HDAC5 Catalytic Activity Suppresses Cardiomyocyte Oxidative Stress and NRF2 Target Gene Expression

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ABSTRACT

Histone deacetylase 5 (HDAC5) and HDAC9 are class IIa HDACs that function as signal-responsive repressors of the epigenetic program for pathological cardiomyocyte hypertrophy. The conserved deacetylase domains of HDAC5 and HDAC9 are not required for inhibition of cardiac hypertrophy. Thus, the biological function of class IIa HDAC catalytic activity in the heart remains unknown. Here, we demonstrate that catalytic activity of HDAC5, but not HDAC9, suppresses mitochondrial reactive oxygen species (ROS) generation and subsequent induction of NF-E2 Related Factor 2 (NRF2)-dependent antioxidant gene expression in cardiomyocytes. Treatment of cardiomyocytes with TMP195 or TMP269, which are selective class IIa HDAC inhibitors, or shRNA-mediated knockdown of HDAC5 but not HDAC9, leads to stimulation of NRF2-mediated transcription in a ROS-dependent manner. Conversely, ectopic expression of catalytically active HDAC5 decreases cardiomyocyte oxidative stress and represses NRF2 activation. These findings establish a role for the catalytic domain of HDAC5 in the control of cardiomyocyte redox homeostasis, and define TMP195 and TMP269 as a novel class of NRF2 activators that function by suppressing the enzymatic activity of an epigenetic regulator.

Heart failure (HF) is estimated to afflict ~7 million adults in the United States alone (1). Despite recent advances in the treatment of HF, patients with this condition continue have poor long-term outcomes, highlighting a large unmet medical need (2). A hallmark of HF is cardiomyocyte hypertrophy, a process whereby cardiac muscle cells grow but do not proliferate.
Cardiac hypertrophy has traditionally been viewed as a compensatory mechanism that normalizes wall stress and enhances cardiac performance in the face of stress. However, suppression of left ventricular hypertrophy (LVH) reduces morbidity and mortality in patients with cardiovascular disease (3,4), and thus cardiac hypertrophy is now recognized as a target for novel therapeutic intervention (5).

In the early 2000s, histone deacetylases (HDACs), which are epigenetic regulatory enzymes, were shown to serve critical roles in the control of cardiac hypertrophy (6-8). The 18 mammalian HDACs are grouped into four classes: class I (HDACs 1, 2, 3 and 8), class II (HDACs 4, 5, 6, 9 and 10), class III (SirT1 – 7), and class IV (HDAC11). Class II HDACs are further divided into two subclasses, IIA (HDACs 4, 5, 7 and 9) and IIB (HDACs 6 and 10) (9). Class IIA HDAC5 and HDAC9 associate with pro-hypertrophic transcription factors such as myocyte enhancer factor 2 (MEF2) and block transcription of genes that are required for stress-induced growth of the heart (8,10). In response to signals for cardiac hypertrophy, HDAC5 and HDAC9 undergo phosphorylation-dependent nuclear export, which leads to derepression of downstream target genes (11,12).

The biological functions of class IIA HDAC catalytic domains have remained elusive. Class IIA HDACs are unable to deacetylate histones (13), and bona fide endogenous substrates of these enzymes have yet to be identified; class IIA HDAC catalytic activity can only be monitored using an artificial substrate (14). Furthermore, with regard to the heart, the deacetylase domains of HDAC5 and HDAC9 are dispensable for inhibition of cardiomyocyte hypertrophy.

In the current study, we employed chemical biology and genetic approaches to elucidate roles for class IIA HDAC catalytic domains in cardiomyocytes. The findings reveal a previously unrecognized function for HDAC5 catalytic activity in the suppression of oxidative stress and antioxidant gene expression governed by NF-E2 Related Factor 2 (NRF2).

