INHIBITION OF CLASS I HDACs BLUNTS CARDIAC HYPERTROPHY

VIA TSC2-DEPENDENT mTOR REPRESSION

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DEDICATION

To Dr. Joseph A. Hill for your support all these years, and for your constant confidence in me. You have lead by example, with your hard work for the division, and with your commitment to basic science. Thank you Joe, I have learned a lot from your expertise inside and outside the lab, and I will embrace these lessons in my future endeavors.

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ABSTRACT

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Stress-induced pathological hypertrophy is observed in most forms of heart disease. If left unchecked, pathological remodeling can lead to heart failure. Histone deacetylases (HDACs) participate in the progression of pathological cardiac growth, and small molecule inhibitors of HDACs can both reduce and regress pathological hypertrophy. The mammalian target of rapamycin complex 1 (mTORC1) is an important regulator of cell growth. It has been shown that mTORC1 is active during cardiac hypertrophy, leading to increased protein synthesis. Inhibiting mTORC1 can repress pathological remodeling. Interestingly, pan-HDAC inhibitors target mTOR activity in some cancer models. Therefore, we hypothesized that class I HDACs regulate cardiac hypertrophy in an mTOR-dependent manner.

To test this hypothesis, neonatal rat ventricular myocytes (NRVMs) were exposed to a variety of growth stimuli, and class I HDACs were inhibited by either pharmacological means or by knockdown of individual HDAC isoforms. We found that HDAC1, HDAC2 and HDAC3 act together to facilitate pathological and physiological cardiomyocyte hypertrophy. In addition, inhibition of class I HDACs decreases mTOR activation by hypertrophic growth stimuli. HDAC inhibition also decreased mTOR activity in the setting of pressure overload using an in vivo surgical model of transverse aortic constriction (TAC). Adult mice with conditional cardiomyocyte-specific knockout of both HDAC1 and HDAC2 together had improved function following TSC surgery as well as decreased mTOR activity. Tuberin (TSC2) is a component of the tuberin-hamartin complex, which inhibits mTOR. We found that inhibition of class I HDACs by either genetic knockdown or using small molecules increased expression of TSC2 in both NRVMs and embryonic stem cell-derived cardiomyocytes. Furthermore, using siRNA we observed that TSC2 is required for HDAC-dependent inhibition of mTOR in NRVMs.

These findings point to mTOR, and TSC2-dependent control of mTOR, as critical components of the mechanism through which HDAC inhibitors blunt pathological cardiac growth. Together, these results enhance our understanding of the function of HDACs in cardiac pathology and facilitate the ultimate translational application of HDAC inhibitors in the treatment of heart disease.

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LIST OF ABBREVIATIONS

4EBP1: eukaryotic translation initiation factor 4E-binding protein 1
AMPK: AMP-activated protein kinase
ANF: atrial natriuretic factor
Api: apicidin
ATG13: autophagy-related gene 13
BNP: brain natriuretic factor
BW: body weight
DEPTOR: DEP-domain-containing mTOR-interacting protein
eIF4E: translation initiation factor 4E
ERK: extracellular signal-regulated kinase
ESC-CM: embryonic stem cell derived cardiomyocytes
ET-1: endothelin-1
FIP200: focal adhesion kinase family-interacting protein of 200 kDa
FKBP12: FK506 binding protein 12
FS: Fractional shortening
GAP: GTPase-Activating Protein
HDAC: histone deacetylase
HW: heart weight
IGF-1: Insulin growth factor 1
IVS: interventricular septum thickness
LVID: left ventricular internal diameter
LVIDd: left ventricular internal diameter at end-diastole
LVIDs: left ventricular internal diameter at end-systole
LVPW: left ventricular posterior wall thickness
MEF: mouse embryonic fibroblast
MEF2: myocyte enhancer factor-2
MEK: mitogen-activated protein kinase kinase
mLST8: mammalian lethal with Sec13 protein 8
mSIN1: mammalian stress-activated protein kinase interacting protein
mTOR: mammalian target of rapamycin
mTORC1: mammalian target of rapamycin complex 1
mTORC2: mammalian target of rapamycin complex 2
NRVMs: neonatal rat ventricular cardiomyocytes
PE: phenylephrine
PFA: paraformaldehyde
PI3K: phosphoinositide-3-kinase
PIP2: phosphatidylinositol 4,5-bisphosphate
IP3: inositol 1,4,5-triphosphate
PRAS40: proline-rich AKT substrate 40 kDa
Protor-1: protein observed with Rictor-1
Rap: rapamycin

Raptor: regulatory-associated protein of mTOR
RCAN: regulator of calcineurin
Rictor: rapamycin-insensitive companion of mTOR
RMS: rhabdomyosarcomas
S6: S6 ribosomal protein
SAHA: suberoylanilide hydroxamic acid
TAC: thoracic aortic constriction
TBC1D7: TBC1 domain family, member 7
TSA: trichostatin A
TSC1: hamartin
TSC2: tuberin
ULK1: unc-51-like kinase 1
α-MHC: alpha myosin heavy chain
β-MHC: beta myosin heavy chain

CHAPTER 1: INTRODUCTION

Heart failure

Cardiovascular diseases are the leading cause of death in industrialized nations, an alarming statistic soon to be extended worldwide. Heart failure is the most common form of heart disease, and it is estimated that 5 million individuals in the United States suffer from it [1]. Despite the development of new therapies and the use of cardiac support devices, morbidity and mortality remain high. In the US, approximately 380,000 males and 400,000 females died from cardiovascular diseases in 2010. In comparison, all cancers combined were responsible for the deaths of close to 300,000 males and 270,000 females [2]. Between 2000 and 2010, the number of hospitalizations due to heart failure doubled, reaching almost 1 million per year, resulting in an estimated expenditure of 315 billion health care dollars [1].

Heart failure is broadly defined as an inability of the heart to pump blood to meet the demands of the body [3]. In response to stress, the heart adjusts in order to maintain cardiac output. To compensate, heart morphology undergoes remodeling, that can include either physiological or pathological hypertrophy. Pathological growth of the myocardium accompanies most forms of heart disease [3]. Cardiac hypertrophy is a major risk factor for heart failure, myocardial infarction, arrhythmia and sudden death [4, 5]. However, the mechanisms that underlie pathological progression are poorly understood.

Pathological hypertrophy and progression to heart failure

As a muscle, when the heart is exposed to increased workload, it grows. The beating unit of the heart is the cardiomyocyte. Adult cardiomyocytes are post-mitotic cells and rarely re-enter the cell cycle to divide. Growth of the adult heart results therefore not through cell division but instead via cellular hypertrophy [6]. Depending on the nature of the stress, cardiomyocytes grow either in a concentric or eccentric manner [7]. Initially, if the heart is subjected to pressure overload, concentric growth is observed, meaning that the cardiomyocyte sarcomeric units are

added in parallel, resulting in a thicker cell and a thicker left ventricular wall [7]. In volume overload, sarcomeric units are generally added in series and cardiomyocytes adopt an elongated form [7]. Cardiac hypertrophy can be classified as either physiological or pathological [3]. Concentric and eccentric morphology can be either physiological or pathological depending on the nature of stressor and the efficacy of compensation.

Physiological hypertrophy is observed during pregnancy, in response to exercise, and during neonatal growth. This form of growth leads to an increase in heart mass and size, formation of new sarcomere units, normal gene expression, enhanced contractile function, and it is reversible [8]. While fatty acid oxidation is the main source of energy in an adult heart, physiological hypertrophy is characterized by an increase in both fatty acid oxidation and glycolysis [9]. Physiological growth is usually considered adaptive, improving heart function and not progressing to disease. On the other hand, pathological hypertrophy occurs during pressure or volume overload in the context of disease. While the left ventricle also enlarges, as in physiological growth, pathological hypertrophy is accompanied by loss of cardiomyocytes, development of fibrosis, decreased cardiac function, derangement of sarcomeres, increased expression of the fetal gene program, deregulation of calcium handling and contraction, increased glycolysis, and decreased fatty acid oxidation. Pathological hypertrophy is generally considered irreversible [8].

Persistent pathological stress, causing continual maladaptive growth of the myocardium, results in the transition to heart failure. In this scenario, exacerbated enlargement of the heart is observed with thinning of the left ventricle caused by a switch to eccentric growth of cardiomyocytes [3]. This transition is believed to be in part due to a deficient supply of blood to cardiomyocytes, which may then undergo eccentric rearrangement of sarcomeric and extracellular structures to improve oxygen diffusion rates [10, 11]. Consequently, the thinned and weakened ventricular wall cannot contract and relax efficiently, severely compromising cardiac function.

In a wide range of animal models, therapeutic inhibition of cardiomyocyte hypertrophy affords benefit and prevents the transition to heart failure [12, 13]. While it was once believed that hypertrophy was an essential compensatory measure to meet increased demand, evidence in animal models suggests that the heart can continue to perform against a specific stressor and meet the body demands even in the absence of hypertrophy [14]. Therefore, pathological hypertrophy has been considered a potential target for the treatment of cardiovascular diseases. However, molecular mechanisms involved in cardiomyocyte growth are known to be compensatory and redundant [15]. Because of this, translational application of the observations in cell and animal models has been challenging.

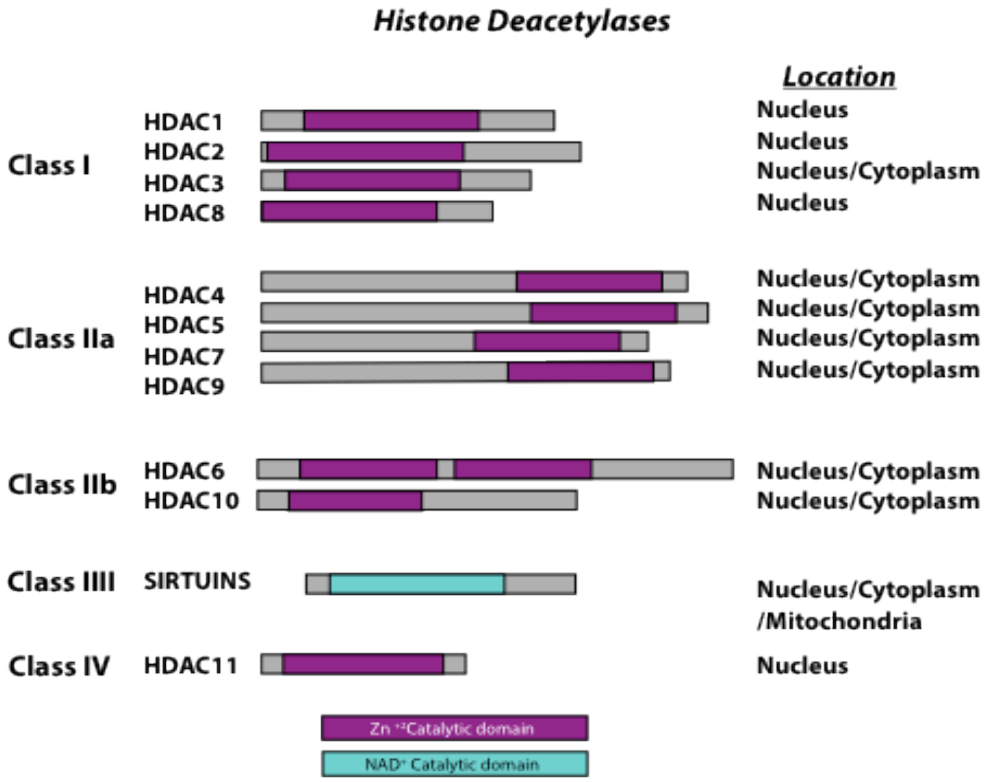

Figure 1.1. HDAC classification based on catalytic domain homology and mechanism of action.

Class I and IIa HDACs and their role in pathological cardiac hypertrophy

Histone deacetylases (HDACs) remove acetyl groups from ε- acetyl lysine amino acids in many protein targets, including histones. HDACs are classified in four classes according to their sequence identity, domain organization and catalytic mechanism based on Zinc or NAD+ [16]. Zinc-dependent HDACs include class I (HDAC1, HDAC2, HDAC3, HDAC8), class IIa (HDAC4, HDAC5, HDAC7, HDAC9), class IIb (HDAC6, HDAC10) and class IV (HDAC11). Class III includes Sirtuins 1 to 7, which require NAD+ for their activity, making them mechanistically different [16]. Although the most studied function of HDACs is as epigenetic regulators, increasing evidence has shown HDAC activity towards a diversity of non-histone protein targets [17].

HDACs play important roles in cardiac development and physiology. Class IIa HDACs are expressed most abundantly in the heart and are considered protective against pathological remodeling [16]. Class IIa isoforms shuttle from the nucleus to the cytosol upon phosphorylation, where they are believed to be inactive [18]. In the nucleus, class IIa HDACs bind to the myocyte enhancer factor-2 (MEF2) transcription factor and repress expression of pro-hypertrophic genes [19]. This can be observed in HDAC5 or HDAC9 knockout mice that develop spontaneous hypertrophy and show increased pathological remodeling after pressure overload [20]. Consistent with this, over-expression of HDAC4, HDAC5 or HDAC9 repress MEF2 activity [21]. Interestingly, recent evidence suggests that class IIa HDACs do not posses enzymatic activity, or that it is minimal compared to other HDAC isoforms [22]. In addition, class IIa HDACs have been shown to be insensitive to pan-HDAC inhibitors [23]. Rather, it is believed that their catalytic function is based on their ability to bind and form complexes with Class I HDACs and other transcriptional regulators [22].

On the other hand, HDACs from class I confer the majority of the HDAC activity in cardiomyocytes [24]. HDAC1, HDAC2 and HDAC3 are the best studied class I HDACs in cardiac hypertrophy, while the role(s) of HDAC8 is/are unknown. In general, class I HDACs are

found in the nucleus; however, a pool of HDAC3 and HDAC8 has been reported to function in the cytosol [25]. HDAC1 and HDAC2 are 92% identical in their catalytic domain, and they share several binding proteins in core complexes involved in gene transcription modulation (REST/CoREST, Sin3a/NuRD, SHIP and NODE complexes) [25]. This is not surprising, as HDAC1 and HDAC2 are considered a duplication of the same gene [16]. This is also evidence of redundancy in their functional roles, as HDAC1 and HDAC2 function redundantly in several contexts. Cardiomyocyte-specific knockouts of HDAC1 or HDAC2 driven by α-Myosin Heavy Chain Cre-recombinase are viable and have a normal phenotype. Indeed, a single copy of either HDAC1 or HDAC2 is sufficient to maintain regular physiology and respond to pathological stress [26]. However, deletion of both HDAC1 and HDAC2 is lethal at P14 with severe cardiac abnormalities [26]. Redundancy of HDAC1 and HDAC2 is also evident in neuronal differentiation and several cancer models in which HDAC1 deletion leads to an up-regulation of HDAC2, and vice versa [27, 28].

HDAC2-specific functions are suggested in experiments by Trivedi *et al* in which deletion of HDAC2 alone was sufficient to inhibit pathological cardiac growth by activating GSK3β [29]. In this model, a *lacZ* cassette was inserted between exons 8 and 10 of HDAC2. As the catalytic domain of HDAC2 is formed by the first 4 exons, it has been suggested that this strategy may not generate complete gene knockout. Still, HDAC1 and HDAC2 are also known to have distinct functions in gene regulation and differentiation. Whole body knockout of HDAC1 is lethal at E9.5, while whole body knockouts of HDAC2 survive until P1 [27]. Whether HDAC1 and HDAC2 can compensate for one another may be dependent on the cell type and cellular context. Further evidence is needed to define whether HDAC1 and HDAC2 have redundant roles in cardiac hypertrophy.

HDAC3 seems to have functions independent of either HDAC1 or HDAC2. Whole body knockout of HDAC3 is lethal at E9.5 [30]. Conditional cardiomyocyte-specific deletion mediated by α-MHC-Cre results in spontaneous hypertrophy with significantly increased expression of

genes involved in fatty acid oxidation and oxidative phosphorylation [30]. Conversely, α-MHC-Tg HDAC3 hearts have enlarged ventricles and septa characteristic of concentric hypertrophy due to an increase in cardiomyocyte proliferation rather than hypertrophy of individual myocytes [31]. Additionally, HDAC3 has been localized to sarcomeres, where it can deacetylate α-MHC and β-MHC isoforms. The reversible acetylation of both MHC isoforms was reported to increase their sliding velocity, but whether this phenomenon is compensatory or detrimental remains to be determined [32].

Table 1: Cardiac phenotype of mouse models of HDACs at baseline and hypertrophy

Genetic Model	Hypertrophy Model	Cardiac Outcome	Ref
		CLASS I HDACs	
HDAC2 -/-		Increased cardiomyocyte proliferation, abnormal sarcomeres	29
	TAC, Isoproteranol	Resistant to hypertrophy	
aMHC-HDAC2 Tg		Spontaneous hypertrophy	29
HDAC1 -/-		Embryonic lethal E9.5	26
aMHC-Cre HDAC1 f/f		Normal cardiac phenotype	26
	TAC	Normal hypertrophic response	
HDAC2 -/-		Lethal P1, increased cardiomyocyte proliferation	26
aMHC-Cre HDAC2 f/f		Normal cardiac phenotype	26
aMHC-Cre HDAC1 f/f and HDAC2 f/f		Lethal at P14, increase calcium handling genes	25
aMHC-Cre HDAC1 f/+ and HDAC2 f/f	TAC	Normal hypertrophic response	25
aMHC-MCM HDAC1 f/f and HDAC2 f/f		Normal cardiac phenotype	This study
	TAC	Reduced hypertrophy	
HDAC3 -/-		Embryonic lethal E9.5	30
aMHC-Cre HDAC3 f/f		Lethal at 16 weeks	30
		Spontaneous hypertrophy and severe fibrosis	
		Compromised cardiac function at 12 weeks	
		Increased expression of oxidative phosphorylation related genes	
aMHC HDAC3 Tg		Increased cardiomyocyte proliferation	31
		Spontaneous hypertrophy	
	Isoproteranol	Normal hypertrophic response	
		CLASS II HDACs	
HDAC5 -/-		Increased heart weight at months	20
	HDAC5-/- x Cn-Tg	Hypersensitive to calcineurin-induced hypertrophy	
	TAC	Hypersensitive to pressure overload	
HDAC9 -/-		Age-dependent hypertrophy	21
	TAC	Hypersensitive to pressure overload	
	HDAC9-/- x Cn-Tg	Hypersensitive to calcineurin-induced hypertrophy	
HDAC5 -/- and HDAC 9 -/-		Increased heart weight to body weight and fetal gene program	20
		Decreased body weight	
		HDAC Inhibitors	
WT + Apicidin	TAC	Reduced hypertrophy and fibrosis	34
WT + TSA	TAC	Reduced hypertrophy and fibrosis	75

Studies from our lab and others have shown in mouse models that pharmacological inhibition of class I and class II HDAC activity reduces cardiomyocyte hypertrophy caused by pressure overload [33]. Since class IIa are considered to be insensitive to pan-HDAC inhibitors, it is believed that these small molecules exert their effect by targeting class I HDAC activity. Furthermore, it was recently shown that specific pharmacological inhibition of class I HDACs can repress pressure overload-induced hypertrophy in a similar manner to pan-HDAC inhibitors [34].

HDAC inhibitors

There are several small molecule inhibitors of HDACs. Because HDACs act on a common substrate, acetylated lysine, the structure of their catalytic binding pockets are similar. Thus, competitive inhibitors usually target all of the zinc-dependent isoforms [35]. In addition to pan-inhibitors, structurally unique compounds can bind and inhibit HDACs in an allosteric manner, thus providing class-specific inhibition [36]. Examples of allosteric inhibitors include entinostat (MS-275) and apicidin, which inhibit class I only (**Table 1**) [37]. Inhibition of HDAC activity through the use of small molecules has been successful in impeding the proliferation of cancer cells *in vitro* and *in vivo* [38]. Three HDAC inhibitors are currently FDA approved for the treatment of T-cell lymphoma. Vorinostat (SAHA), a hydroxamic acid-based pan-HDAC inhibitor, was the first HDAC inhibitor used in the clinic with a maximal dose of 400mg daily for two weeks. This regimen is considered to be well tolerated, and the most common adverse effects in patients are fatigue (62%), nausea (56%) and diarrhea (49%) [39, 40]. Because different HDAC isoforms perform different biological functions, the identification and development of specific inhibitors for a particular class of HDACs and/or isoform could result in reduced side effects [41].

Table 2: HDAC inhibitors classification and clinical trial use

Class	Compound	Name	Potency range	HDAC Specificity	Clinical Stage
Hydroxamates	SAHA	Vorinostat	μM	Pan-inhibitor	Approved for CTCL
	PXD101	Belinostat	μM	Pan-inhibitor	Phase II
	LBH589	Panobinostat	nM	Pan-inhibitor	Phase III
	ITF2357	Givinostat	nM	Pan-inhibitor	Phase II
	4SC-201	Resminostat	μM	Pan-inhibitor	Phase II
	PCI 24781	Abexinostat	na	Pan-inhibitor	Phase II
	Trichostatin A		nM	Pan-inhibitor	none
Cyclic peptides	Depsipeptide	Romidepsin	nM	Class I	Approved for CTCL and PTCL
	Apidicin		nM	Class I	none
Benzamides	MS-275	Entinostat	μM	Class I	Phase II
	MGCD0103	Mocetinostat	μM	Class I	Phase II
Aliphatic fatty acids	Valproic acid		mM	Class I and IIa	Phase II
	Butyrate		mM	Class I and IIa	Phase II

Adapted from New et.al. 2012 [38]
Abbreviations: CTCL: Cutaneous T-cell lymphoma. PCTL: Peripheral T-cell lymphoma

Signaling cascades in cardiac hypertrophy

There are three major signaling cascades implicated in mediating pathological and physiological cardiac growth: 1) PI3K-AKT, 2) MAPK-ERK1/2, and 3) Calcium-calmodulin. In response to pathological stress, cardiomyocytes secrete paracrine and autocrine humoral factors, such as angiotensin II, endothelin-1 and norepinephrine to induce growth. Angiotensin II binds to Ang II receptor type 1, endothelin-1 binds to endothelin receptors 1 and 2, and norepinephrine binds to α-adrenergic receptors. All of these receptors are within the G-protein coupled receptor (GPCR) family [42-44]. GPCRs activate phospholipase C that cleaves PIP2 to diacyglycerol and IP3. This event results in the activation of the MAPK pathway, activation of mTOR, increased cyclic-AMP, and increased contraction [45]. On the other hand, insulin-like growth factor 1 (IGF1) binds to the tyrosine kinase receptor IGF1R. IGF1 is secreted during exercise and physiological cardiac growth in mice, and in conditioned athletes, leading to the activation of PI3K and AKT pathways [46, 47].

