote muscle gene expression (28). Treatment of skeletal muscle cells with trichostatin A, an HDAC inhibitor, causes myotube hypertrophy due to upregulation of follistatin, an antagonist of myostatin. Similarly, trichostatin A has been shown to stimulate expression of follistatin and of markers of muscle regeneration when delivered to adult mice following muscle injury (10). Conversely, the histone acetyltransferase p300/CBP and PCAF form a multimeric protein complex with MyoD, and induce activation of myogenic program (29). The class III HDAC, Sir2, which acts as a NAD-dependent histone deacetylase inhibits myogenesis (6). Thus, the activation of skeletal muscle differentiation requires the

coordinated activities of multiple HDACs and HATs acting in concert with different sets of downstream transcription factors that control the expression of muscle structural genes as well as the genes involved in termination of cell proliferation.

In summary, we have shown that HDAC5 inhibits myoblast differentiation *in vivo* dependent on the MEF2-binding domain. The inhibitory role of HDAC5 on myogenic differentiation is likely to be mediated through coordinated regulation of genes related to signaling, transcription, cell cycle progression, myogenic differentiation and other important cellular events required for the transition from cell proliferation to differentiation. High-density microarray analysis provides a global gene expression profile under the influence of HDAC5/MEF2 interaction in both proliferating and differentiated myocytes.

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Chapter VI

VITAE

Shurong Chang was born in Yancheng, Jiangsu Province, China on November 22nd, 1978, daughter of Meiyun Feng and Yunguan Chang, and elder sister of Shuyan Chang. After completing her study at Yancheng Middle School in 1996, she entered College of Life Sciences, Peking University in Beijing, China. In June 1999, she joined the laboratory of Dr. Zhangliang Chen at National Laboratory of Protein Engineering and Plant Genetic Engineering for her thesis study. The title of her thesis is "Identification and Characterization of the Nuclear Localization Sequence of S4 protein". She received the degree of Bachelor of Science with a major in biotechnology from Peking University in July 2000. In August 2000, she entered the Graduate School of Biomedical Sciences at the University of Texas Southwestern Medical Center at Dallas. She joined the laboratory of Dr. Eric N. Olson in May 2001, where she developed her interest and enthusiasm for science. She married Shijie Li in July 2003. She graduated with a Ph.D. degree in November 2005.