**RESULTS**

**Class IIA HDAC catalytic domain inhibition has no effect on hypertrophy of cardiomyocytes**

Class IIA HDACs function as signal-responsive repressors of cardiac hypertrophy. Prior studies demonstrated that ectopic expression of truncated versions of HDAC5 and HDAC9 lacking catalytic domains leads to inhibition of cardiomyocyte hypertrophy (8). To rule out possible confounding issues related to HDAC overexpression and to corroborate these findings at the level of endogenous class IIA HDACs, cultured neonatal rat ventricular myocytes (NRVMs) were treated with a recently discovered small molecule, TMP195, which selectively inhibits class IIA catalytic activity but does not suppress other HDAC isoforms (15). Cell area of NRVMs was quantified based on α-actinin immunostaining (Fig. 1A). The results revealed that TMP195 treatment did not reduce phenylephrine (PE)-mediated hypertrophic growth of NRVMs, nor did it alter cell size when administered in the absence of PE (Fig. 1B). Consistent with this, quantitative PCR analysis demonstrated that TMP195 treatment failed to impact agonist-dependent induction of expression of classical hypertrophic marker genes, including *Nppa* and *Mysl*7 (Fig. 1, C and D). In contrast, the class IIA HDAC inhibitor did repress expression of *Xirp2*, which is a direct target of the MEF2 transcription factor (Fig. 1E).

Thus, although pharmacological inhibition of class IIA HDAC catalytic activity is capable of influencing cardiac gene expression, it is not sufficient to suppress agonist-dependent hypertrophy of cardiomyocytes. **Class IIA HDAC catalytic domain inhibition alters gene expression in cardiomyocytes**

To further explore the role of class IIA HDAC catalytic activity in cardiomyocytes, whole-transcriptome RNA-seq analysis was performed using RNA from NRVMs treated with TMP195 or vehicle control for 48 hours. TMP195 significantly downregulated expression of 623 mRNA transcripts and upregulated expression of 594 mRNA transcripts, as illustrated by the heat map (Fig. 2A). Pathway enrichments were computed using Gene Set Enrichment Analysis (GSEA) and databases available through the Molecular Signatures Database (MsigDB). Any enrichment with a normalized P-value less than 0.05 was considered to be significant. Across multiple databases (e.g. Oncogenic Signatures,
KEGG and Reactome), there was a significant enrichment of metabolic and oxidative pathways (Fig. 2B and data not shown); pathways enriched in TMP195 treated NRVMs are displayed with positive log $P$-value and normalized enrichment score (NES), whereas pathways that are downregulated upon exposure of cells to the class IIa HDAC inhibitor are shown as negative log $P$-value and NES.

Further evaluation revealed that gene sets enriched in TMP195 treated NRVMs showed striking conservation of *cis*-regulatory elements predicted to bind to the Cap‘N’Collar transcription factors NF-E2 and NF-E2 Related Factor 2 (NRF2) (Fig. 2C). Network interaction analysis of this subset indicated that antioxidant genes that are known to be stimulated by NRF2, including *Hmox1*, *Srxn1* and *Txnrd1*, were dramatically upregulated in TMP195 treated NRVMs compared to controls (Fig. 2D). As such, we focused the remainder of this study on elucidating the mechanisms and consequences of class IIa HDAC-mediated control of NRF2-dependent transcription.

**Inhibiting Class IIa HDACs triggers NRF2-dependent gene expression**

Quantitative PCR and immunoblot analyses of independent samples confirmed that TMP195 stimulates *Hmox1* mRNA and HOX1 protein expression in NRVMs (Fig. 3, A-C). Induction of *Txnrd1* and *Srxn1* mRNA transcripts by TMP195 was also validated (Fig. 3, D and E). TMP195-mediated antioxidant gene expression was attenuated by knockdown on endogenous NRF2, further suggesting that the class IIa HDAC inhibitor stimulates the activity of this transcription factor (Fig. 3, F and G).

To begin to address whether TMP195 stimulates NRF2-mediated gene expression via inhibition of class IIa HDAC catalytic activity as opposed to an off-target action, NRVMs were treated with a structurally distinct class IIa HDAC inhibitor, TMP269 (15) (Fig. 3H). Similar to TMP195, TMP269 induced expression of the NRF2 target genes *Hmox1* and *Srxn1* (Fig. 3, I and J).