PI3K-AKT

Activation of PI3K is coupled with both tyrosine kinase receptors and GPCRs [48]. The main downstream target of PI3K is AKT, which regulates mTOR activity though the TSC1/TSC2 complex [49]. As such, AKT is also activated by both receptor tyrosine kinases and GPCRs; however, it may play different roles depending on the stimuli. AKT1 knockout mice have a blunted hypertrophic response to exercise, while their pathological response to aortic constriction is preserved [50]. Because of this, it is believed that the IGF1/AKT/mTOR axis generally mediates physiological hypertrophy; however, mTOR is also active in models of pathological growth [51].

Mitogen Activated Protein Kinase (MAPK)

The family of MAPKs is composed of extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK), and p38-MAPK [52]. The MAPK pathway is activated when GPCRs are stimulated during hypertrophic growth conditions in vitro, in animal models, and in failing human hearts [53, 54, 55]. The most prevalent MAPK cascade in pathological hypertrophy involves the activation of Raf1/MEK1 after Gq dissociation from GPCRs. ERK1/2 is a target of MEK and its activation is required for cardiac hypertrophy [56]. Nevertheless, reduced expression of ERK1 and ERK2 fails to block hypertrophy elicited by aortic constriction or swim exercise [54]. Another relevant MAPK mediator of hypertrophy is p38-MAPK, which is active in pressure overload and sufficient to induce hypertrophy [57]. p38 phosphorylates MEF2 and NFAT3 transcription factors to promote hypertrophy [58, 59].

Calcium-calmodulin

Calcium signaling is central to the control of myocyte contraction, which can be governed by GPCRs [60]. Elevated cytoplasmic calcium levels enhance the binding of calmodulin to the phosphatase calcineurin. Calcineurin activity is increased after surgical

thoracic aortic constriction and in failing hearts of human patients [61, 62]. Mice over-expressing calcineurin develop severe pathological cardiac hypertrophy with a rapid transition to heart failure [63]. Inhibition of calcineurin with cyclosporine A or FK506 prevents hypertrophy of NRVMs and in constitutively active calcineurin transgenic mice [64].

Calcineurin promotes hypertrophy through the dephosphorylation of NFAT (nuclear factor of activated T-cells), which translocates to the nucleus and associates with MEF2 to promote transcription of hypertrophic genes [65]. Over-expression of constitutively active NFAT3 triggers hypertrophy similar to calcineurin transgenics [63]. MCIP1 (myocyte-enriched calcineurin-interacting protein 1) is a downstream target of NFAT that binds and inhibits calcineurin. Over-expression of MCIP inhibits pathological hypertrophy of calcineurin transgenics, aortic banded mice, or treatment with isoproterenol [66]. NFAT activity is up-regulated in pathological remodeling models of TAC and myocardial infarction, but not in physiological growth after exercise or IGF1 treatment. However, over-expression of MCIP is able to inhibit exercise-induced hypertrophy [66].

Calcium can also regulate calmodulin-dependent protein kinase II (CaMKII) during cardiac growth. Increased CaMKII levels have been observed in animal models of pathological hypertrophy and patients with heart failure [67, 68]. Over-expression of the nuclear form of CaMKII induces spontaneous cardiac hypertrophy, while CaMKII null mice do not develop pathological hypertrophy after aortic constriction [69, 70]. CaMKII can phosphorylate HDAC4, which then translocates out of the nucleus. This causes activation of MEF2, which promotes pathological hypertrophy [71].

Mammalian Target of Rapamycin

The kinase mammalian target of rapamycin (mTOR) is the main catalytic subunit of two distinct kinase complexes: mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2). mTORC1 functions as a sensor-regulator of cell growth and protein synthesis [72]. mTORC1 is

composed of mTOR kinase, regulatory-associated protein of mTOR (Raptor), mammalian lethal with Sec13 protein 8 (mLST8), proline-rich AKT substrate 40 kDa (PRAS40) and DEP-domain-containing mTOR-interacting protein (DEPTOR) [73, 74]. On the other hand, mTORC2 is mainly involved in cytoskeletal regulation during cell growth. The mTORC1 and mTORC2 complexes share in common mTOR kinase, mLST8 and DEPTOR. However, mTORC2 includes rapamycin-insensitive companion of mTOR (Rictor), mammalian stress-activated protein kinase interacting protein (mSIN1) and protein observed with Rictor-1 (Protor-1) [75, 76].

Downstream targets of mTORC1 phosphorylation include p70S6 kinase (S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) [72, 77, 78]. p70S6K phosphorylates and activates S6 ribosomal protein, which leads to ribosomal biogenesis. On the other hand, hypo-phosphorylated 4EBP1 binds to translation initiation factor 4E (eIF4E), inhibiting translation. mTOR-dependent phosphorylation of 4EBP1 releases its binding from eIF4E and targets 4EBP1 for degradation. Phosphorylation of S6K, S6 and 4E-BP1 are commonly used as markers of mTOR activity [72, 78]. mTORC1 can also phosphorylate and inhibit the unc-51-like kinase 1 (ULK1), autophagy-related gene 13 (ATG13) and focal adhesion kinase family-interacting protein of 200 kDa (FIP200) complex, which promotes autophagosome formation. As a result, mTORC1 is an inhibitor of autophagy [79].

Rapamycin is a potent and specific inhibitor of mTORC1 [80]. Rapamycin forms a complex with the immunophillin FK506 binding protein 12 (FKBP12), and binds to mTORC1 to prevent the mTOR-raptor interaction that is necessary for binding of mTOR to its downstream targets [80]. mTORC2 activity is not directly affected by rapamycin [81]. However, in some cell types, long term exposure to rapamycin can also inhibit mTORC2 through a mechanism that is not fully understood. It is believed that rapamycin-FKBP12 can bind to mTOR alone, preventing the formation of mTORC2 over time [82].

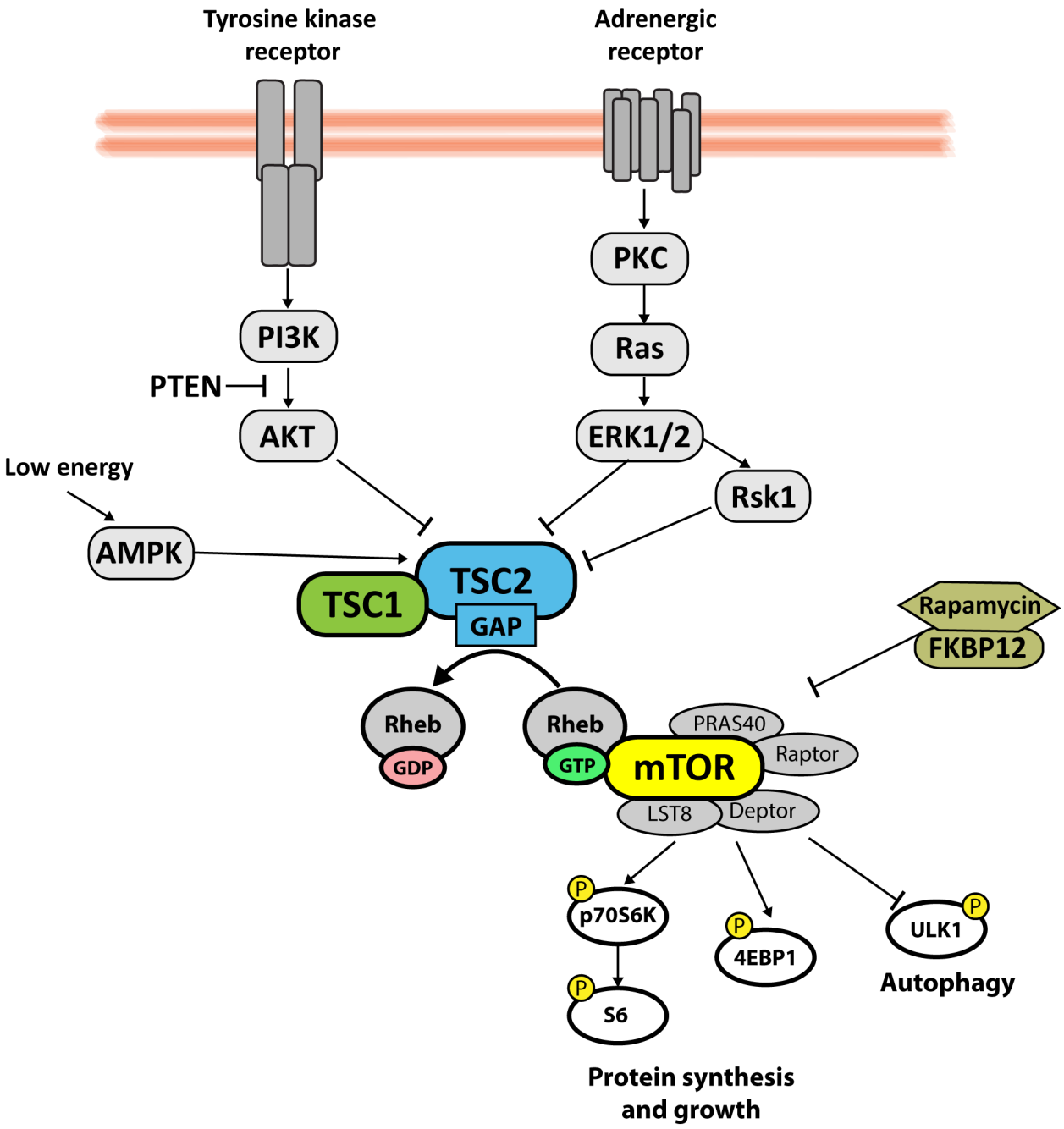

Figure 1.2. Major signaling pathways involved in the regulation of mTOR complex 1.

mTOR in pathological hypertrophy

mTOR is active during physiological and pathological cardiac growth [83]. In the context of pathological cardiac hypertrophy due to pressure overload, rapamycin reduces the increase in heart mass, reduces the level of fibrosis formation, and improves cardiac function [51, 84]. Rapamycin is also capable of partially rescuing pre-existing pathological hypertrophy [85]. Sirolimus, another name for rapamycin, is approved by the FDA as an immunosuppressant agent for transplant recipients. While sirolimus reduces graft rejection and improves renal function post-transplantation, 70% of patients suffer from adverse side effects such that 30% of patients must stop the medication. Some of these reactions include abdominal pain, diarrhea, propensity to infection and increased blood pressure [86]. After kidney transplant, treatment with sirolimus results in decreased left ventricular mass in patients with hypertrophy [87]. It is not known whether this is caused by direct action of sirolimus on the heart or due to improved renal function and blood pressure, or both.

TSC1/TSC2 complex

TSC1 (hamartin) and TSC2 (tuberin) form a complex in which TSC2 functions as the catalytic subunit. TSC2 hydrolyzes GTP and inhibits the small GTPase Rheb; a major, if not the only, downstream target of the TSC1/TSC2 complex [88, 89]. TSC1 is crucial for the activity of TSC2, and by virtue of forming a complex, can regulate the stability of TSC2 [90]. Rheb is a direct and necessary component for the activation of mTORC1 [91, 92]. As such, the TSC1/TSC2 complex is an endogenous inhibitor of mTOR activity [93]. TSC1- or TSC2-deficiency is associated with many types of pathological cell growth, as is observed in mTOR over-activation models [94, 95]. Mice deficient in cardiac TSC1 develop spontaneous cardiac hypertrophy and increased mTOR activity that can be rescued by treatment with rapamycin [96]. Neonatal cardiomyocytes from Eker rat embryos (TSC2-/+), and TSC2 dominant-negative

transgenic mice, harbor increased markers of hypertrophy (βMHC, ANF, BNP levels), as well as increased DNA synthesis, suggestive of cardiomyocyte growth [97, 98].

Tuberous sclerosis complex is a genetic disease characterized by formation of hamartomas in multiple organs, especially in muscle, brain, kidney, heart, eye, lung, and skin. Around 1 in 6000 newborns are affected by TSC, which is caused by the loss of function of the tumor suppressor genes TSC1 or TSC2 [99-101]. Complete loss of the TSC genes is embryonic lethal. Rhabdomyosarcomas (RMS) are benign tumors of the heart and skeletal muscle observed in 47-67% percent of patients with TSC, and 80% of these patients will develop RMS during pre-natal development [99, 102, 103]. Often, cardiac tumors are the earliest symptomatic manifestation of TSC. Clinically, spontaneous regression of RMS is observed in most patients, and the hamartomas that do not regress often do not continue to grow. Life-threatening conditions are observed, however, in rare cases where the size or location of the tumor impacts the hemodynamic performance or rhythm of the heart.

Central Thesis

Small molecule inhibitors of histone deacetylases (HDACs) attenuate pathological cardiac remodeling, including hypertrophic growth, fibrosis, and declines in ventricular function [33, 104]. Of the 4 classes of HDACs, class I HDACs (HDAC1, 2, 3, and 8) are suggested to mediate pathological cardiac remodeling [105]. Small molecule inhibitors specific for class I HDACs blunt pathological hypertrophy similar to pan-HDAC inhibitors [34]. As three HDAC inhibitors are now FDA approved for human use, this avenue holds promise for clinical translation. However, mechanisms underlying the ability of HDAC inhibitors to repress cardiac hypertrophy are unknown.

The mammalian target of rapamycin (mTOR) kinase is a central regulator of cell growth in many contexts including cardiac hypertrophy [84]. Inhibition of mTOR complex I by genetic

and pharmacological means leads to decreased cardiac remodeling under pathological stress [85, 106]. In light of this, we **hypothesized** that **Class I HDACs regulate cardiac hypertrophy in an mTOR-dependent manner.**

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CHAPTER 2: INHIBITION OF CLASS I HDACs BLUNTS HYPERTROPHIC ACTIVATION OF mTOR BY INCREASING TSC2 EXPRESSION

INTRODUCTION

Inhibition of HDACs with small molecules has been shown to reduce pathological growth of the heart in response to either pressure-overload stress or adrenergic agonists [1, 2]. There are 17 different HDAC isoforms categorized into four classes [3]. Recent evidence suggests class I HDACs (including HDAC1, HDAC2, HDAC3 and HDAC8) promote growth in cardiomyocytes. Apicidin, a class I HDAC specific inhibitor, can blunt load-induced hypertrophy, reducing activation of the fetal gene program and blunting fibrosis [4]. Nevertheless, the molecular mechanisms through which pan-HDAC and class I-specific HDAC inhibitors repress cardiac hypertrophy are unknown.

mTOR is active during both physiological and pathological cardiac growth [5]. HDAC inhibitors have been shown to inhibit mTOR in several cancer models in which mTOR is overactive [6]. In addition, the mTOR inhibitor rapamycin can reduce hypertrophic growth [7]. Therefore, we performed the following studies to test whether HDAC inhibitors alter mTOR activity during cardiac growth.

RESULTS

Class I HDACs redundantly regulate NRVM hypertrophy

We compared the broad-spectrum HDAC inhibitor trichostatin A with the class I-specific inhibitor apicidin. Total cellular HDAC activity was reduced in the presence of TSA and to a lesser extent with apicidin (**Figure 2.1.A**). The 70% reduction in HDAC activity with TSA compared to the 50% reduction with apicidin may be due to the difference in specificities. The fact that apicidin can decrease total cellular HDAC activity by half suggests that the contribution of class I HDACs to total HDAC activity is relatively high in NRVMs.

Using leucine incorporation to quantify cell growth, apicidin, the class I-specific HDAC inhibitor, elicited greater suppression of phenylephrine-induced growth compared to TSA (**Figure 2.1.B**). It is possible that TSA's ability to inhibit other HDAC isoforms may counteract the effect of its action on class I HDACs. Alternatively, 60 nM of TSA may not inhibit class I HDACs to the same extent as 200 nM apicidin. Distinguishing between the two would require that we specifically measure class I activity. As reported before [4], representative images illustrate that NRVMs treated with apicidin are smaller compared to control and PE-treated NRVMs (**Figure 2.1.C**). Transcript levels of BNP and RCAN1.4, which are induced during phenylephrine treatment, are likewise inhibited with apicidin. This is consistent with previous findings [4].

To control for off-target effects and identify the pertinent HDAC, we performed siRNA knockdown of individual HDAC isoforms. Western blot analysis confirmed effective protein knockdown of HDAC1, HDAC2 and HDAC3 (**Figure 2.2.A**). Knockdown of individual HDAC isoforms did not alter total cellular HDAC activity (**Figure 2.2.B**). However, knockdown of HDAC1 and HDAC2, or HDAC1, HDAC2 and HDAC3 together resulted in a 25% reduction of total cellular HDAC activity (**Figure 2.2.B**). As shown in Figure 2.1.A, apicidin treatment of NRVMs resulted in a 50% decrease in total cellular HDAC activity. The residual pool of class I HDACs left after knockdown likely accounts for the difference in HDAC activity, reinforcing the notion that class I HDAC activity is robust in NRVMs. Although apicidin is a potent and highly specific inhibitor; it is also possible that off-target effects contribute to the magnitude of repression of HDAC activity.

Similar to the effects on HDAC activity, silencing of HDAC1, HDAC2 or HDAC3 individually did not affect PE-induced leucine incorporation. However, PE-induced growth was inhibited by knocking down class I isoforms in combination (**Figure 2.2.C**). Selective silencing of HDAC1 and HDAC2 together was sufficient to achieve this effect (**Figure 2.2.C**). At baseline, a

trend toward reduction in leucine incorporation was observed after knockdown of individual class I HDACs; however, this trend was not statistically significant. Similar results were observed in the fetal gene program response (**Figure 2.2.D**).

Diverse mechano-humoral signals activate a wide range of cellular events in cardiomyocytes to induce hypertrophy. Therefore, we tested whether HDAC inhibitors could blunt NRVM growth induced by different stimuli. As pathological models, we used phenylephrine and endothelin-1 (ET-1). Insulin-like growth factor 1 (IGF-1) is an established model for physiologic NRVM growth. Lastly, a recent model using 50% hypo-osmotic solution is reported to mimic mechanical stretch conditions [8]. Tritiated leucine incorporation increased in all of these conditions, and exposure to apicidin significantly inhibited leucine incorporation in all these models (**Figure 2.3.A**). Similar to apicidin, knockdown of HDAC1, HDAC2 and HDAC3 in combination inhibited hypertrophy in all the models (**Figure 2.3.B**).

Hypertrophic activation of mTOR is suppressed by class I HDAC inhibitors

Inhibition of mTOR with rapamycin in hypertrophy suggests that it may be a promising target to regulate pathological remodeling [9]. We compared leucine incorporation in NRVMs exposed to apicidin and/or rapamycin at baseline and under growth conditions. As expected, both apidicin and rapamycin reduced the growth response under resting and PE-induced growth conditions (**Figure 2.4.A**). However, after PE treatment, suppression of leucine incorporation was greater in the setting of HDAC inhibition than with rapamycin (**Figure 2.4.A**). This suggests that HDACs may control other pro-growth mechanisms in addition to mTOR.

As an indirect measure of mTOR activity, we performed Western blot analysis of two downstream targets: S6 ribosomal protein and 4EBP1. PE-induced mTOR activity measured this way was inhibited by rapamycin and by apicidin. In this context, inhibition of mTOR was

almost complete with rapamycin, whereas apicidin only reduced phosphorylation of S6 and 4EBP1 by 50% (**Figure 2.4.B and Figure 2.4.C**).

In addition, inhibition of PE-induced mTOR activity measured by S6 phosphorylation was abrogated when more than one class I HDAC isoform was silenced (**Figure 2.5.A**). Similar effects were seen in NRVMs exposed to ET-1, IGF-1, or 50% HS (**Figure 2.5.B**). Together, these data highlight functional redundancy among class I HDAC isoforms in the regulation of mTOR and NRVM growth.

Inhibition of class I HDACs reduces TAC-induced mTOR activity

To corroborate the observation that apicidin reduced mTOR activity *in vivo*, we subjected wild type mice to TAC for three days. At the same time, we administered apicidin or vehicle in three daily doses. We chose 3 days of TAC compared to the 3-week standard protocol since mTOR is active very early following TAC surgery, peaking within one week after the surgery.

While differences in heart mass of sham surgery versus TAC mice were not detectable after three days, vehicle-treated mice showed up-regulation of genetic markers of hypertrophy. At this time, the expression of ANF, BNP and RCAN.4 was attenuated in apicidin-treated animals (**Figure 2.6.A**). In TAC mice, phosphorylation of S6 and 4EBP1 was increased, suggesting an increase in mTOR activity (**Figure 2.6.B**). Consistent with our observations in NRVMs, this increase in mTOR activity was blunted in mice treated with apicidin (**Figure 2.6.B and Figure 2.6.C)**.