Using LV lysates from adult rats as a source of cardiac HDACs for enzymatic assays, we confirmed that TMP195 is a selective class IIa HDAC inhibitor (Fig. S1). Nonetheless, at higher concentrations the compound is capable of suppressing class Iib HDAC activity. To rule out the possibility that TMP195 triggers NRF2-dependent antioxidant gene expression by targeting other HDAC isoforms, NRVMs were treated with the hydroxamic acid HDAC inhibitor, trichostatin A (TSA), which potently suppresses class I and Iib HDAC activity, but has minimal effects on class IIa HDACs (16). In contrast to TMP195 and TMP269, TSA failed to stimulate *Hmox1* or *Srxn1* mRNA expression (Fig. 3, K and L). Taken together, the results suggest that selective inhibition of class IIa HDAC catalytic activity stimulates NRF2-dependent gene expression in cardiomyocytes.

**HDAC5 suppresses cardiomyocyte NRF2 activity**

Class IIa HDACs consist of HDACs 4, 5, 7 and 9. HDACs 4, 5 and 9 are well-established regulators of cardiomyocyte gene expression, while HDAC7 appears to be more relevant in endothelial cells (17,18). To address the contributions of the three cardiomyocyte class IIa HDACs to the control of NRF2 function, shRNA-encoding adenoviruses were employed to knockdown expression of endogenous HDAC4, HDAC5 and HDAC9 in NRVMs (Fig. 4, A-C). Reduced expression of HDAC5, but not HDAC4 or HDAC9, led to dramatic induction of *Hmox1* mRNA and HOX1 protein expression, which correlated with increased NRF2 abundance (Fig. 4, D and E). Adenovirus encoding shRNA that targets a distinct region of the HDAC5 mRNA transcript also triggered NRF2 target gene expression, confirming an on-target mechanism of action (Fig. 4F).

**Class IIa HDACs inhibitors do not activate NRF2 via a common electrophile-mediated pathway**

Most small molecule NRF2 activators function by covalently modifying cysteine residues in KEAP1 to disrupt the interaction between KEAP1 and NRF2, thereby preventing ubiquitin-dependent NRF2 degradation (19). Although the findings described above demonstrate that HDAC5 inhibition is sufficient to stimulate NRF2 target gene expression in cardiomyocytes, it remained possible that class IIa HDAC inhibitors also function via direct
KEAP1 inhibition. Thus, to address the potential TMP195 stimulate NRF2 activity via KEAP1, a series of experiments was performed to compare the action of this class IIa HDAC inhibitor to a prototypical thiol-reactive NRF2 activator, AI-1.

Quantitative PCR analysis revealed distinct kinetics of NRF2-target gene expression in NRVMs treated with TMP195 or AI-1. AI-1 rapidly induced expression of Hmox1 and Srxn1, while the response to TMP195 was delayed and more prolonged (Fig. 5, A and B). The differential kinetics of target gene induction by AI-1 and TMP195 correlated with the rate of induction of NRF2 protein abundance (Fig. 5C), suggesting that the compounds stimulate NRF2 activity through different mechanisms. In further support of this notion, combined treatment of NRVMs with AI-I and TMP195 led to an additive increase in HMOX1 protein expression (Fig. 5D). Additionally, AI-1 stimulated NRF2 target gene expression in all cell types tested (NRVMs, rat aortic smooth muscle cells and cardiac fibroblasts), while TMP195 was only able to increase NRF2 activity in muscle cells (Fig. 5, E and F). Finally, mass spectrometry analysis of recombinant KEAP1 failed to reveal evidence of covalent modification by TMP195, but confirmed that AI-I is capable of coupling to cysteine-151, which is a residue in KEAP1 that is commonly targeted by electrophile activators of NRF2 (Fig. 5, G - I) (20,21). Together, the data suggest that the primary mechanism by which TMP195 stimulates NRF2 in cardiomyocytes is through HDAC5 inhibition rather than by electrophilic adduction to KEAP1.