Cardiomyocyte-specific silencing of HDAC1 and HDAC2 reduces TAC-induced growth and mTOR activity

Cardiomyocyte-specific deletion of genes coding for HDAC1 and HDAC2 driven by αMHC-Cre is lethal at P14 [10]. While apicidin can inhibit heart remodeling, genetic studies to test HDAC1 and HDAC2 in pathological growth of the adult heart are lacking. We engineered conditional HDAC1 and HDAC2 double knockout (DKO) mice by crossing $HDAC1^{ff/ff}$ and $HDAC2^{ff/ff}$ mice with αMHC-Mer-Cre-Mer mice. At 6 weeks of age, these mice were treated with 5 daily doses of 20mg/kg of tamoxifen to activate the Cre-recombinase. We observed these mice for 3 weeks after tamoxifen injection, and they appeared to be normal and healthy (**Figure 2.7.A**). Real time-PCR analysis revealed a reduction in the mRNA levels of HDAC1 and HDAC2 in whole heart extracts of DKO mice after tamoxifen. (**Figure 2.7.B**). DKO mice did not manifest changes in heart mass or cardiac function for at least 3 weeks after tamoxifen (**Figure 2.7.C**).

HDAC1/2 DKO mice were subjected to TAC for 3 weeks. As controls, we studied $HDAC1^{ff/ff}$ and $HDAC2^{ff/ff}$ mice. Declines in ventricular performance observed in TAC-treated control mice were not observed in DKO animals (**Figure 2.8.A**). In addition, DKO mice manifested blunted hypertrophic growth as determined by HW/BW ratios and expression of fetal gene markers (**Figure 2.8.B, Figure 2.8.C and Figure 2.8.D**).

In this model, mTOR activity was normal in DKO mice under sham conditions. After 3 weeks of load stress, DKO mice showed reduced mTOR activity compared to control mice (**Figure 2.9.A and Figure 2.9.B**). Taken together, we conclude that class I HDAC inhibition reduces stress-induced activation of mTOR in the heart.

Suppression of mTOR by HDAC inhibition requires transcription

Class I HDACs are primarily nuclear localized, but a small population has been reported in the cytosol [11]. Therefore, we considered the possibility that class I HDACs could directly deacetylate mTOR in the cytosol. We performed immunoprecipitation studies of mTOR to test whether the molecule is reversibly acetylated. NRVMs were treated with TSA for 24 hours. There was no detectable acetylation of mTOR after immunoprecipitation using an anti-acetylated lysine antibody (**Figure 2.10.A**). As control, tubulin acetylation was observed in the input. The converse experiment using acetylated lysine antibody did not pull down mTOR, while tubulin was significantly precipitated from TSA treated NRVMs (**Figure 2.10.A**). Based on this, we conclude that HDAC-dependent control of mTOR does not involve mTOR as an acetylated HDAC substrate.

To define the kinetics of the inhibition of mTOR by HDAC inhibitors, we conducted a detailed time course analysis of mTOR repression. NRVMs exposed to apicidin or TSA displayed a similar progressive, time-dependent decline in mTOR activity (measured as S6 phosphorylation), requiring 6 hours to reach statistical significance (**Figure 2.11.A**). On the other hand, rapamycin completely inhibited mTOR activity within 3 hours (**Figure 2.11.A**). This 6 hour delay in mTOR repression suggested transcriptional regulation as a possible mechanism.

To test this, NRVMs were treated with cycloheximide or actinomycin D to block transcriptional or translational processes, respectively. In these experiments, we found that TSA did not inhibit mTOR activity in the presence of either cycloheximide or actinomycin D (**Figure 2.12.A and Figure 2.12.B**). Considering that blocking translation activates mTOR activity [12], these data suggest that gene transcription is required for HDAC inhibitor-dependent repression of mTOR.

Class I HDAC inhibition increases TSC2 mRNA levels

Given the hypothesis that gene transcription is involved in the inhibition of mTOR, we screened the mRNA levels of several direct binding components of the mTOR complex 1, mTOR complex 2, and the TSC1/TSC2 complex. The screen was performed in NRVMs treated with TSA or apicidin under resting and growth conditions. mRNA levels of specific components of the mTORC2 did not change (**Figure 2.13.A**). From mTORC1, PRAS40 mRNA was significantly down-regulated. PRAS40 functions as a weak inhibitor of mTOR [13]. As such, it would be expected to increase upon HDAC inhibition. In addition, mRNA of PRAS40 is similarly reduced by rapamycin. Therefore, we concluded that this response is a downstream consequence of mTOR inhibition (**Figure 2.13.B**).

Common to mTORC1 and mTORC2 is DEPTOR, which is increased with TSA and apicidin treatments equally, and is not affected by rapamycin. DEPTOR helps maintain the activity of an already active mTOR, and when mTOR is inhibited, DEPTOR prevents the activation of mTOR [14]. In this context, we considered that increasing DEPTOR mRNA may be a mechanism by which HDACs promote mTOR activity (**Figure 2.13.A and Figure 2.13.B**).

We also found that TSC2 mRNA levels increased in response to class I HDAC inhibition. TSC2 is the catalytic subunit of the TSC1/TSC2 complex, which inhibits Rheb by hydrolyzing its GTP form. As a consequence, mTOR is inhibited. An increase in TSC2 mRNA was not observed in NRVMs treated with rapamycin, suggesting that this effect is not secondary to the inhibition of mTOR (**Figure 2.14.A**). Consistently, TSC2 mRNA levels also increased in NRVMs silenced for HDAC1, HDAC2 and HDAC3 (**Figure 2.14.B**). A 2-fold increase in TSC2 mRNA when HDACs were inhibited in NRVMs is consistent with a similar-fold increase in TSC2 protein detected by Western blot (**Figure 2.14.C**).

To pursue this observation *in vivo*, we measured TSC2 mRNA in both the 3-day TAC model, and the double knockout (HDAC1 and HDAC2) mouse. Mouse hearts subjected to TAC

for 3 days and treated with apicidin manifested elevated levels of TSC2 (**Figure 2.14.D**). This effect was also observed in DKO mice after 3 weeks of TAC (**Figure 2.14.E**). Protein levels of TSC2 from left ventricles of the 3-day TAC mice showed a similar 2-fold increase (**Figure 2.14.F**). Of note, TSC2 mRNA levels were not inhibited during growth conditions either *in vitro* or *in vivo*.

Role of TSC2 in mTOR control in NRVM hypertrophy

We found that inhibition of HDACs promoted expression of TSC2 and DEPTOR, both repressors of mTOR. To test whether TSC2 or DEPTOR are required for HDAC inhibitor suppression of mTOR activity, we performed siRNA knockdowns in NRVMs. We found that mTOR activity in DEPTOR-deficient NRVMs was repressed by HDAC inhibitors similarly to control in siRNA treated cardiomyocytes (**Figure 2.15.A and Figure 2.15.B**). We concluded that while HDAC inhibitors increase DEPTOR mRNA, these changes were not required for the HDAC-mediated repression of mTOR.

Knockdown of TSC2 did not increase mTOR activity in NRVMs either at baseline or under growth conditions (**Figure 2.15.A and Figure 2.15.B**). This finding is contrary to that previously reported in other muscle cell types [15]. Nevertheless, in the absence of TSC2, HDAC inhibitors failed to decrease mTOR activity (**Figure 2.15.A and Figure 2.15.B**). A similar response was observed in siRNA knockdown of TSC1 in NRVMs, demonstrating that the activity of the TSC1/TSC2 complex is required for HDAC inhibition to blunt mTOR activation (**Figure 2.16.A and Figure 2.16.B**). Interestingly, in TSC1-deficient NRVMs, mTOR activity tended to increase at baseline and during growth. This may be due to the role of TSC1 as a chaperone of TSC2 protein [16]. As observed by Western blot, siRNA of TSC1 significantly reduced TSC2 levels, possibly resulting in stronger inhibition of the TSC1/TSC2 complex and activation of mTORC1 (**Figure 2.16.A and Figure 2.16.B**).

To test further the role of TSC2 in cardiomyocyte hypertrophy, the growth response of NRVMs subjected to TSC2 knockdown was assessed by leucine incorporation. Consistent with the degree of mTOR activation, knockdown of TSC2 did not induce growth at baseline, nor did it exacerbate phenylephrine-induced growth. However, silencing of TSC2 partially rescued the growth response after exposure to HDAC inhibitors (**Figure 2.17.A**), pointing to TSC2 as an important mediator of HDAC-dependent suppression of mTOR activity.

The inhibition of mTOR by TSC1/TSC2 has important clinical implications. A dysfunctional TSC1/TSC2 complex due to a variety of mutations results in tuberous sclerosis complex and is observed in several forms of cancer. Therefore, we set out to test whether the biology observed in NRVMs is similar and relevant in other cell types. $TSC2^{+/+}$ and $TSC2^{+/-}$ MEFs were treated with apicidin, each manifested a 2-fold increase in TSC2 steady-state levels (**Figure 2.18.D**). Consistent with our prior results, apicidin inhibited mTOR activity in WT MEFs and did not reduce mTOR activity in TSC2 knockout MEFs. A partial inhibition of mTOR by apicidin was observed in $TSC2^{+/-}$ MEFs (**Figure 2.18.A and Figure 2.18.B**). Cell growth in WT MEFs measured as leucine incorporation was reduced in the presence of HDAC inhibition, and this effect was lost in the TSC2 knockout MEFs (**Figure 2.18.C**).

To test the effect of HDAC inhibitors in human cells, we studied H9 embryonic stem cells differentiated into cardiomyocyte-like cells (ESC-CM) [17]. Upon differentiation, ESC-CM cells beat, harbor sarcomeric structures, and express the cardiogenic markers troponin-I and α-actinin (**Figure 2.19.A**). This cardiac-like phenotype is not abolished by treatment with apicidin (**Figure 2.19.B**). However, treatment with apicidin reduced mTOR activity and resulted in a 2-fold increase in TSC2 mRNA levels, similar to what we observed in other models (**Figure 2.20.A to Figure 2.20.C**).

Class I HDAC inhibitors repress ERK1/2 independent of mTOR

The MAPK pathway is activated when α-adrenergic receptors are stimulated during hypertrophic growth [18]. ERK1/2 is a target of MEK, and its activation is required for cardiac hypertrophy [19]. Our initial studies suggested that HDAC inhibitors target additional mTOR-independent mechanisms to repress growth. Therefore, we tested the effect of HDAC inhibitors on ERK phosphorylation, a common readout of its activity.

NRVMs treated with either apicidin or TSA showed a significant reduction in phenylephrine-induced phosphorylation of ERK1/2 (**Figure 2.21.A**). The S6 ribosomal subunit is a target of p70S6K, which is itself a target of both mTORC1 and ERK. In addition, ERK can activate mTOR through TSC2-dependent and –independent mechanisms [19]. Because of these reasons, we tested whether HDAC inhibitors can reduce mTOR activity through ERK1/2. NRVMs treated with the ERK1/2 inhibitor U0126 showed reduced S6 phosphorylation, but there was no effect in 4EBP1 phosphorylation (**Figure 2.21.B and Figure 2.21.C**). In the presence of ERK1/2 inhibitors, HDAC inhibitors still reduced phosphorylation of 4EBP1 and S6 (**Figure 2.21.C**). Therefore, we concluded that HDAC inhibitors blunt ERK1/2 and mTOR activities in parallel through independent mechanisms.

HDAC inhibitors do not alter AKT or AMPK phosphorylation

Because AKT is a key upstream regulator of mTOR, we tested whether HDAC inhibitors regulate mTOR through AKT. We exposed NRVM to PE for 6 hours in the presence of TSA. Western blot analysis of AKT phosphorylation manifested a modest increase in AKT activation by PE. However, treatment with TSA did not affect AKT phosphorylation. To further test the role of HDAC inhibitors in AKT activation, we treated NRVMs with IGF1 or insulin, both strong activators of AKT. We pre-treated NRVMs for 12 hours and then challenged them with IGF1 or insulin for 10 minutes before stopping the experiment. This time, AKT was hyper-

phosphorylated by IGF1 or insulin treatments. Similar to what we observed in PE conditions, AKT phosphorylation was not affected by HDAC inhibitors. Despite this, S6 phosphorylation was still reduced by inhibition of HDACs.

On the other hand, AMPK activation leads to the suppression of mTOR by inhibiting the TSC1/TSC2 complex. To test whether HDAC-dependent regulation of mTOR is mediated by AMPK, we performed Western blot analysis of the phosphorylation of AMPK in NRVMs treated with PE and HDAC inhibitors. Our results uncovered no significant difference in the phosphorylation of AMPK under any of the treatment conditions. This suggests that AMPK is not likely to be responsible for the inhibition of mTOR by HDAC inhibitors.

FIGURE 2.1

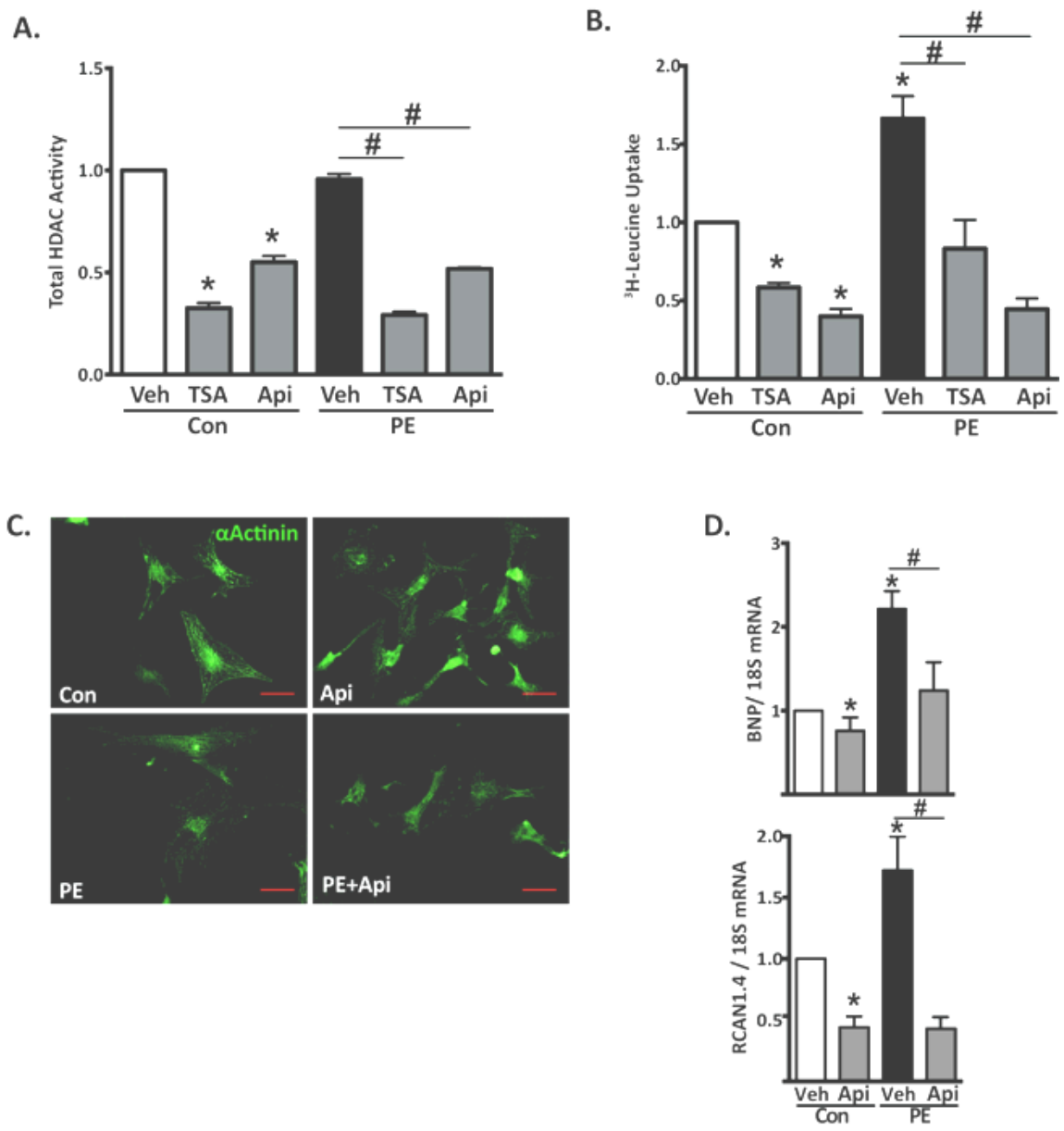

Figure 2.1. Class I HDAC inhibitor apicidin represses PE-induced NRVMs hypertrophy. A. Total HDAC activity in NRVMs exposed to pan-HDAC inhibitor TSA (60 nM) or apicidin (Api) (200 nM) for 24 h. B. ^{3}H-Leucine incorporation of NRVMs treated with TSA (60 nM) or apicidin (200 nM) under control and PE-stimulated (50 μM) conditions. C. Representative images of NRVMs exposed to PE (50 μM) and apicidin (200 nM) for 48h and probed with α-actinin. Scale bar: 40 μm. D. mRNA levels of BNP and RCAN1.4 in cardiomyocytes exposed to PE (50 μM) in the presence of apicidin (200 nM). Values are mean ± SEM, analyzed by one-way ANOVA followed by Tukey's test (n=4-6). (*) $p<0.05$ vs control; (#) $p>0.05$ vs PE.

FIGURE 2.2

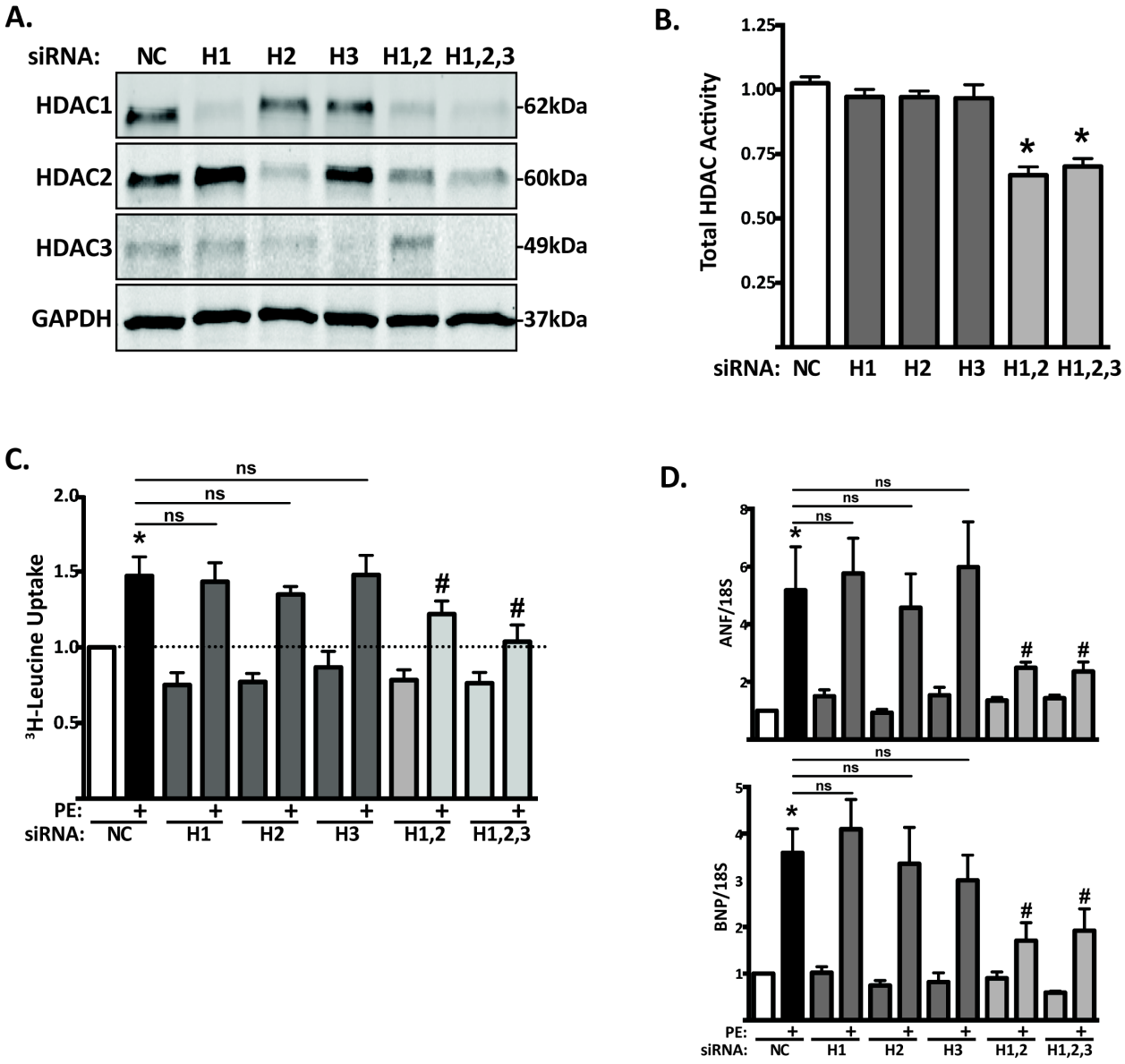

Figure 2.2. Knockdown of class I HDACs represses PE-induced NRVMs hypertrophy. A. Western blot analysis of HDAC1, HDAC2 and HDAC3 knockdowns in NRVMs. B. Total HDAC activity of NRVMs depleted of HDAC1, HDAC2 and/or HDAC3. C. ^{3}H-Leucine incorporation of NRVMs depleted of HDAC1, HDAC2 and/or HDAC3 in the presence of PE (50μM). D. mRNA of ANF and BNP. Values are mean ± SEM, analyzed by one-way ANOVA followed by Tukey's test (n=4-5). (*) $p<0.05$ vs control; (#) $p>0.05$ vs PE.