**HDAC5 inhibition stimulates cardiac NRF2 activity by triggering oxidative stress**

To further address the mechanism by which inhibition of HDAC5 catalytic activity leads to induction NRF2 in NRVMs, experiments were performed to determining whether HDAC5 and NRF2 physically associate. Using a mammalian two-hybrid approach, ectopic expression of HDAC5 failed to block reporter gene expression driven by a hybrid protein containing the complete open reading frame of NRF2 fused to the GAL4 DNA-binding domain (Fig. S2A). In contrast, HDAC5 potently suppressed GAL4-MEF2-driven reporter gene expression (Fig. S2B), which is consistent with the ability of HDAC5 to directly bind to MEF2 (10). Likewise, endogenous NRF2 failed to co-immunoprecipitate with FLAG-tagged HDAC5 in transiently transfected HEK293 cells, even under conditions where NRF2 abundance was elevated due to treatment of cells with the proteasome inhibitor, MG132 (Fig. S2, C and D). Conversely, endogenous MEF2A was readily co-immunoprecipitated with HDAC5 (Fig. S2D). Consistent with this, HDAC5 also failed to co-immunoprecipitate with KEAP1, while NRF2 was detected in association with its inhibitor (Fig. S2, E and F). These findings indicate that HDAC5 does not physically interact with NRF2:KEAP1 complexes, and suggest that the class IIa HDAC represses NRF2 activity through an indirect mechanism.

ROS can stimulate NRF2 by modifying cysteine residues in KEAP1 (21). Thus, we addressed the possibility that HDAC5 inhibition promotes NRF2-dependent transcription by triggering cardiomyocyte oxidative stress. Indeed, treatment of NRVMs with TMP195 for 48 hours led to a significant increase in mitochondrial ROS levels, as did selective knockdown of HDAC5 using shRNA (Fig. 6, A-C). Furthermore, pre-treatment of NRVMs with the antioxidant N-acetylcysteine (NAC) completely blocked TMP195-induced expression of Hmox1 and Srxn1 mRNA, and also reduced basal levels of these transcripts in unstimulated cardiomyocytes (Fig. 6, D and E). These data suggest that HDAC5 inhibition stimulates NRF2 in cardiomyocytes, at least in part, by triggering oxidative stress.

**HDAC5 catalytic activity reduces cardiomyocyte oxidative stress**

To determine if increasing HDAC5 catalytic activity is sufficient to alter cardiomyocyte redox signaling, NRVMs were infected with adenoviruses encoding FLAG-tagged versions of wild-type (WT) HDAC5 or HDAC5 harboring a histidine-to-alanine substitution at amino acid 1006 within the catalytic domain (H1006A) (Fig. 7A) (13). *In vitro* enzymatic assays employing FLAG immunoprecipitates from the cells demonstrated that the catalytic activity of HDAC5 (H1006A) was attenuated (decreased by 77%) but not abolished (Fig. 7, B and C). For comparison, 3 μM TMP195 reduced class IIa
HDAC catalytic activity in living NRVMs by 89% (Fig. 7D).

Next, mitochondrial ROS levels were quantified in NRVMs ectopically expressing WT HDAC5, HDAC5 (H1006A), or a β-galactosidase control via adenoviruses. As shown in Fig. 7E, mitochondrial ROS was significantly decreased in cardiomyocytes expressing WT HDAC5 compared to β-galactosidase. HDAC5 (H1006A) also reduced ROS levels, albeit to a lesser extent than HDAC5 WT, which is consistent with its impaired catalytic function. Furthermore, ectopic WT HDAC5, but not HDAC5 (H1006A), suppressed Hmox1 mRNA expression in NRVMs (Fig. 7F). These data further suggest that HDAC5 catalytic activity suppresses cardiomyocyte redox signaling.

**DISCUSSION**

Here, we describe a role for HDAC5 catalytic activity in the repression of cardiomyocyte oxidative stress and NRF2-dependent antioxidant gene expression. These functions were initially uncovered using a new generation of HDAC inhibitors that selectively target class Ila HDAC catalytic domains, and were validated using HDAC5 gain- and loss-of-function approaches in cultured cardiomyocytes. The data suggest that alterations in HDAC5 abundance and/or catalytic activity could contribute to cardiac mitochondrial dysfunction and oxidative stress, which are drivers of HF pathogenesis (22). Thus, HDAC5 action in the heart extends beyond transcriptional repression of pro-hypertrophic gene expression.