FIGURE 2.3

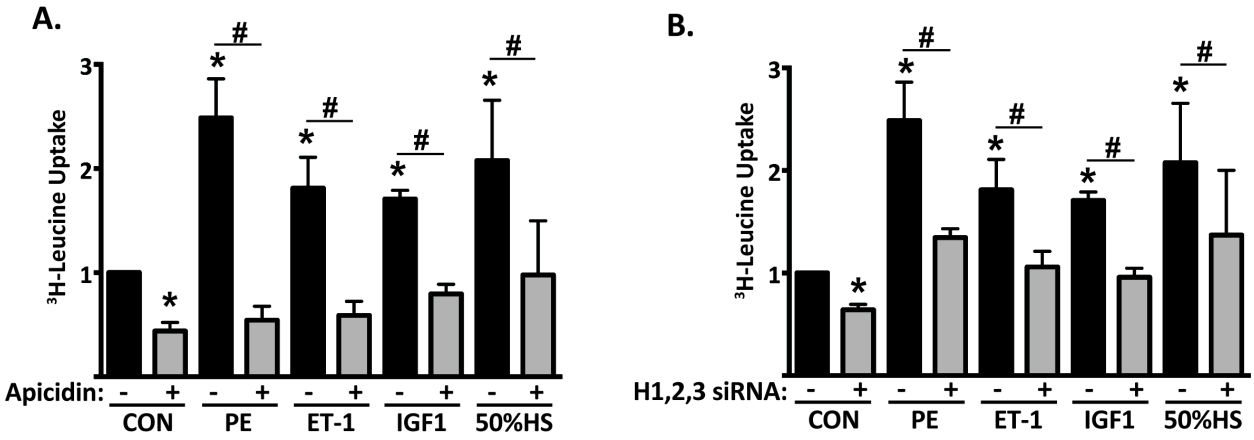

Figure 2.3. Knockdown of class I HDACs represses NRVMs hypertrophy induced by different stressors. A. ^{3}H-Leucine incorporation of NRVMs stimulated with PE (50 μM), ET-1 (200 nM), IGF1 (10 nM) or 50% hypo-osmotic solution and exposed to apicidin. B. ^{3}H-Leucine incorporation in NRVMs depleted of HDAC1, HDAC2 and HDAC3 stimulated with PE (50 μM), ET-1 (200 nM), IGF1 (10 nM) or 50% hypo-osmotic solution. Values are mean ± SEM, analyzed by one-way ANOVA followed by Tukey's test (n=3-4). (*) $p<0.05$ vs control; (#) $p>0.05$ vs growth stimuli.

FIGURE 2.4

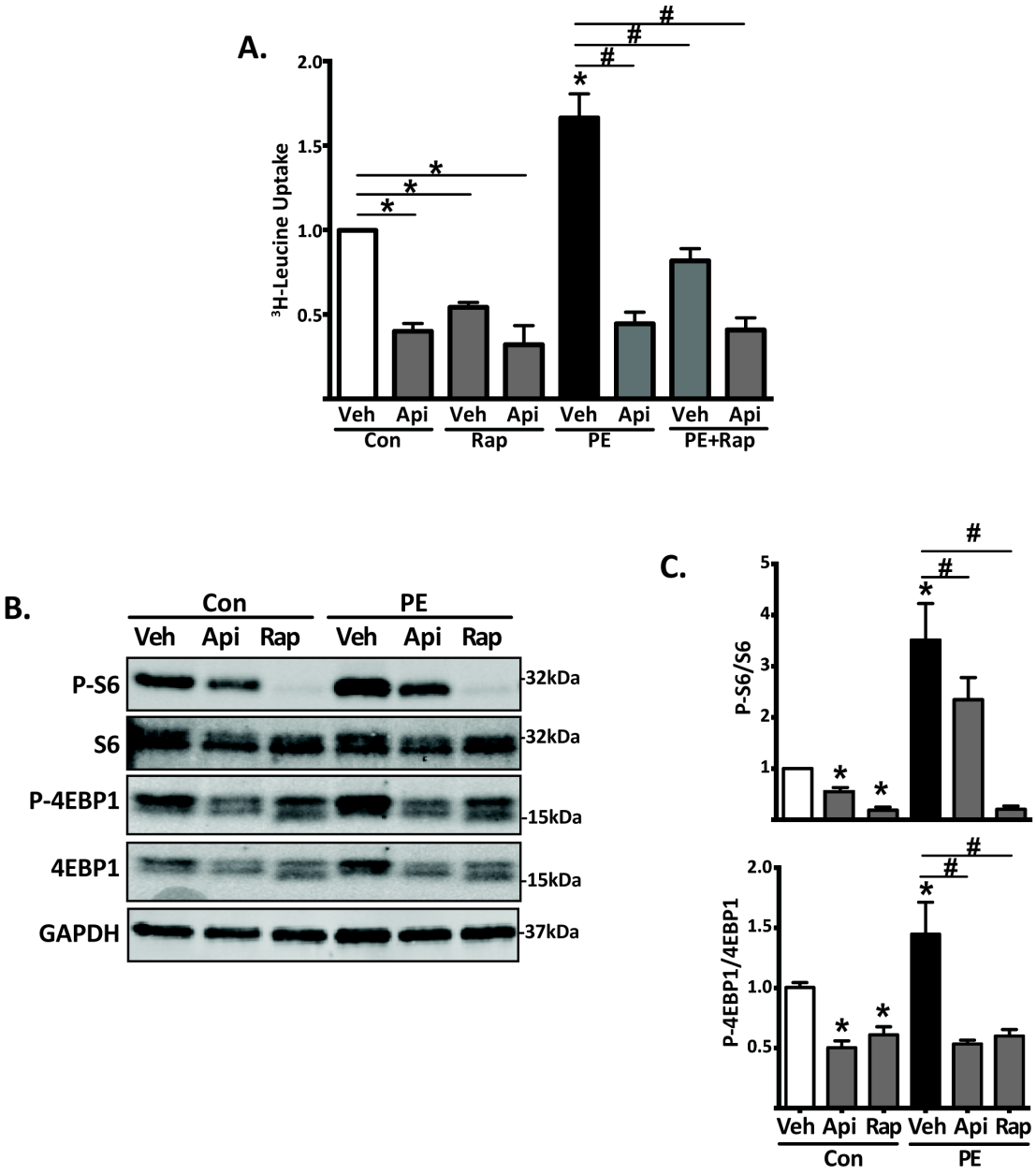

Figure 2.4. Inhibiting class I HDACs reduces mTOR activity in PE-induced growth. A. ^{3}H-Leucine incorporation in NRVMs exposed to apicidin (200 nM) and/or rapamycin (100 nM). B. Western blot of downstream targets of mTOR in NRVMs treated with apicidin (200 nM) or rapamycin for 6 h. C. Quantification of S6 and 4EBP1 phosphorylation. Values are mean ± SEM, analyzed by one-way ANOVA followed by Tukey's test (n=3-6). (*) $p<0.05$ vs control; (#) $p>0.05$ vs PE.

FIGURE 2.5

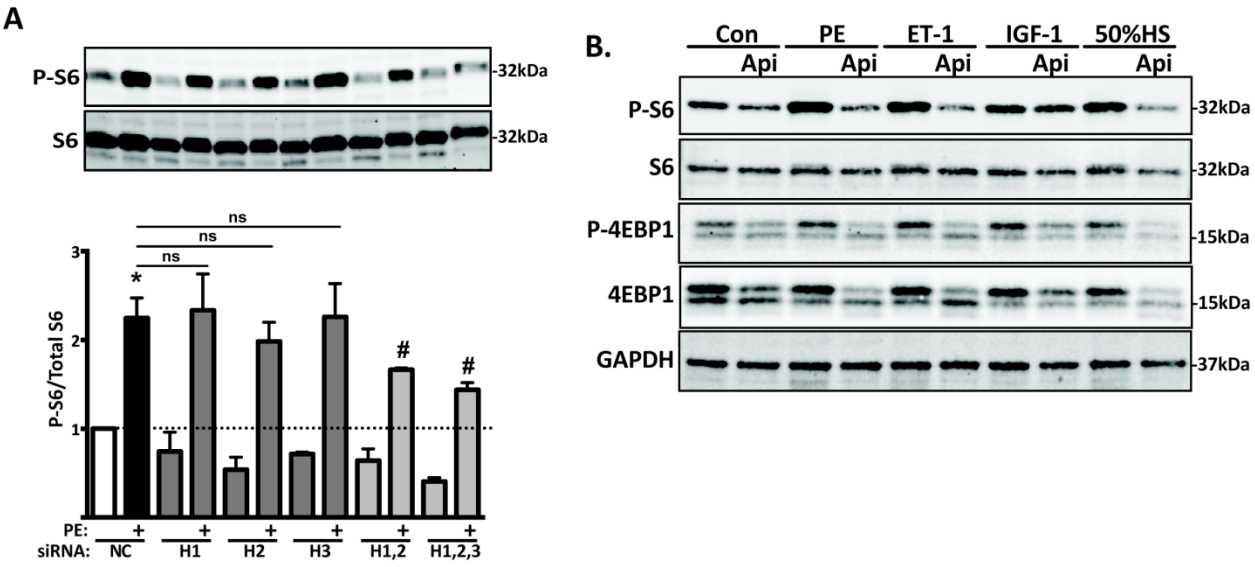

Figure 2.5. mTOR activity is reduced by inhibition of class I HDACs under several conditions. A. Western blot and quantification of S6 phosphorylation in NRVMs knockdown for HDAC1, HDAC2 and/or HDAC3. B. Western blot for mTOR activity in NRVMs treated with PE (50 μM), ET-1 (200 nM), IGF-1 (10 nM) and 50%HS with HDAC inhibitor apicidin. Values are mean ± SEM, analyzed by one-way ANOVA followed by Tukey's test (n=3-4). (*) $p<0.05$ vs control; (#) $p>0.05$ vs growth stimuli.

FIGURE 2.6

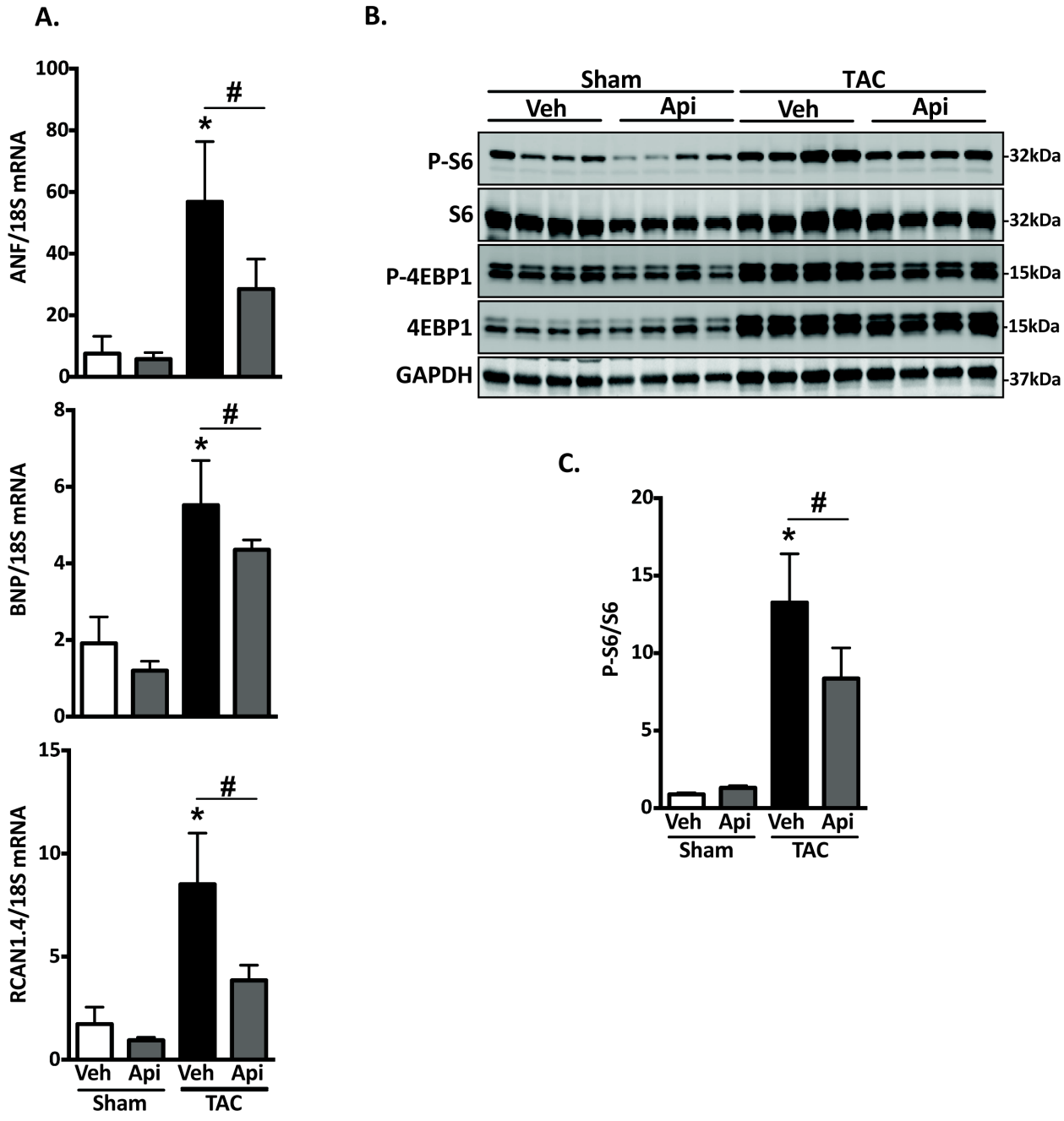

Figure 2.6. Inhibition of class I HDACs blunts TAC-induced mTOR activity. A. mRNA of hypertrophy markers ANF, BNP and RCAN1.4 from LV after 3 days of TAC and treatment with apicidin (3mg/kg). B. Western blot analysis of mTOR activity from LV after TAC for 3 days. C. Quantifications of western blot. Values are mean ± SEM, analyzed by one-way ANOVA followed by Tukey's test (n=6-8). (*) $p<0.05$ vs Sham; (#) $p>0.05$ vs TAC.

FIGURE 2.7

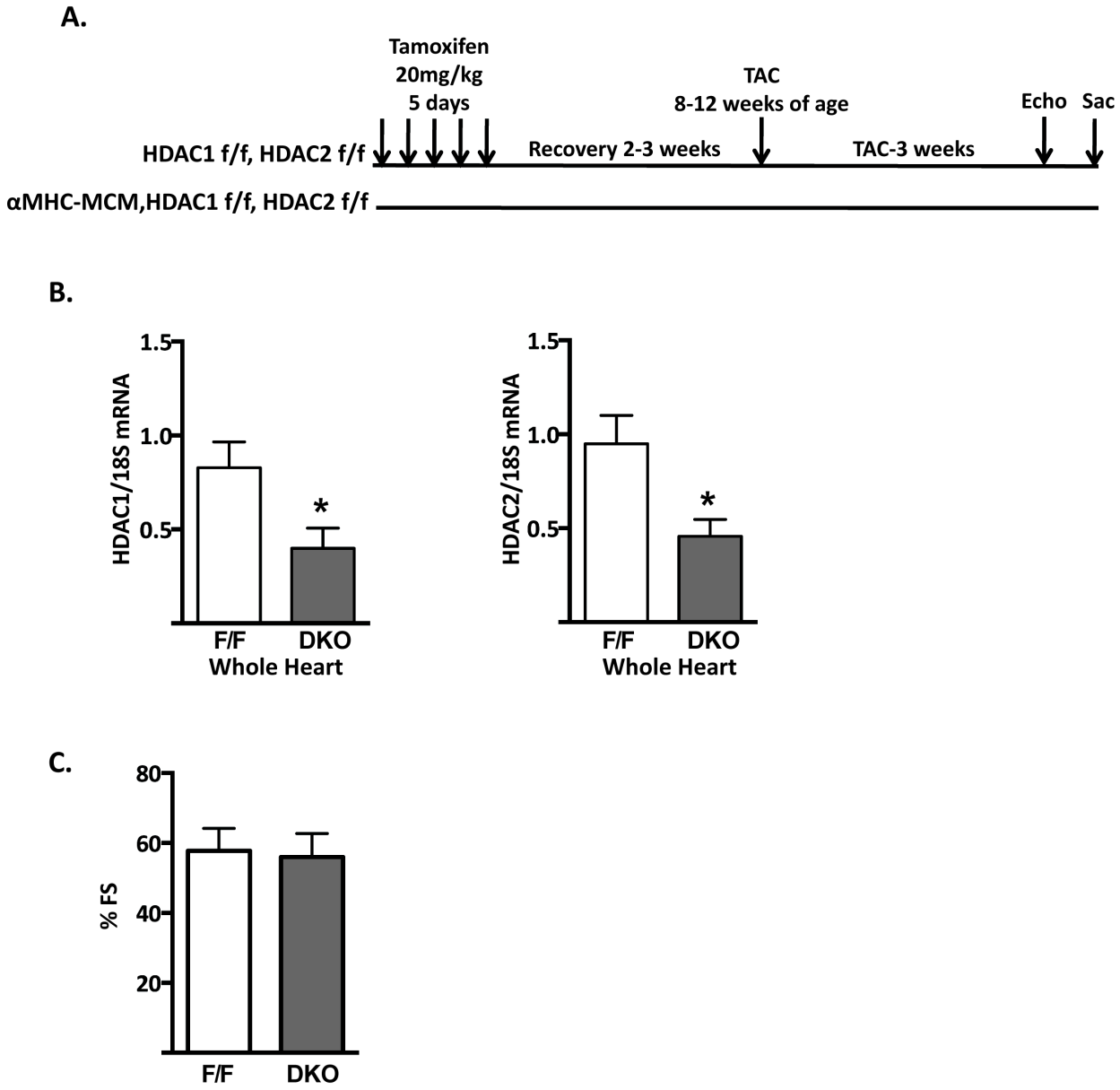

Figure 2.7. Experimental model of conditional, cardiomyocyte-specific HDAC1 and HDAC2 double knockout mice. A. DKO mice were treated with tamoxifen 5x20 mg/kg and let to recover for 2-3 weeks. TAC was performed for 3 weeks after. B. mRNA of HDAC1 and HDAC2 from whole hearts of control and DKO mice. C. Percentage fractional shortening of DKO mice 2-3 weeks after tamoxifen injections. Values are mean ± SEM, analyzed by one-way ANOVA followed by Tukey's test (n=6-8). (*) $p<0.05$ vs F/F.

FIGURE 2.8

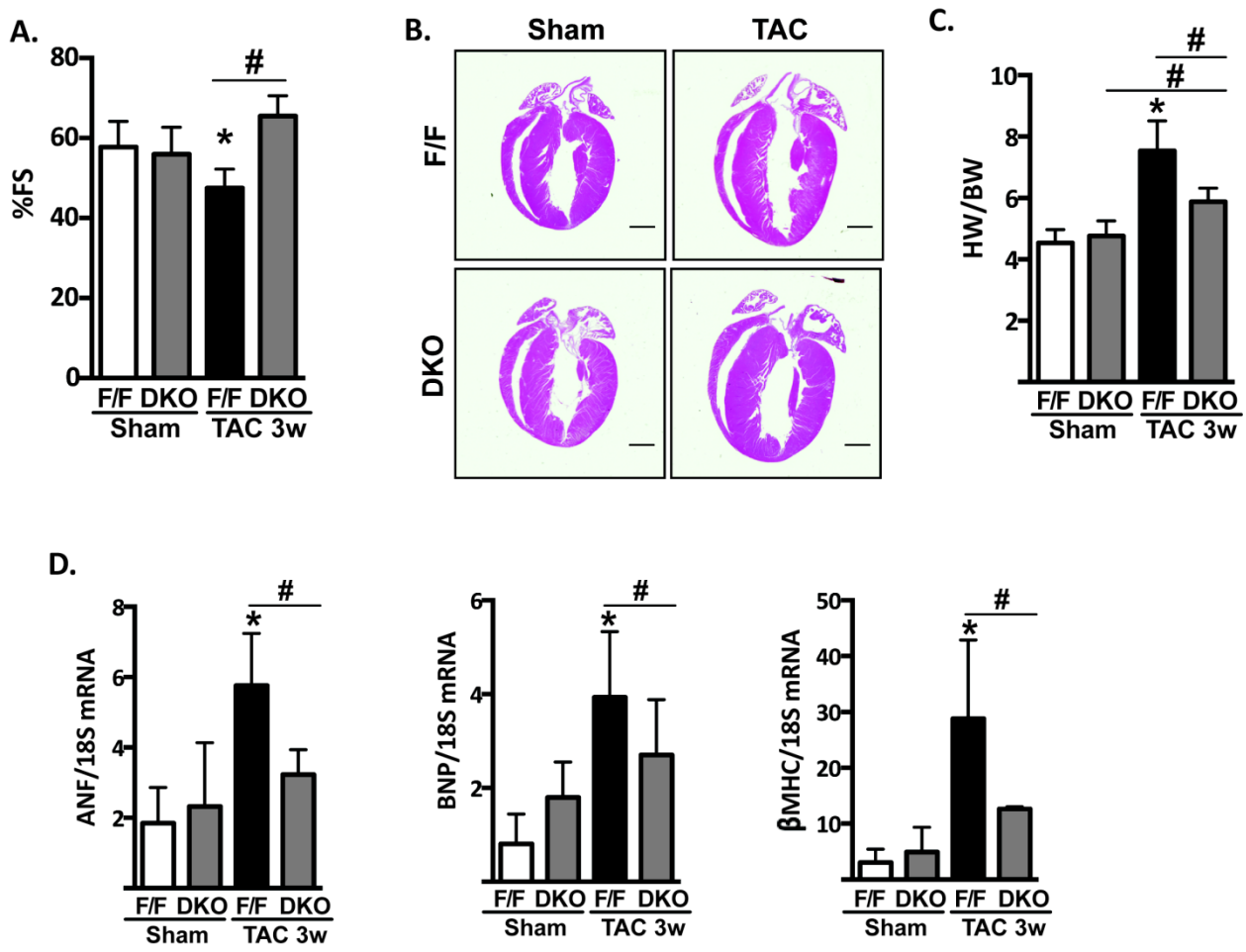

Figure 2.8. TAC-induced hypertrophy is repressed in HDAC1 and HDAC2 DKO mice. A. Percentage fractional shortening of control and DKO mice after 3 weeks of TAC. B. Hematoxylin and eosin staining of four-chamber heart sections after TAC. Scale bar: 2 mm. C. Heart weight/Body weight ratio. D. mRNA levels of hypertrophy markers ANF, BNP and βMHC. Values are mean ± SEM, analyzed by one-way ANOVA followed by Tukey's test (n=6-8). (*) $p<0.05$ vs Sham F/F; (#) $p>0.05$ vs TAC F/F.

FIGURE 2.9

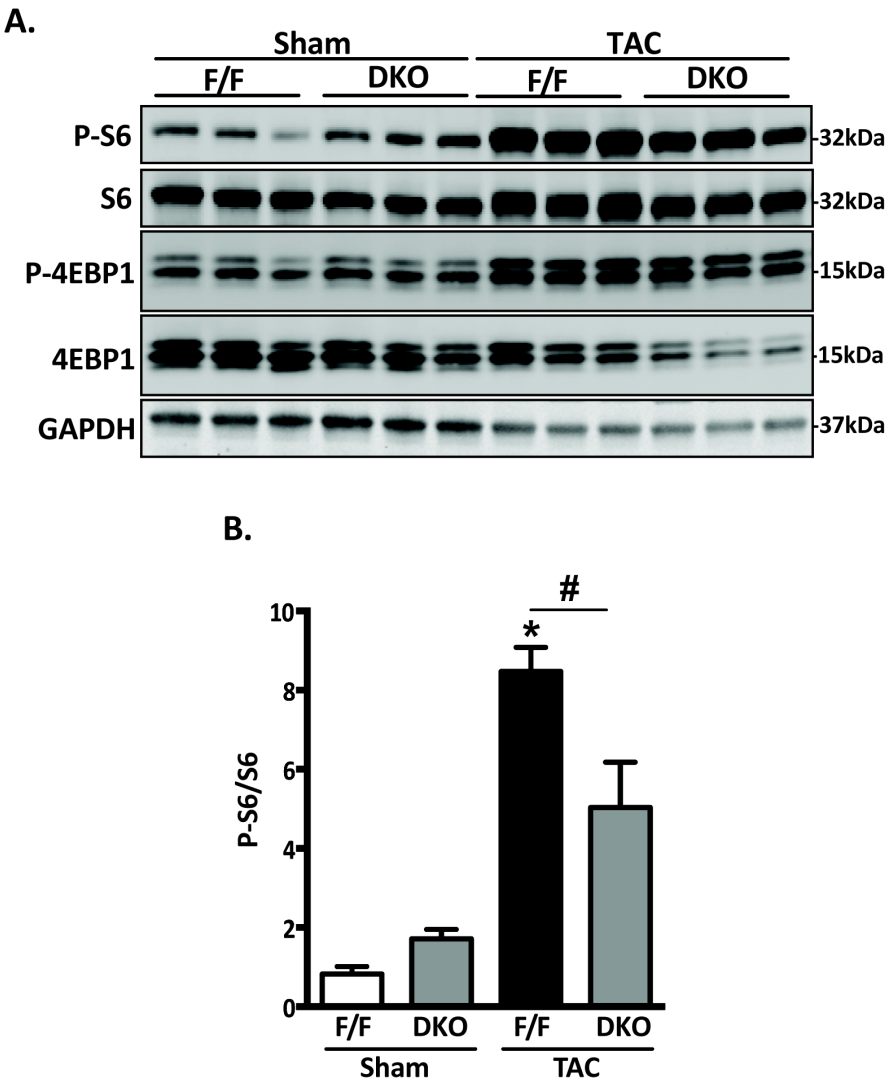

Figure 2.9. TAC-induced mTOR activity is reduced in HDAC1 and HDAC2 DKO mice. A. Western blot of S6 and 4EBP1 phosphorylation. B. Quantification of western blot. Values are mean ± SEM, analyzed by one-way ANOVA followed by Tukey's test (n=6-8). (*) $p<0.05$ vs Sham F/F; (#) $p>0.05$ vs TAC F/F.

FIGURE 2.10

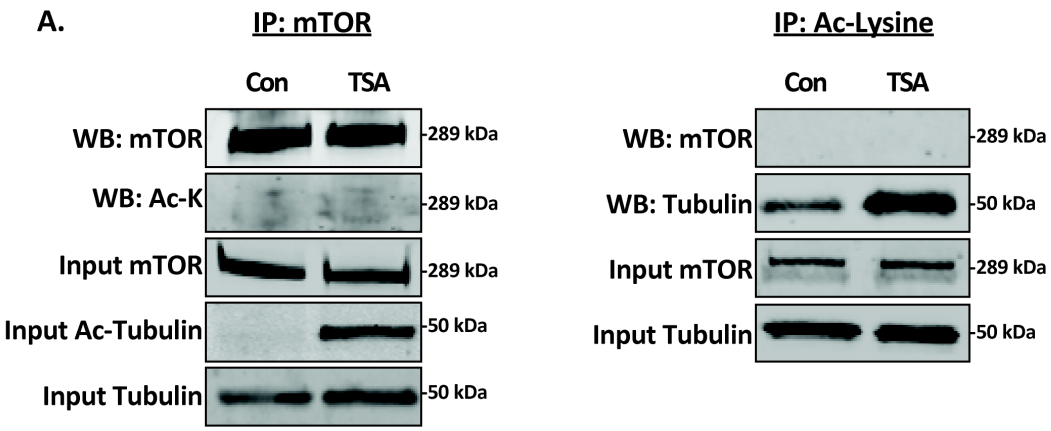

Figure 2.10. mTOR is not acetylated in NRVMs treated with HDAC inhibitors. A. Immunoprecipitation with anti-mTOR antibody and Western blot for acetylation and tubulin as positive control. NRVMs were treated with TSA (60 nM) for 24h. B. Reverse immunoprecipitation with anti-acetylated-lysine antibody and Western blot for mTOR.

FIGURE 2.11

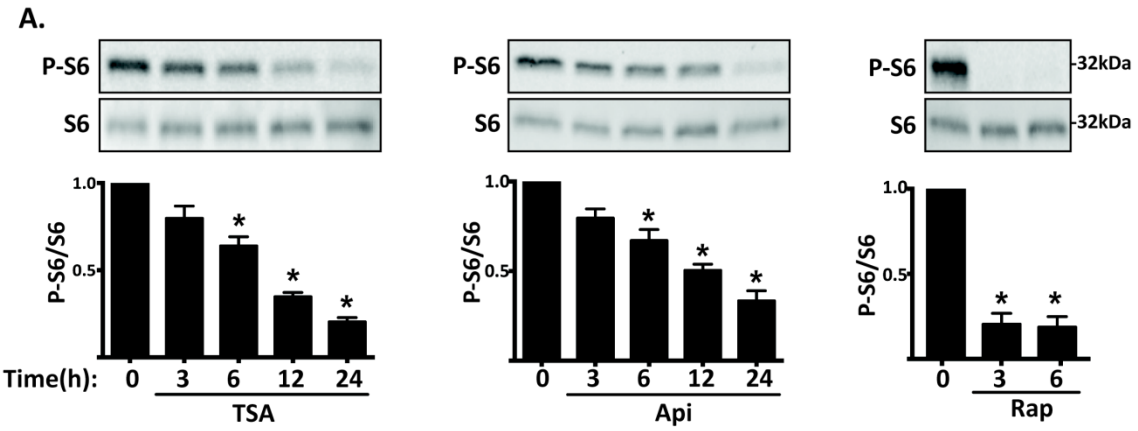

Figure 2.11. Significant reduction of S6 phosphorylation in NRVMs exposed to HDAC inhibitors is measured at 6 hours. A. Western blot of a time course of S6 phosphorylation in NRVMs treated for the noted times with apicidin (200 nM), TSA (60 nM) or rapamycin (100 nM). Values are mean ± SEM, analyzed by one-way ANOVA followed by Tukey's test (n=4). (*) $p<0.05$ vs control

FIGURE 2.12

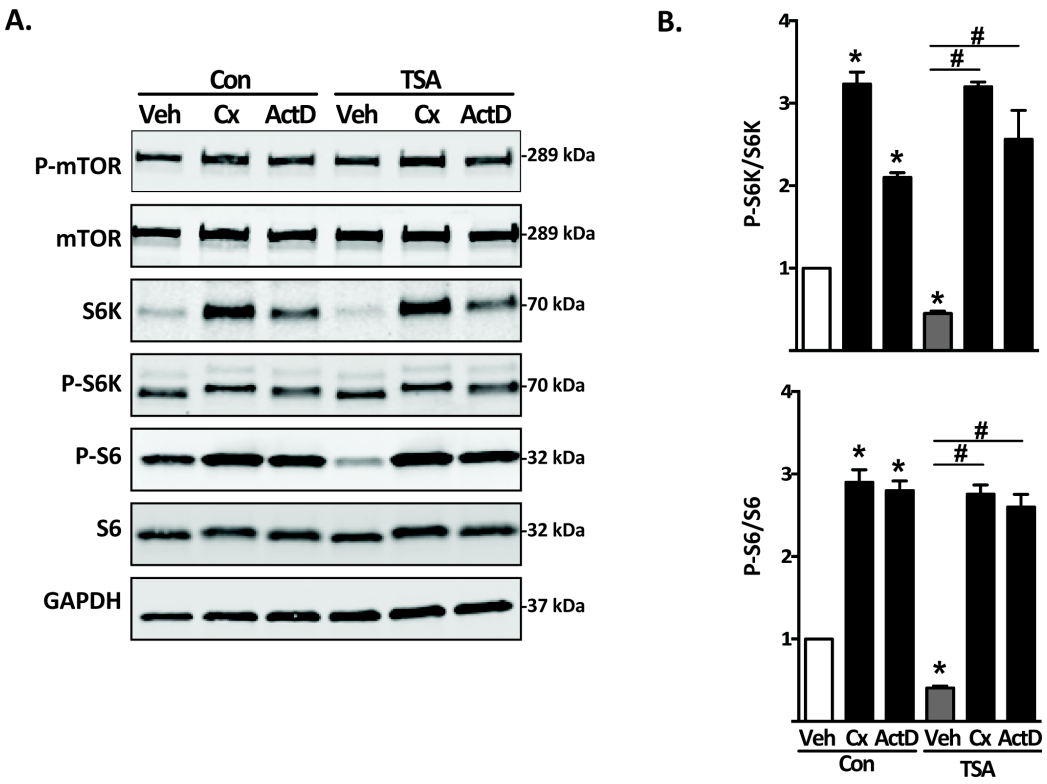

Figure 2.12. Inhibition of mTOR by HDAC inhibitors likely depends on transcription. A. Western blot and quantifications of mTOR targets in NRVMs exposed to cycloheximide (70 μM) or actinomycin D (8 μM) and TSA (60 nM) for 6h. B. Quantification of western blot. Values are mean ± SEM, analyzed by one-way ANOVA followed by Tukey's test (n=4). (*) $p<0.05$ vs control; (#) $p>0.05$ vs TSA.

FIGURE 2.13

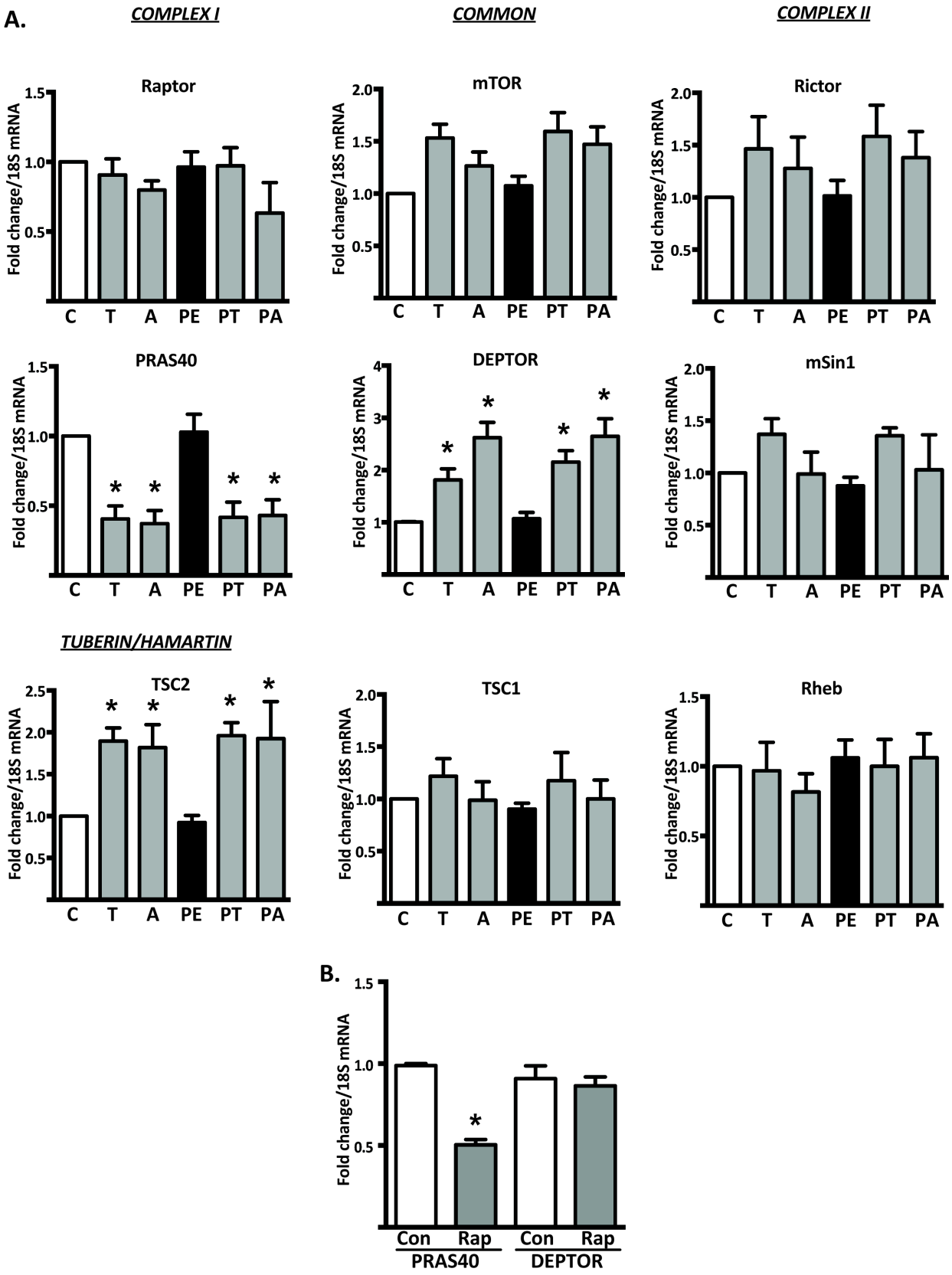
A.
COMPLEX I
COMMON
COMPLEX II
Raptor
mTOR
Rictor
PRAS40
DEPTOR
mSin1
TUBERIN/HAMARTIN
TSC2
TSC1
Rheb
Fold change/18S mRNA
C T A PE PT PA
B.
Con Rap Con Rap
PRAS40 DEPTOR

Figure 2.13. RT-PCR screen of mTOR Complex I, mTOR Complex II and TSC1/TSC2 complex. A. Real time PCR analysis of NRVMs treated with apicidin (200 nM), TSA (60 nM) and PE (50 μM) for 6h. B. mRNA levels of PRAS40 and DEPTOR in NRVMs treated with rapamycin (100 nM) for 6h. Values are mean ± SEM, analyzed by one-way ANOVA followed by Tukey's test (n=3-6). (*) $p<0.05$ vs control

FIGURE 2.14

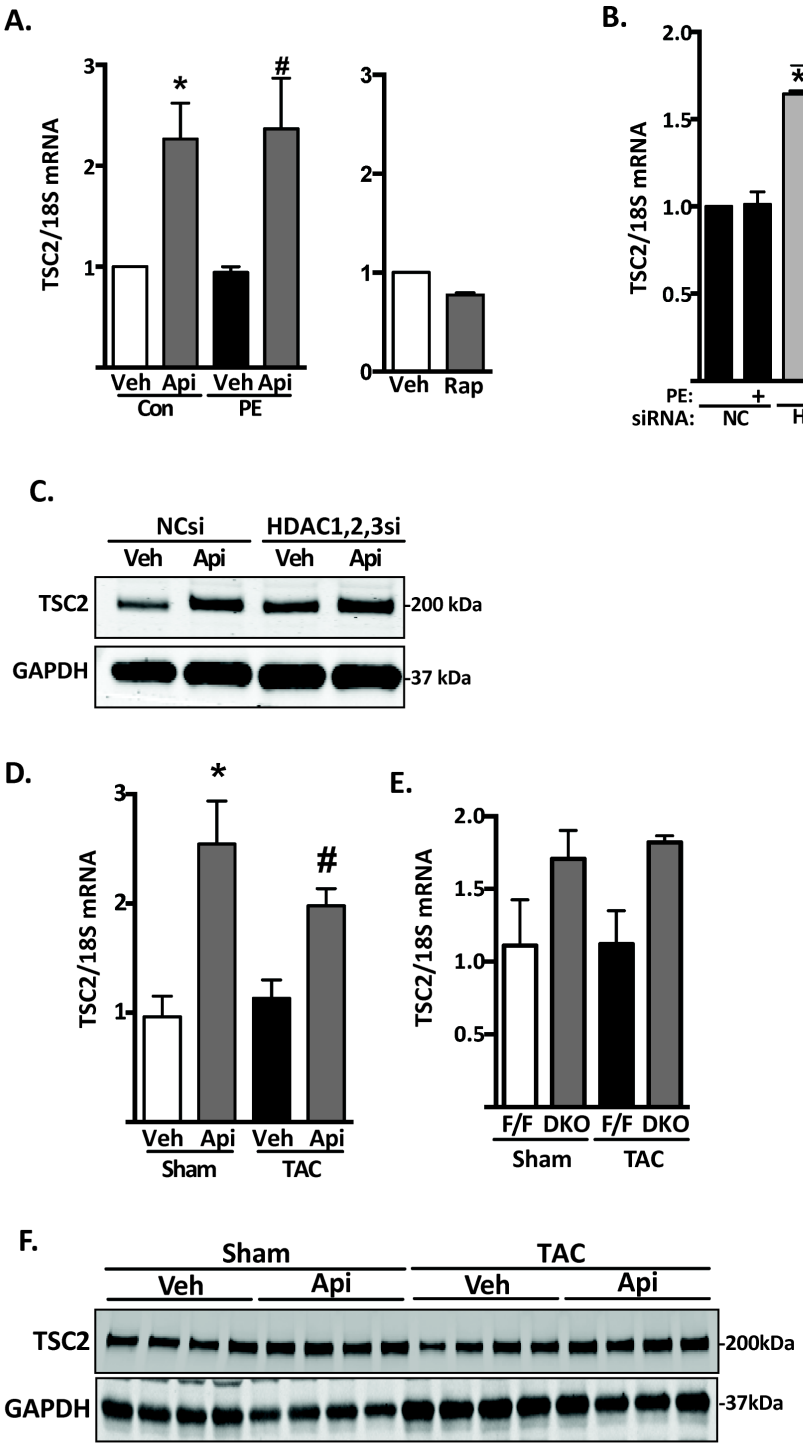
A.
TSC2/18S mRNA
Veh Api Veh Api
Con PE
Veh Rap
B.
TSC2/18S mRNA
ns
ns
PE:
siRNA: NC H1,2 H1,2,3
C.
NCsi HDAC1,2,3si
Veh Api Veh Api
TSC2
200 kDa
GAPDH
37 kDa
D.
TSC2/18S mRNA
Veh Api Veh Api
Sham TAC
E.
TSC2/18S mRNA
F/F DKO F/F DKO
Sham TAC
F.
Sham TAC
Veh Api Veh Api
TSC2
200kDa
GAPDH
37kDa

Figure 2.14. mRNA levels of TSC2 are induced with the inhibition of class I HDACs. A. RT-PCR analysis of NRVMs exposed to apicidin (200 nM) and rapamycin (100 nM) under basal and PE-induced (50 μM) growth for 6h. B. TSC2 mRNA levels in NRVMs knockdown for HDAC1, HDAC3 and/or HDAC3. C. Western blot of TSC2 in NRVMs knockdown for Class I HDACs and treated with apicidin (200 nM). D. mRNA levels of TSC2 in WT mice subjected to TAC for 3 days in the presence of Class I HDAC inhibitor apicidin (3 mg/kg). E. TSC2 mRNA levels in HDAC1 and HDAC2 DKO mice after 3 weeks of TAC. F. Western blot for TSC2 in WT mice subjected to TAC for 3 days in the presence of Class I HDAC inhibitor apicidin (3 mg/kg). Values are mean ± SEM, analyzed by one-way ANOVA followed by Tukey's test (n=4-8). (*) $p<0.05$ vs control; (#) $p>0.05$ vs PE or TAC.

FIGURE 2.15

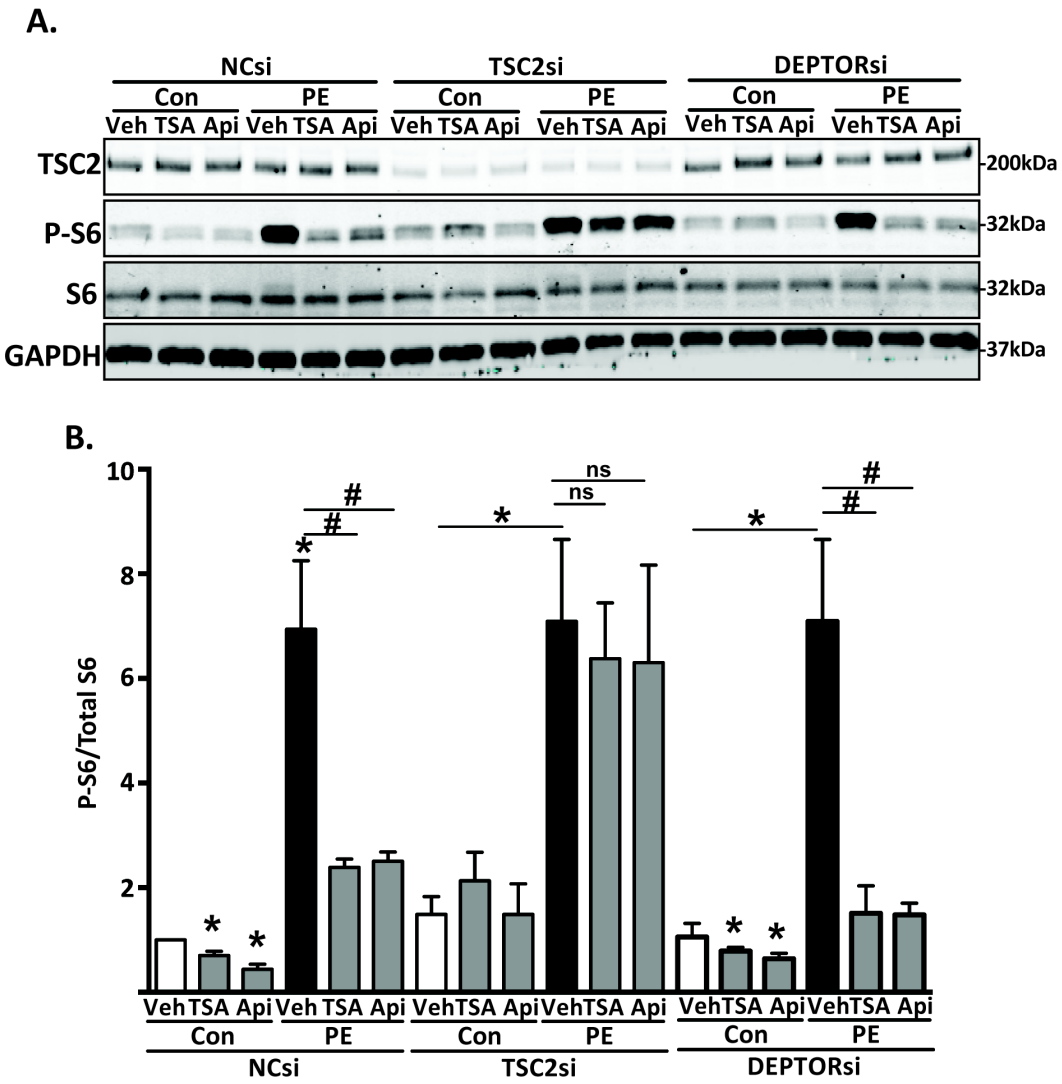

Figure 2.15. TSC2 is required for the HDAC-dependent inhibition of mTOR. A. Western blot analysis of mTOR activity in NRVMs knockdown for TSC2 and DEPTOR. Treatments with PE (50 μM) and HDAC inhibitors TSA (60 nM) and apicidin (200 nM) were performed for 24 h. B. Quantification of S6 ribosomal subunit phosphorylation. Values are mean ± SEM, analyzed by one-way ANOVA followed by Tukey's test (n=3-5). (*) $p<0.05$ vs control; (#) $p>0.05$ vs PE.

FIGURE 2.16

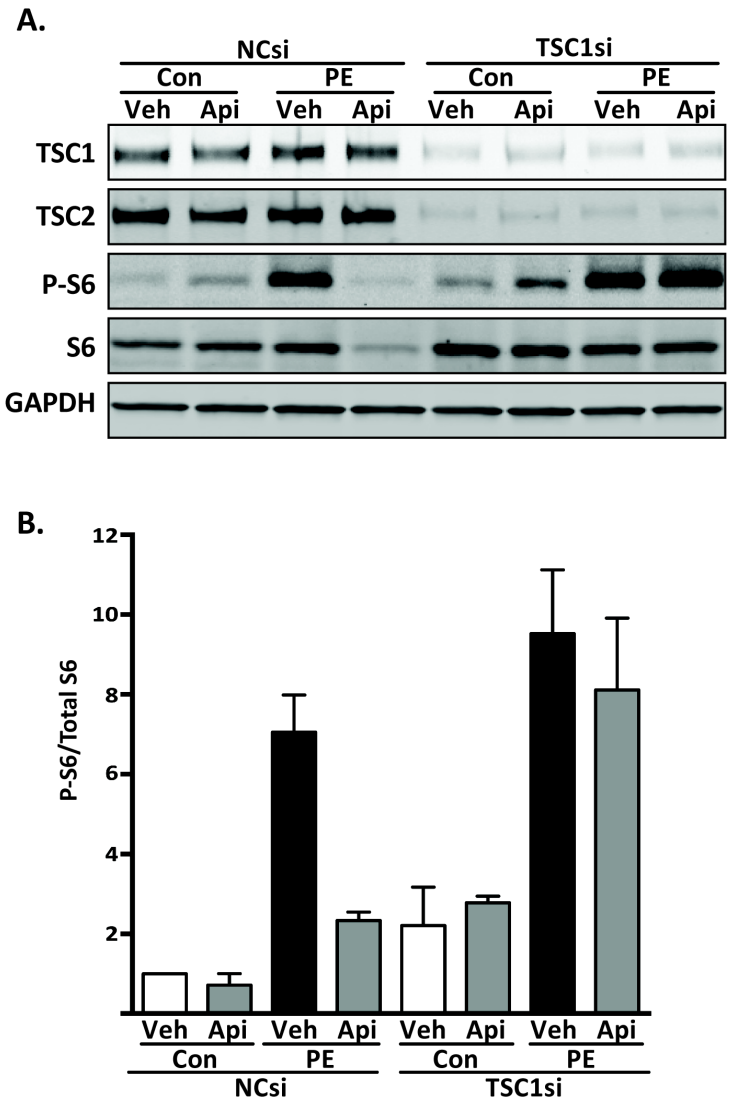

Figure 2.16. TSC1 is required for the HDAC-dependent inhibition of mTOR. A. Western blot analysis of mTOR activity in NRVMs knockdown for TSC1. Treatments with PE (50 μM) and apicidin (200 nM) were performed for 24 h. B. Quantification of S6 ribosomal subunit phosphorylation. Values are mean ± SEM, analyzed by one-way ANOVA followed by Tukey's test (n=2).

FIGURE 2.17

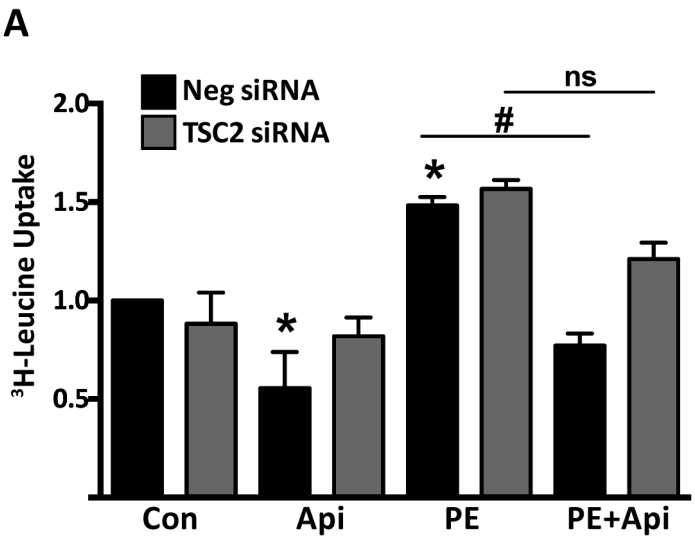

Figure 2.17. TSC2 siRNA partially recovers the PE-induced response reduced by apicidin. A. Leucine incorporation in NRVMs deficient of TSC2 under basal conditions and PE (50 μM) treatment for 48h. Apicidin (200 nM). Values are mean ± SEM, analyzed by one-way ANOVA followed by Tukey's test (n=4-5). (*) $p<0.05$ vs control; (#) $p>0.05$ vs PE.

FIGURE 2.18

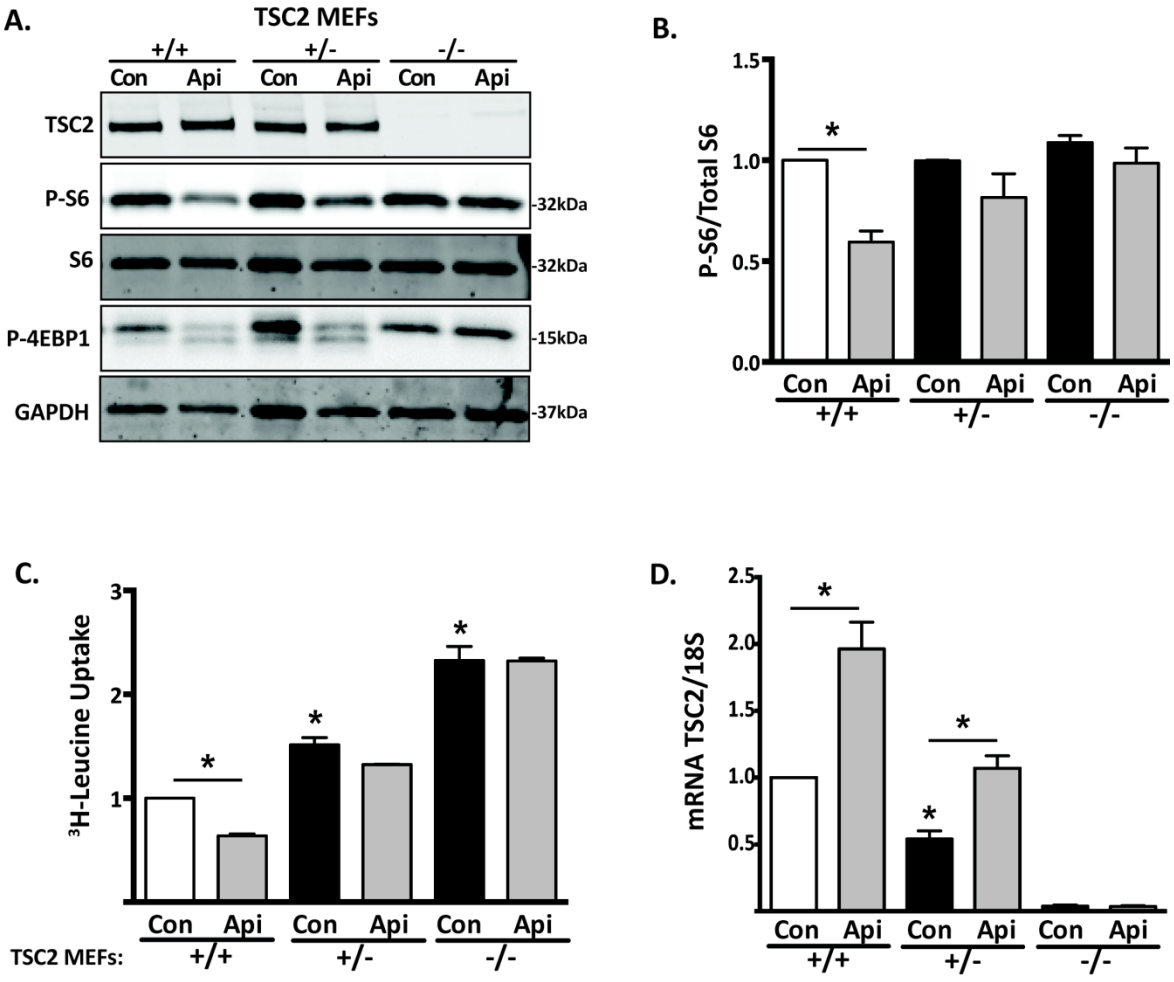

Figure 2.18. HDAC inhibitors induce TSC2 mRNA and reduce mTOR activity in MEFs. A. Western blot for mTOR activity from MEFs WT, heterozygous or knockout for TSC2 treated with apicidin (200 nM) for 6h. B. Quantification of S6 phosphorylation. C. Tritiated leucine incorporation in MEFs deficient in TSC2 treated with apicidin (200 nM) for 48h. D. mRNA levels of TSC2 in MEFs exposed to HDAC inhibitor apicidin (200 nM). Values are mean ± SEM, analyzed by one-way ANOVA followed by Tukey's test (n=4-6). (*) $p<0.05$.

FIGURE 2.19

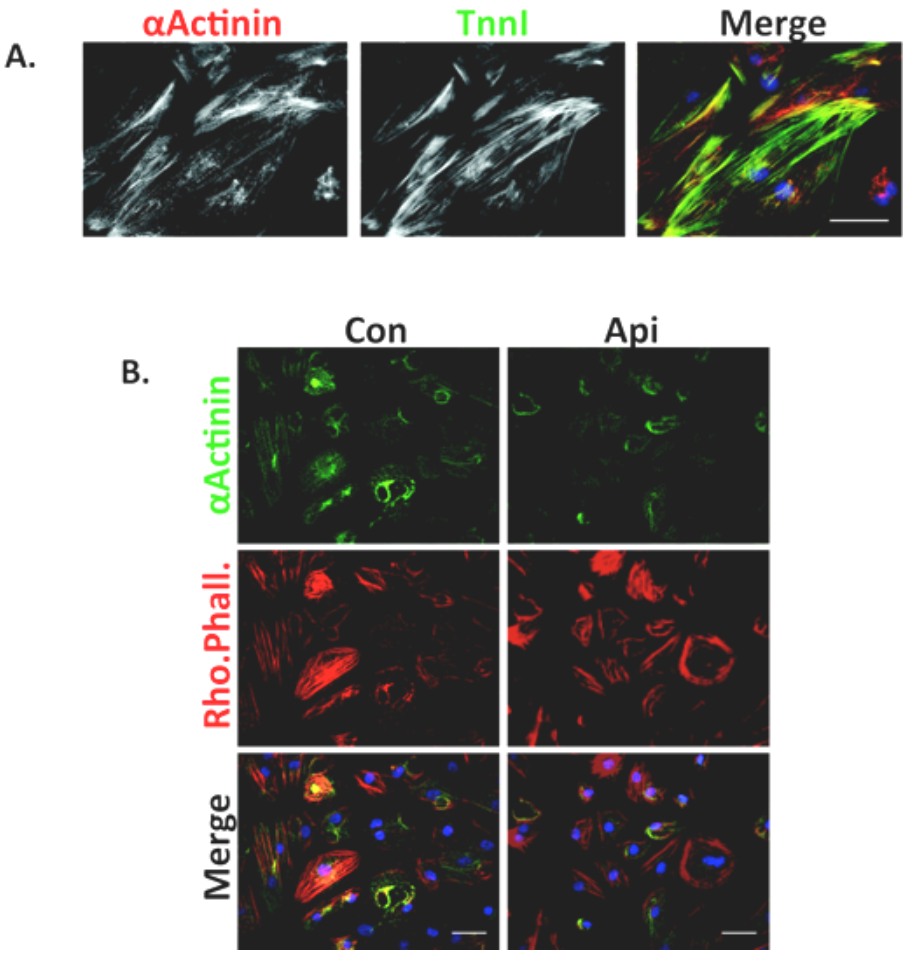

Figure 2.19. Human embryonic stem cell-derived cardiomyocytes (ESC-CM) express cardiac markers. A. Immunostaining of ESC-CM for α-actinin and troponin I. B. Representative images of ESC-CM exposed treated with apicidin (200 nM) for 24h and probed with α-actinin and rhodamine phalloidin. Scale bars: 40 μm.

FIGURE 2.20

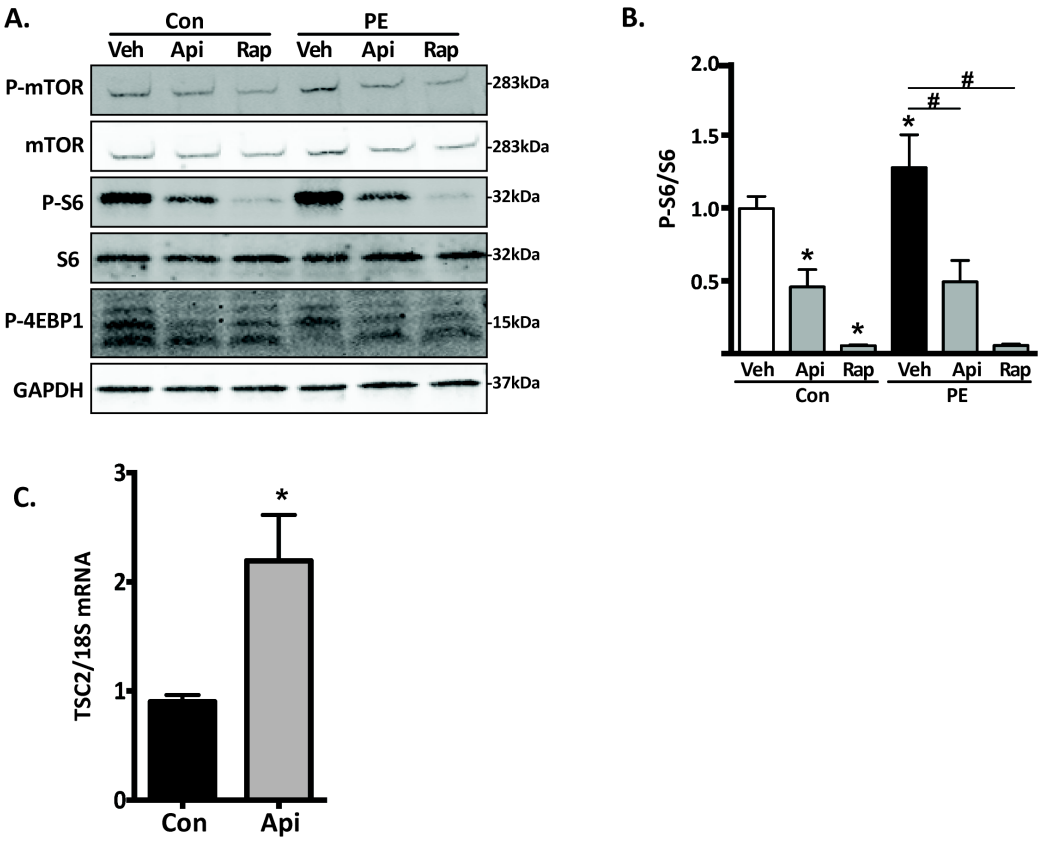

Figure 2.20. Inhibition of class I HDACs with apicidin induces TSC2 mRNA and reduces mTOR activity in ESC-CM. A. Western blot for mTOR downstream targets from ESC-CM treated with apicidin (200 nM) and rapamycin (100 nM) for 24h. B. Quantification of S6 phosphorylation. C. mRNA levels of TSC2 in ESC-CM exposed to apicidin (200 nM) for 24h. Values are mean ± SEM, analyzed by one-way ANOVA followed by Tukey's test (n=3). (*) $p<0.05$ vs control; (#) $p>0.05$ vs PE.

FIGURE 2.21

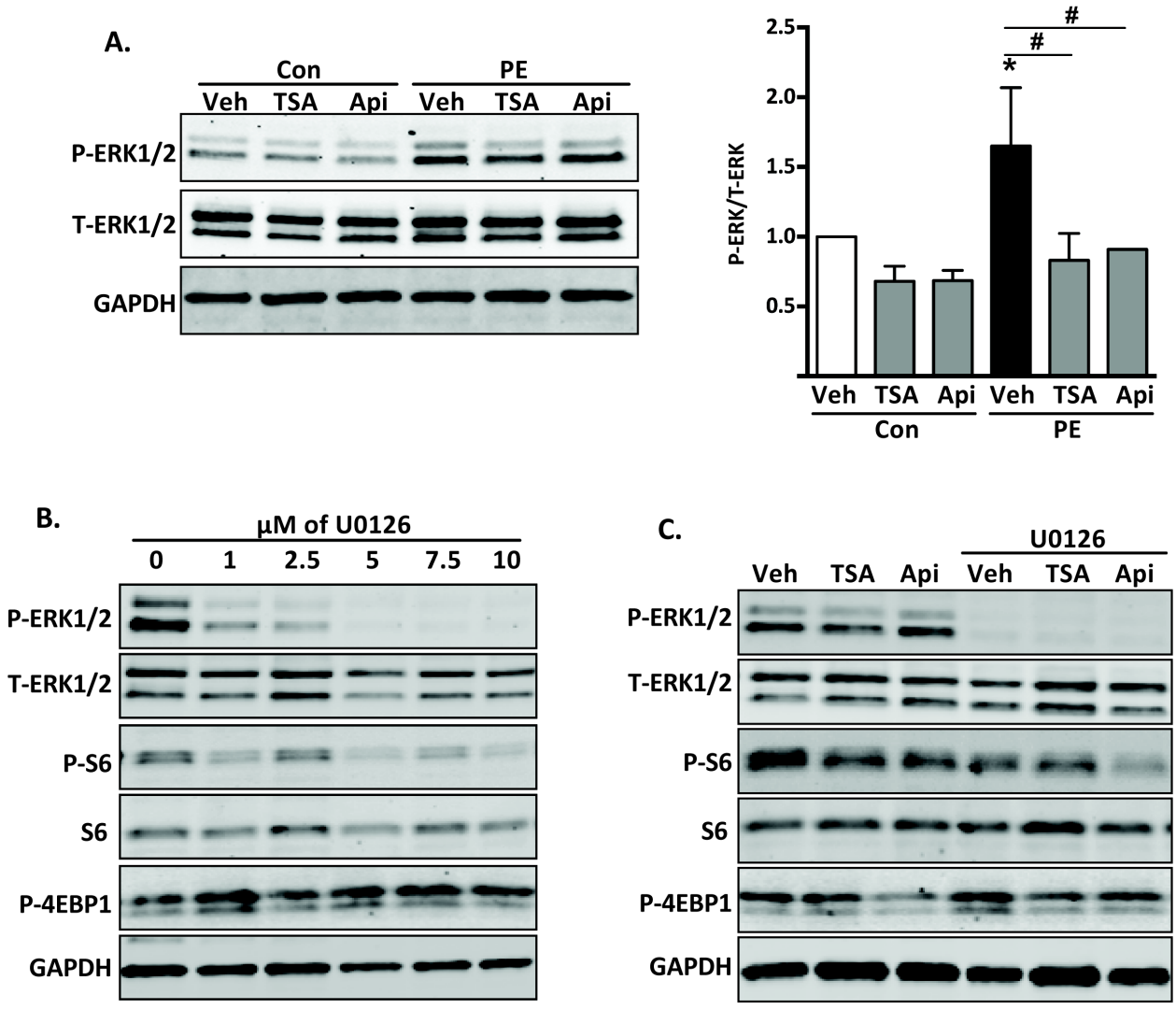

Figure 2.21. HDAC inhibitors reduce ERK1/2 phosphorylation. A. Western blot and quantification for ERK1/2 phosphorylation from NRVMs treated with TSA (60 nM) or apicidin (200 nM) for 24h. B. Western blot of NRVMs treated with U0126. C. Western blot of NRVMs co-treated with U0126 (5 µM), TSA (60 nM), or apicidin (200 nM) for 24h to compare ERK and mTOR activities. Values are mean ± SEM, analyzed by one-way ANOVA followed by Tukey's test (n=3). (*) $p<0.05$ vs control; (#) $p>0.05$ vs PE.

FIGURE 2.22

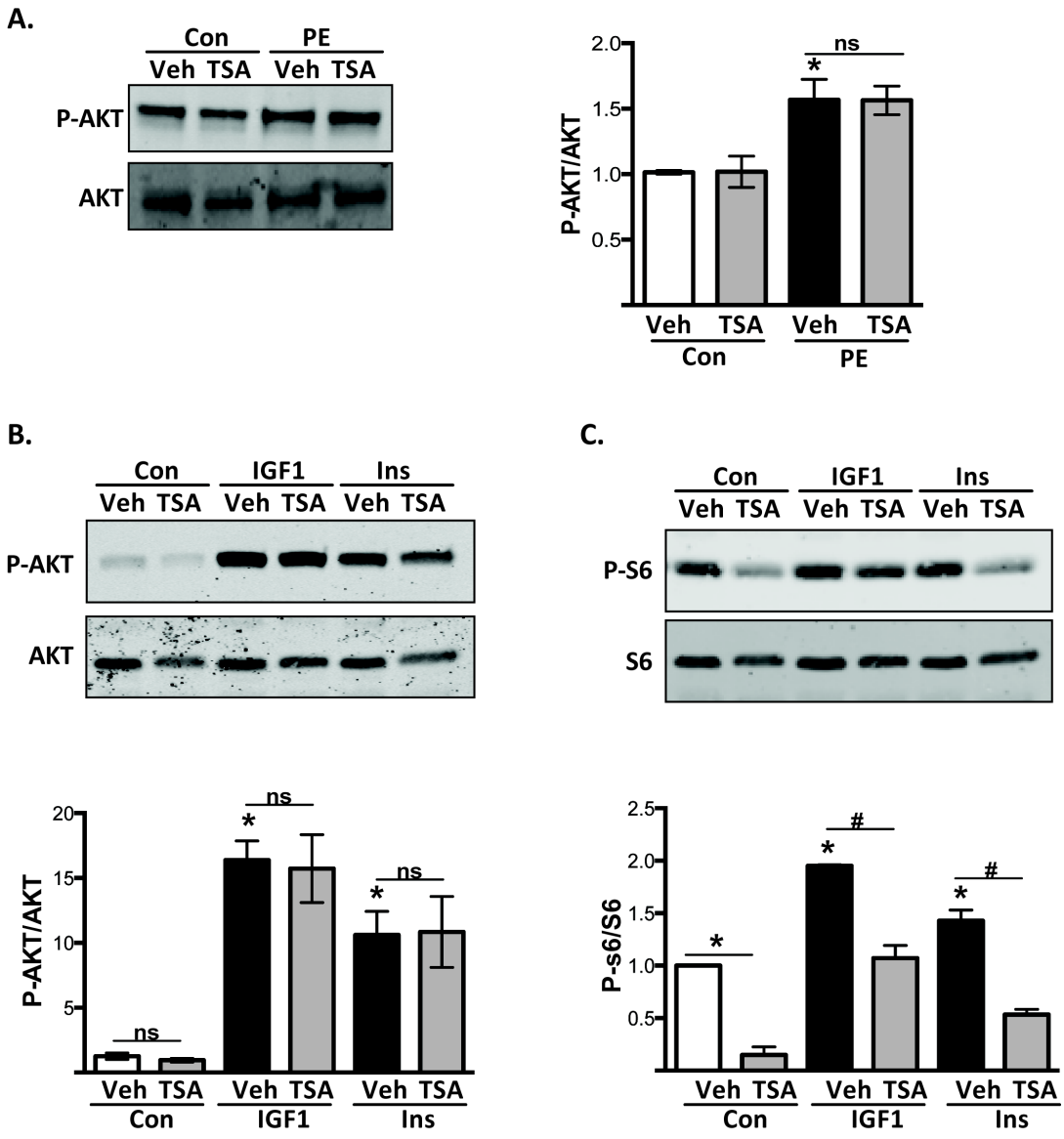

Figure 2.22. HDAC inhibitors do not alter Ser473-AKT phosphorylation in NRVMs. A. Western blot and quantification for AKT phosphorylation from NRVMs treated with TSA (60 nM) and PE (50 μM) for 6h. B.and C. Western blot of NRVMs pre-treated with TSA for 12 h and the challenged with IGF1 or insulin for 10 min. Values are mean ± SEM, analyzed by one-way ANOVA followed by Tukey's test (n=3). (*) $p<0.05$ vs control; (#) $p>0.05$ vs PE.

FIGURE 2.23

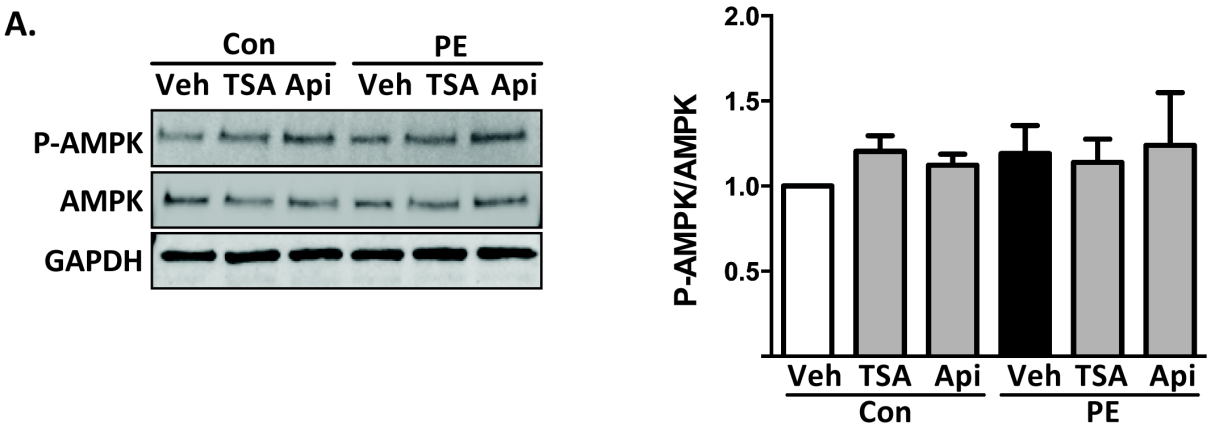

Figure 2.23. HDAC inhibitors do not alter AMPK phosphorylation in NRVMs. A. Western blot and quantification for AMPK phosphorylation from NRVMs treated with TSA (60 nM) or apicidin (200 nM) and PE (50 μM) for 6 h. Values are mean ± SEM, analyzed by one-way ANOVA followed by Tukey's test (n=3). $p<0.05$

MATERIALS AND METHODS

All studies conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011) and were approved by the Institutional Ethics Review Committees of the University of Texas Southwestern Medical Center.

Neonatal rat ventricular myocytes (NRVMs) and siRNA knockdowns

Cardiomyocytes were isolated from the left ventricle of 1-2-day-old Sprague-Dawley rats. Lysates were digested with collagenase, and the resulting cell suspension was pre-plated to remove fibroblasts. Myocytes were then plated at a density of 1250 cells/mm in DMEM 4.5 g/L glucose medium containing 10% FBS and 100 μmol/L bromodeoxyuridine. For siRNA knockdown experiments, NRVMs were transfected 36 hours after plating with siRNA constructs (Sigma) using Lipofectamine RNAiMax (Invitrogen) in Optimem (6h). Experiments were launched 24h after knockdown. For hypertrophy studies, cells (48h after plating) were exposed to serum-free DMEM containing: phenylephrine (PE) [50 mM], endothelin-1 (ET-1) [200 nM], insulin growth factor-1 (IGF1) [10 nM], or 50% hypo-osmotic solution. HDAC inhibitors were employed as follows: apicidin (Api) at 0.2 mM; trichostatin A at 60 nM [20].

Western blot analysis

Whole cell protein lysates were obtained from NRVMs or from tissue with M-PER mammalian extraction buffer (Thermo Scientific) containing protease and phosphatase inhibitors (Roche). Tissue protein extracts were passed through glass wool to remove DNA. Cell lysates were separated by SDS/PAGE, transferred to nitrocellulose membrane, and subjected to immunoblot analysis. Antibodies used were: mouse anti-S6 ribosomal protein, rabbit anti-

phospho-S6 ribosomal protein (Ser235/236), rabbit anti-4EBP1, rabbit anti-phospho-4EBP1 (Thr37/46), rabbit anti-S6Kinase, mouse anti-phospho-S6Kinase (Thr389), rabbit anti-TSC2, mouse anti-HDAC1, rabbit anti-HDAC3, rabbit anti-acetyl lysine, mouse anti-mTOR, and rabbit anti-phospho-mTOR (Ser2448) all from Cell Signaling; mouse monoclonal anti-GAPDH (Fitgerald Industries Int.); mouse anti-α-tubulin (Sigma); rabbit anit-HDAC2 (Invitrogen). Blots were scanned, and bands were quantified using an Odyssey Licor (version 3.0) imaging system.

Real-time PCR

Total RNA was harvested from NRVMs or left ventricles using TRIzol (Invitrogen) according to the manufacturer's protocol. cDNA was prepared from RNA using a high capacity cDNA reverse transcription kit (Applied Biosystems). Real-time PCR was performed using SYBR green on an ABI 7000 Prism Sequence Detection System (Applied Biosystems). To confirm amplification specificity, PCR products were subjected to melting curve analysis. Negative controls containing water instead of cDNA were run concomitantly. Data for each transcript were normalized to reactions performed with 18S rRNA primers, and fold change was determined using the comparative threshold method (**Supplemental Table I**).

^{3}H-Leucine incorporation

NRVMs were cultured with ^{3}H-leucine (1 mCi/mL, Perkin Elmer) at the time of treatment. NRVMs were washed (3x) with ice-cold PBS and incubated with 10% trichloroacetic acid (30 min, 4^{o}C) followed by three washes with ice-cold 95% ethanol. Samples were incubated in 0.5N NaOH (6h, 37^{o}C) with gentle agitation, then neutralized with 0.5N HCl, and subjected to scintillation counting (Beckman).

HDAC activity

Total cellular HDAC activity was measured with the Fluor-de-Lys HDAC Fluorometric Cellular activity assay kit (Enzo Life Sciences, BML-AK503-0001). In brief, NRVMs plated in 96 wells were treated with HDAC inhibitor or HDAC RNAi and subsequently incubated with 200 mM of Fluor-de-lys substrate (4h). The cell-permeable acetylated substrate fluoresces when deacetylated. Fluor-de-lys lysis buffer, trichostatin A, and developer solution were added 15 min before the end of the experiment and fluorescence was measured in a microplate reader (Ex 360 nm, Em 460 nm).

In vivo cardiac hypertrophy and histology

Male C57/BL6 mice (8–10 weeks old) were subjected to thoracic aortic constriction (TAC) for 3 weeks as previously described [2]. Control animals underwent sham operations. HDAC1 and HDAC2 conditional cardiomyocyte-specific double knockout (DKO) mice were generated by crossing HDAC1 and HDAC2 floxed mice [10] with C57BL6 Mer-Cre-Mer mice. To drive Cre expression, 5 daily doses of tamoxifen (20 mg/kg) were delivered IP at 4-6 weeks of age. For HDAC1/2 DKO mice, cardiac function was determined after tamoxifen exposure, as well as pre- and post-surgery. For histology, hearts were fixed (4% PFA, RT with agitation) followed by routine paraffin processing. Hematoxylin and eosin and Masson's trichrome staining were performed according to routine procedures.

Echocardiography

Echocardiograms were performed on conscious, gently restrained mice using a Vevo 2100 system and an 18 MHz linear probe. A short axis view of the left ventricle at the level of the papillary muscles was obtained, and M-mode recordings were performed from this view.

Measurements of interventricular septum thickness (IVS), left ventricular internal diameter (LVID), and left ventricular posterior wall thickness (LVPW) were obtained from 2D parasternal short axis views in diastole. Left ventricular mass was calculated by the cubed method as 1.05 x $((IVS + LVID + LVPW)^3 - LVID^3)$ (mg) [21], which we have verified independently [22]. Left ventricular internal diameter at end-diastole (LVIDd) and end-systole (LVIDs) were measured from M-mode recordings. Fractional shortening was calculated as (LVIDd - LVIDs) / LVIDd (%).

Embryonic stem cell differentiation to cardiomyocytes

Embryonic stem cells (H9 cell line, from "WiCell") were maintained on matrigel in mTeSR culturing medium. Cardiac differentiation was performed according to (Lian et al, 2013), with modifications as in (Boheler et al., 2014). When cells reached 85-90% confluency, media was switched to RPMI 1640 (Life Technologies, 11875-093) with B27 Supplement, minus insulin (Life Technologies, A1895601) (day 0 to 8), supplemented with small molecule inhibitors: 6 μM CHIR99021 (Selleckchem, S2924) on day 0 for 48 hours and 5 μM IWR-1 (Sigma) on day 4 for 48 hours. Medium was changed to RPMI 1640 with B27 Supplement, serum free (Life Technologies, 17504-044) on day 8. Cells were metabolically selected for 10 days with media without glucose, replated and used for experiment after day 30.

Immunofluorescence staining

For immunofluorescence staining, cells were fixed with 4% formaldehyde, permeabilized with 0.1% triton X-100 in PBS, blocked with 3% FBS, incubated overnight with primary antibody (α-actinin (mouse, Sigma, 7811) and Troponin-I, (rabbit, Santa Cruz Biotechnology, sc-15368) at $4^{O}C$ diluted 1:400 in primary antibody, washed with PBS, followed by incubation with appropriate secondary antibody and counter-stained with DAPI.

Statistical methods and data handling

Data are presented as mean ± SEM. The unpaired Student's t test was used for comparison between two independent groups, and ANOVA followed by Tukey post-hoc test for pair wise comparisons. For all statistical comparisons, $p < 0.05$ was considered significant. All statistical analyses were performed using GraphPad Prism (version 6.01) software.

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CHAPTER 3: DISCUSSION

Pharmacological suppression of HDACs is a recently identified and highly promising therapeutic strategy to target heart disease. Because of their potential clinical importance, we set out to determine the mechanisms through which HDAC inhibitors blunt pathological cardiac hypertrophy. In the present study, we employed *in vitro* and *in vivo* models of pathological cardiac growth, finding that class I HDACs act together to facilitate cardiac hypertrophy induced by a variety of growth factors. We found that HDAC inhibitors suppress the activity of mTOR, a generalized regulator of cell growth. We further defined mechanisms of HDAC-dependent control of mTOR, which we find involves governance of TSC2 expression, a negative regulator of mTOR. All together, these data define a novel mechanism for therapeutic manipulation of pathological cardiac hypertrophy involving class I HDACs, TSC2, and mTOR. Furthermore, the data reported here are the first to delineate this novel mode of action that could be exploited therapeutically to modulate mTOR activity in other disease contexts.

Class I HDACs regulate physiological and pathological growth

Inhibitors of class I and class II HDACs have been shown to be protective in the context of cardiac hypertrophy and ischemia/reperfusion injury. The idea of using HDAC inhibitors clinically for the treatment of cardiovascular disease has been proposed for many years. Building upon existing scientific research, we have developed a deeper understanding of how HDACs promote the hypertrophic response. Part of this effort focuses on the idea of using isoform-specific inhibitors for different classes of HDACs to reduce side effects and limit undesired outcomes. Therefore, understanding the role of HDACs in cardiac physiology and pathology, and determining the specific HDAC isoforms involved in pathogenesis has been of considerable interest.

Recent literature suggests that class I HDACs are responsible for pathological hypertrophy. This has been tested by using inhibitors specific for class I HDACs [1]. Genetic models of HDAC1 and HDAC2 knockout demonstrate that these isoforms can compensate for one another in their ability to promote cardiomyocyte growth. However, double knockout of HDAC1 and HDAC2 is lethal at P14 and characterization of these enzymes in an adult heart has been lacking [2]. An additional model in which HDAC2 is silenced specifically in the heart manifests inhibition of hypertrophy [3]. Therefore, additional characterization of the role of class I HDACs and their potential mechanisms is warranted.

In this study, we reveal that genetic and pharmacological inhibition of class I HDACs in NRVMs can repress hypertrophic growth induced by phenylephrine and by other stimuli, including physiological hypertrophy induced by IGF1. While previous studies have focused on pathological remodeling, our results *in vitro* indicate that HDACs promote cardiac growth in both normal and disease states. Whether HDAC inhibitors can repress physiological growth during development, exercise or pregnancy requires further examination. These results further imply and reinforce the idea that inhibiting protein synthesis alone, or potentially cell size, in pathological remodeling, may be sufficient to arrest the development of other phenotypic markers of disease, such as detrimental metabolic changes, the development of fibrosis, and the transition to heart failure.

Furthermore, we demonstrate that deficiency of a single class I HDAC isoform is not sufficient to reduce total HDAC activity or reduce hypertrophy. Knockdown of HDAC1 and HDAC2 together does have a substantial effect on total HDAC activity in the heart, further supporting a model in which HDAC1 and HDAC2 can compensate for each other. Knockdown of HDAC3 indicates that this isoform also contributes to type I HDAC activity in the heart. HDAC3 knockout mice quickly develop a heart failure phenotype and have increased expression of genes involved in fatty acid oxidation [4]. This phenotype is not observed in HDAC1 and HDAC2 double knockout mice, suggesting that HDAC3 has biological functions

independent of HDAC1 and HDAC2. It may be possible that HDAC1 and HDAC2, which seem to have 25% of total HDAC activity in NRVMs, play redundant roles in cell size regulation and that additional inhibition of HDAC3 has a complementary effect on cardiomyocyte growth by promoting fatty acid oxidation.

We bypassed the lethally caused by HDAC1 and HDAC2 deletion during development with a conditional loss-of-function model. Surprisingly, after tamoxifen administration to trigger recombination, HDAC1 and HDAC2 DKO mice do not manifest changes in cardiac function for 3 weeks. The adult myocardium is not actively growing, but maintains a stable heart weight to body weight ratio unless there is a change in cardiac demand. This may explain why HDAC1 and HDAC2 deletion in the adult heart does not cause a change in cardiac morphology. It is also possible that the *in vivo* deletion of HDAC1 and HDAC2 is only partial. Still, after TAC, these mice show a reduction in HW/BW ratio and fetal gene markers compared to control. Importantly, they also have improved cardiac function, supporting our *in vitro* results.

Both pan-HDAC inhibitors and apicidin can effectively inhibit phenylephrine-induced growth; however, apicidin shows greater inhibitory capacity. Class IIa HDACs, which act to suppress cardiac growth through their interaction with MEF2, are also considered targets of pan-HDAC inhibitors. This may be why TSA is less effective than apicidin because of their potential to release type II HDAC inhibition of MEF2-dependent hypertrophic growth. However, it is thought that class IIa HDACs may actually be relatively insensitive to pan-HDAC inhibitors and that their catalytic activity is not required to suppress hypertrophy [5, 6]. It is also possible that the differences observed between treatments with TSA versus apicidin are due to HDAC6, HDAC10 and HDAC11, which are sensitive to pan-HDAC inhibitors, but whose role in cardiac hypertrophy is not well defined.

Blunting hypertrophic mTOR activation by HDAC inhibitors

Studies using rapamycin to suppress pathological cardiac growth demonstrate that mTORC1 is a primary mediator of hypertrophy. However, studies using transgenic manipulation of mTOR kinase in the heart are difficult to interpret, because deletion of mTOR affects both mTOR complex I and complex II.

Mice with cardiomyocyte-specific over-expression of mTOR manifest no change in cardiac function and cardiomyocyte area relative to wild-type littermates. After TAC, mTOR transgenics have improved cardiac function and reduced hypertrophy compared to WT. This would appear contrary to our hypothesis that HDAC inhibitors act by blunting mTOR. In the mTOR transgenics, S6 and 4EBP1 are hyperphosphorylated, as is AKT, despite reduced hypertrophy [7]. It is possible that the over-expression of mTOR kinase results in the activation of both mTORC1 and mTORC2. AKT is a target of mTORC2 suggesting that the interplay of both complexes plays a role in hypertrophy.

Tamoxifen-induced deletion of mTOR in cardiomyocytes at 2 weeks of age leads to a rapid transition to heart failure and death [8]. Similarly, cardiomyocyte-specific deletion of raptor, needed for mTORC1 activity, driven by αMHC-Cre, results in increased fetal gene expression and increased cardiac mass at 3 weeks of age which progresses rapidly to heart failure [9]. In contrast to studies with rapamycin, *in vivo* mTOR deletion illustrates the importance of mTOR baseline activity in the heart, and that the timing and context in which mTOR is active or inhibited is crucial for proper physiological functions.

In this study, we use the phosphorylation of mTORC1 downstream targets S6 and 4EBP1 as markers of mTOR activity; S6 being a more robust marker compared to 4EBP1. Interestingly, the hearts of mice with cardiomyocyte-specific double knockout for S6K1 and S6K2 still hypertrophy in response to a swim exercise regime or TAC stress [10]. This S6K-independent growth response has only been reported in the heart since S6K is required for the growth response in other cell types [11]. It may be that other mechanisms compensate for the

lack of S6 kinases and that an orchestrated response downstream of mTOR and other upstream kinases lead to the efficient blockage of growth. On the other hand, deletion of 4EBP1 rescues the lethal phenotype observed in mTOR knockout mice [8].

Broad-spectrum HDAC inhibitors blunt mTOR activity in some cancer models [12]; however, the underlying mechanisms have not been defined. Here, we propose that inhibition of class I HDACs specifically can decrease mTOR activity during cardiac growth. This was observed under baseline conditions and in physiological and pathological growth of NRVMs. In the context of pathological growth, inhibition of mTOR was observed *in vivo* after TAC with both treatment with apicidin or the genetic ablation of HDAC1 and HDAC2. We did not directly study the effect of HDAC inhibitors on mTORC2 activity, but in NRVMs we know that HDAC inhibitors do not alter gene expression of mTORC2. HDAC inhibitors provide a way to partially inhibit mTORC1 specifically without reaching the detrimental effects of complete inhibition of complex I or the effects of perturbing mTORC2.

IGF1 activates the IGF-1 receptor that results in downstream activation of class I PI3K which robustly activates AKT. AKT then phosphorylates TSC2 and inhibits it, resulting in activation of mTOR [13]. Phenylephrine activates α-adrenergic receptors and endothelin-1 binds to the endothelin receptor [14]. Both G protein-coupled receptors activate phospholipase C that cleaves PIP2 to diacyglycerol and IP3. This event results in the activation of the MAP kinase pathway, activation of mTOR, and decreased cyclic-AMP and increased contraction [14]. The mechanism(s) underlying activation of mTOR by hypo-osmotic stress is currently under study.

mTOR is activated by phenylephrine, endothelin-1, IGF-1 and hypo-osmotic stress in NRVMs, and can be inhibited by HDAC inhibitors. This evidence led us to speculate that a proximal regulator of mTOR is likely to be the one affected by HDAC inhibitors. It also raises the question of whether one can draw a discrete line dividing the concepts of pathological and physiological growth. Because of their distinct phenotypes, it is believed that each has distinct

signaling cascades involved in either healthy or pathogenic growth. While this may be the case for some of the molecular signaling events, our evidence supports the idea that physiological and pathological growth share some of the same molecular pathways to promote growth.

Inhibition of mTOR in NRVMs using rapamycin is sufficient to decrease the maladaptive response, yet HDAC inhibitors are even more effective in diminishing the growth response; this is true despite the fact that inhibition of mTOR by HDAC inhibitors is not as complete as with rapamycin. As seen in this study, it has been previously reported that rapamycin cannot completely inhibit phenylephrine-induced protein synthesis, suggesting that phenylephrine promotes growth through mTOR-dependent and -independent mechanisms [15]. All together, this observation suggests that additional mechanisms exist downstream of HDAC inhibitors to repress cardiac hypertrophy independent on mTOR.

Other potential mechanisms

We have reported previously that chronic administration of HDAC inhibitors decreases autophagic activity in cardiomyocytes [16]. Evidence from our lab shows that treating NRVMs with HDAC inhibitors promotes autophagy at early time points (3-6 hours), consistent with the inhibition of mTOR reported here; this is followed by a second phase in which autophagy is inhibited [17]. The mechanism by which HDAC inhibitors regulate autophagy at longer exposure times remains to be determined. However, it is possible that simultaneous inhibition of mTOR and autophagy with HDAC inhibitors may elicit a stronger negative effect on hypertrophy compared to that seen with inhibiting mTOR alone.

Further, we cannot eliminate the possibility that inhibiting HDACs may alter other signaling nodes regulating cardiac growth, including calcium handling, contractility, and other kinases, such as ERK. ERK1/2 is necessary for phenylephrine-induced protein synthesis and

hypertrophy. Our evidence shows that, similar to the degree of mTOR inhibition, apicidin can decrease ERK1/2 phosphorylation. ERK1/2 can change S6 phosphorylation, which is likely to contribute to the reduction of phosphorylation achieved with HDAC inhibition, as both ERL1/2 and mTOR converge in this nodal point. We concluded that HDAC inhibition modulates mTOR, since 4EBP1 phosphorylation is a unique indicator of mTORC1 activity. It is reported that ERK can phosphorylate and inhibit TSC2 [18], drawing a possible mechanism by which HDAC inhibitors repress mTOR. However, in NRVMs, direct inhibition of ERK with U0126 does not blunt mTOR. We conclude that, while the activation of mTOR may be ERK dependent, HDAC inhibitors are likely suppressing mTOR and ERK though separate mechanisms.

Role of TSC1/TSC2 in regulating cardiomyocyte growth

Through the GAP catalytic domain of TSC2, the TSC/TSC2 complex inhibits Rheb. Rheb is active when bound to GTP and is necessary for the activation of mTOR. Hence, the TSC1/2 complex functions as a negative regulator of Rheb and mTOR activity [19]. The TSC1/TSC2 complex is regularly inhibited under growth conditions to facilitate mTOR activation, and is active during starvation [20]. Over-expression of TSC2 in HEK293 cells reduces S6 phosphorylation, and inhibits tumor growth of the B88 cancer cell line [21, 22]. We report here that HDAC inhibitors regulate the activity of the TSC1/TSC2 complex by promoting expression of TSC2, the catalytic component.

Tuberous sclerosis complex (TSC) is a genetic disease characterized by formation of hamartomas in multiple organs, especially in muscle, brain, kidney, heart, eye, lung, and skin. Around 1 in 6000 newborns are affected by TSC, which is caused by the loss of function of TSC1 or TSC2. At an embryonic stage, TSC patients develop cardiac rhabdomyosarcomas that halt and/or regress after birth, at around the time when cardiomyocytes exit cell cycle and

become post-mitotic [23]. The ability of TSC2 to inhibit mTOR using HDAC inhibitors is also observed in mouse embryonic fibroblasts. This suggests that the mechanism of action reported here might be relevant for the understanding of other diseases, such as TSC.

In neonatal rat cardiomyocytes, TSC2 deficiency does not promote hypertrophy on its own, but rather leads to increased DNA synthesis [24]. In mice, silencing TSC1 in adult heart triggers robust cardiac hypertrophy [25]. The role of TSC2 deficiency is adult heart is yet to be determined. In TSC2 knockdown NRVMs, we do not observe an exacerbated hypertrophic response at baseline, nor after phenylephrine treatment. However, we do observe a partial recovery of growth after HDAC inhibition. Apicidin reduced phenylephrine-induced growth by 50%, while only a 20% reduction is measured in TSC2 knockdown NRVMs.

We consistently measure a 2-fold increase in TSC2 levels with HDAC inhibition in all our models, with no changes in TSC1 levels. It has been postulated in the literature that the TSC1/TSC2 complex could have a stoichiometry of 1:2, instead of 1:1 [26]. Nevertheless, this report is based on sucrose gradient studies, and not on actual protein characterization. Further, data suggesting a potentially larger TSC1/TSC2 complex may be due to other proteins associated with TSC1/TSC2. This is the case for TBC1D7 [27]. Additionally, TSC1 can form homodimers *in vitro,* and it has been suggested that TSC2 could also bind to itself to form functional units. However, there is no evidence to support this hypothesis, and the TSC1 homodimer has no known biological relevance. Nevertheless, a study of the binding kinetics of TSC1 to TSC2 suggests that the complex is formed very quickly after TSC2 translation, or even as it is being transcribed [26]. This might have a biological advantage since TSC1 functions as a chaperone of TSC2, and when absent, TSC2 is quickly degraded [28]. Therefore, we propose the hypothesis that TSC1, being more stable than TSC2, exists in excess and forms functional complexes as TSC2 becomes available.

Epigenetic events participate in a wide range of disease states, including cancer and heart disease [29, 30]. As such, there is significant interest in the therapeutic use of small molecule inhibitors to target DNA methylation, histone acetylation, and other chromatin remodeling targets to activate or repress gene activity [31]. A previous study in smooth muscle-like cells from a patient with tuberous sclerosis showed that epigenetic regulation of the TSC2 promoter via methylation represses TSC2 expression [32]. Our data show that TSC2 mRNA levels are increased after HDAC inhibition, but not during growth conditions. This implies that TSC2 mRNA expression is not a target of HDAC activity during growth; however, artificially reducing HDAC activity can release the repression of the TSC2 promoter. Whether the increased TSC2 mRNA levels reported here are due to direct changes in histone acetylation, or derived from indirect mechanisms, remains unknown.

Clinical relevance

Understanding the mechanisms behind the repression of hypertrophy by HDAC inhibitors is of interest due to their translational potential [33]. In this study, we report the reduction of mTOR activity as a consequence of HDAC inhibition. Relevant to the clinic, both HDAC inhibitors and rapamycin can regress established pathological hypertrophy in mice [16, 34]. Sirolimus, another name for rapamycin, has been used clinically for several years as an immunosuppressant to prevent transplant rejection. Common side effects of rapamycin include abdominal pain, diarrhea, propensity to infection and increased blood pressure. Seventy percent of patients taking rapamycin suffer from adverse effects and 30% of patients stop the medication. After kidney transplant, treatment with sirolimus results in decreased left ventricular mass of patients with hypertrophy [35]. It is possible that rapamycin, through the inhibition of mTOR in cardiomyocytes, controls the amount of cardiac remodeling after transplantation.

However, this idea has not been proven. In addition, using rapamycin as an agent against cardiac hypertrophy has been considered in the literature, but never tested on clinical trials.

On the other hand, our results suggest HDAC inhibitors as stronger repressors of cardiac hypertrophy than rapamycin. The FDA approved inhibitor Vorinostat is tolerated in humans, with common side effects of fatigue (62%), nausea (56%) and diarrhea (49%) [36]. HDAC inhibitors have been tested alone or in combination with other therapies for the treatment of T-cell lymphoma. Because of the design and the population of these studies, the effect of HDAC inhibitors alone in the heart has not been characterized.

The evidence that class I HDACs are the main mediators of hypertrophy is relevant to the clinic, since isoform-specific inhibitors are currently available (**Table 1**). The usage of Class I HDAC inhibitors in humans may present an advantage over pan-HDAC inhibitors. While pan-HDAC inhibitors have been considered safe for use for some years, evidence of the consequence of using class I HDAC inhibitors in humans is small. In the future, more data might be available to address this question. However, our data show that class I HDAC inhibitors have a similar role in regulating TSC2 levels and mTOR activity in human embryonic stem cell derived cardiomyocytes. This is a promising for the application of class I HDAC inhibitors.

Perspectives on inhibiting mTOR by HDAC inhibitors in cardiac disease

This study demonstrates the HDAC inhibitors reduce mTOR activity in cardiomyocytes during pathological hypertrophy. mTOR, being a key regulatory kinase of growth, is involved in cellular events other that promoting protein synthesis and inhibiting autophagy. One case is the increased hypoxia-inducible factor 1-alpha (HIF1α) levels caused by mTOR activation [37]. HIF1α is involved in a couple of molecular pathways relevant to cardiac hypertrophy. HIF1α transcriptional activity leads to the expression of proteins involved in glycolysis such as hexokinase 2, phosphoglycerate kinase 1, glucose transporter 1 and others [38]. Fatty acid

oxidation is suppressed during pathological hypertrophy, while glucose consumption is increased [39]. It is believed that switching from glucose to fatty acid utilization in the context of disease might be beneficial to the heart. Therefore, by inhibiting mTOR, HDAC inhibitors might reduce cardiomyocytes' ability to use glucose and force these cells to increase fatty acid oxidation. Literature suggests that HDAC3 levels increase in the hypothalamus of starved mice, presumably to promote glucose utilization, which is the sole source of food in the brain [40]. Along these lines, cardiomyocyte specific HDAC3 KO mice show increased fatty acid oxidation genes [4]. In liver tumors, inhibition of HDAC2 can reduce GLUT4 levels [41]. Lastly, inhibition of Class I HDACs enhances oxidative metabolism in C2C12 myotubes [42]. Therefore, a potential first step would be to determine if inhibition of Class I HDACs could reduce the expression of glycolytic genes during pathological hypertrophy, and promote oxidative metabolism.

Along the same lines, activation of mTORC1 promotes insulin sensitivity by phosphorylation IRS1 and targeting it for degradation [43]. Currently in the lab, we are assessing whether Class I HDAC inhibitors can have beneficial effects in a mouse model of diabetic cardiomyopathy. In this model, mice are fed a high fat diet for 3 weeks, time at which they present insulin insensitivity, decreased glucose uptake and reduced cardiac function. The hypothesis is that HDAC inhibitors, by reducing mTOR activity, will increase IRS levels and restore insulin sensitivity. Previous studies have shown that pan-HDAC inhibitor TSA increased glucose uptake in C2C12 after insulin stimulation [44]. Therefore, repressing mTOR and restoring IRS1 levels might be another relevant cellular event by which HDAC inhibitors reduce pathological hypertrophy.

Another consequence for the inhibition of mTOR via HDAC inhibitors could be based on the regulation of VEGF-A by HIF1α. It has been reported that TSA can reduce the VEGF-A expression promoted by over activating mTOR in TSC2-/- MEFs [45]. Angiogenesis is believed to cross-talk with cardiomyocyte size to facilitate hypertrophy and heart failure [46]. Inhibition of

VEGF-A in physiological hypertrophy caused by over activation of AKT1 results the quick development of a pathological phenotype. In addition, impaired angiogenesis correlates with pathological hypertrophy. On the other hand, inhibiting VEGF-A receptor signaling before stress can actually prevent the development hypertrophy [46]. As such, inhibiting HDACs should decrease VEGF-A expression, and as a consequence, repress cardiomyocyte enlargements by suppressing angiogenesis. On a different case, inhibition of HDACs after an established hypertrophy can regress the pathological phenotype. In this case, it can be expected that reducing capillary density might have detrimental effects. However, capillary density with HDAC inhibition treatment at the time of stress or after hypertrophy has not been determined.

Furthermore, regulation of cardiomyocyte hypertrophy by HDACs is relevant in other cardiac conditions, as in cardiac infarction. In ischemia/reperfusion (I/R) models and in humans after infarction, we observe an increase in cardiomyocyte size that is believed to compensate for the lack of contractility from the area of the heart that was damaged. It is known that HDAC inhibition with pan-HDAC inhibitors improves cardiac recovery and function after I/R, both after treatment at time of ischemia and at time of reperfusion [17]. It is to be determined whether Class I HDACs are the ones responsible in this context. Nevertheless, pan-HDAC inhibitors can reduce mTOR activity and promote autophagy after I/R [17]. Induction of autophagy, a cellular process of bulk degradation, is considered one of the major mechanisms responsible for the positive outcomes after HDAC inhibition during I/R stress. To note, long term exposure to HDAC inhibitors actually inhibit autophagic flux despite the fact that mTOR is inactive in the context of pathological hypertrophy [16, 47]. However, the effect of long-term exposure to HDAC inhibitors on autophagy in vivo after reperfusion is to be determined. How HDAC inhibitors reduce autophagic flux in cardiomyocytes and whether this is beneficial or detrimental needs further investigation. We are planning to expose our HDAC1 and HDAC2 double knockout mice to

ischemia/reperfusion injury to determine the role of Class I HDACs and to asses the autophagic response.

In conclusion, these and other cellular events regulated by mTOR could be inhibited by the treatment of HDAC inhibitors; therefore, highlighting the potential of these small drugs as therapeutic strategy to suppress pathological remodeling.

Future experiments

Future experiments will focus in testing whether the increase in TSC2 mRNA after HDAC inhibition is caused by hyperacetylation of the TSC2 promoter. To this date, no luciferase assays using the promoter of TSC2 have been reported. In fact, cloning of the TSC2 promoter has not been reported. However, we are performing chromatin immunoprecipitation studies to determine whether there is increased acetylation in histones that bind DNA 10 Kb upstream of the TSC2 start site. If no changes in acetylation are observed in this region, potential transcription factors that are know to be regulated by acetylation can be studied. We have a list of transcription factors that can potentially bind 10 Kb upstream of the TSC2 start site based on putative binding sequences. This database could be of great use in the future.

Furthermore, we will investigate the interplay of HDACs and bromodomain containing proteins (BRD). BRD proteins bind to acetylated histones to promote the assembly of transcription elongation factor b (TEF-b) complex with RNA polymerase II, therefore, promoting transcription. Recently, it has been reported that inhibitors of BRDs can repress pathological hypertrophy and that BRD4, a member of the BRD family, is increased after hypertrophic stress [48]. Additionally, preliminary data in our laboratory shows that inhibition of BRDs can reduce mTOR activity. All together, we believe that HDACs may regulate the binding capacity of specific BRDs to specific targets to regulate hypertrophy; one potential target being TSC2. While we will use mTOR as a read-out, our general goal is to understand the interplay of

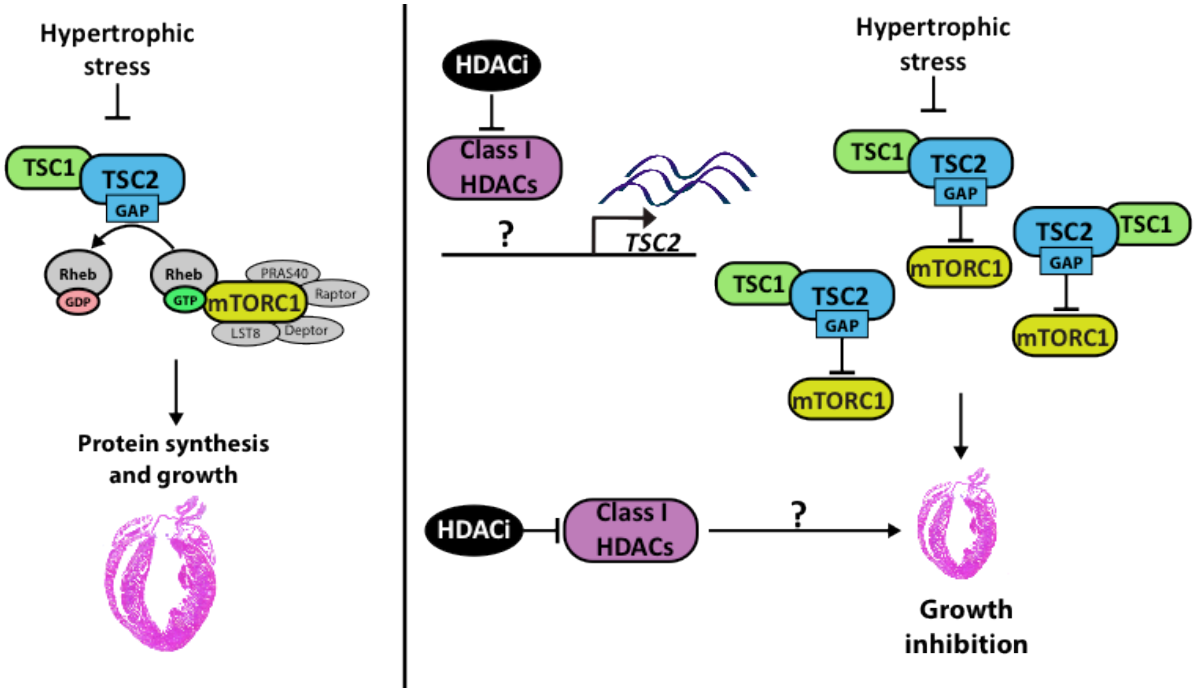
HDACs and BRDs to regulate gene expression in diseased cardiac cells. As such, this information might broaden the therapeutic potential of HDAC and BRD inhibitors.

Conclusion

We and others have shown that broad spectrum-HDAC inhibitors have beneficial effects in models of pathologic cardiac hypertrophy by blunting increases in cardiac size and mass, improving contractile performance and reducing fibrosis [49, 50]. These compounds also reduce the pathological remodeling observed during ischemia/reperfusion injury in both rabbits and rodents, specifically targeting the reperfusion phase of injury [17]. Therefore, we set out here to decipher mechanisms whereby HDAC inhibitors elicit benefits to the heart.

This study shows that repression of class I HDACs can reduce mTOR activity in several forms of cardiac hypertrophy. Inhibition of class I HDACs induces TSC2 mRNA levels, which is necessary for the inhibition of mTOR. Overall, we propose a model in which the expression of TSC2 during hypertrophic conditions is not regulated by HDACs, and allows for the activation of mTOR and progression of pathological remodeling. Nevertheless, when class I HDACs are inhibited, the transcription of TSC2 is induced, resulting in the formation of TSC1/TSC2, reduction of mTOR activity, and a repression of pathological growth. The mechanism by which inhibition of class I HDACs increases TSC2 levels is the focus of future studies.

In conclusion, we have delineated the biology of specific HDACs in the governance of cardiomyocyte growth and remodeling through mTOR. In addition, we uncover one relevant mechanism by which inhibition of class I HDACs regulate mTOR activity and growth in the context of heart disease.



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