Selective inhibitors of class Ila HDAC catalytic activity have only recently become available. Class Ila HDAC catalytic activity is largely resistant to commonly used HDAC inhibitors such as TSA (16). Several years ago, MC1568 was described as the first class Ila HDAC-selective inhibitor (23). However, we found that MC1568 fails to inhibit class Ila HDAC enzymatic activity (24). TMP195 and TMP269 are powerful chemical tools for studying the biological consequences and potential therapeutic benefits of selectively inhibiting class Ila HDAC activity. These compounds were discovered in a high throughput screening campaign employing recombinant HDAC9 (15). They possess a non-chelating zinc binding group, trifluoromethyl oxadiazole (TFMO), which is less promiscuous for off-target actions than the hydroxamic acid, zinc-chelating warheads common to many HDAC inhibitors, including TSA. Our data confirmed the selectivity of TMP195 for class Ila HDACs over other HDACs in the heart.

TMP195 did not alter agonist-mediated growth of cardiomyocytes, which is consistent with prior data showing that versions of HDAC5 and HDAC9 lacking deacetylase domains are still capable of inhibiting MEF2 transcriptional activity and cardiac hypertrophy (8). We note, however, that TMP195 did reduce expression of the gene encoding Xirp2, which is a direct target of MEF2A (25,26). Thus, it is possible that class Ila HDAC catalytic activity promotes expression of a subset of MEF2 target genes.

NRF2 activity has been shown to prevent pathological remodeling of the heart. NRF2-deficient mice develop exaggerated hypertrophy and cardiac dysfunction in response to pressure overload (27), and electrophilic compounds that stimulate NRF2 via covalent attachment to KEAP1 block cardiac hypertrophy and improve ventricular function in mice (28-31). It is likely that direct activation of cardiac NRF2 through KEAP1 targeting will be better tolerated than indirect activation via HDAC5 inhibition, since the latter approach triggers oxidative stress in cardiomyocytes. Nonetheless, despite triggering elevated ROS levels, TMP195 did not elicit overt cytotoxic effects in cultured NRVMs, possibly due to increased expression of compensatory NRF2-dependent antioxidant genes.

TMP195 stimulated NRF2 target gene expression in cardiomyocytes and smooth muscle cells, but not in cardiac fibroblasts, suggesting that HDAC5 regulates this pathway in a cell type-specific manner. Consistent with this, TMP195 treatment failed to induce NRF2 target gene expression in monocytes, B lymphocytes or T lymphocytes (15). This differential regulation could be a reflection of the relative abundance and activity of mitochondria in muscle cells over other cell types.

The mechanism by which HDAC5 inhibition promotes cardiomyocyte oxidative stress remains unknown. HDAC5 is detected in mitochondrial fractions from cultured NRVMs, suggesting the
possibility that this class IIa HDAC directly regulates mitochondrial function through a non-transcriptional mechanism. A recent report demonstrated that HDAC5 depletion with siRNA in HeLa cells resulted in elevated ROS levels in association with reduced expression of FTH1 and FTHL2, which are proteins that regulate iron homeostasis (32). While we cannot rule out the possibility that altered iron abundance contributes to oxidative stress in NRVMs in which HDAC5 has been inhibited or depleted, we note that expression of FTH1 was not reduced in TMP195 treated NRVMs; FTHL2 was not detected in our RNA-seq dataset. Therefore, the mechanisms by which HDAC5 inhibition leads to oxidative stress in HeLa cells and cardiomyocytes appear to be distinct.

In summary, we have described a novel function for a class IIa HDAC in the control of cardiac redox homeostasis, with HDAC5 catalytic function reducing oxidative stress in cardiomyocytes. Previously, oxidation of conserved cysteine residues in class IIa HDACs, including HDAC5, was shown to lead to nuclear export of these transcriptional repressors through a phosphorylation-independent mechanism (33-35). This process results in derepression of pro-hypertrophic genes and exaggerated cardiomyocyte hypertrophy. Our findings reveal a second level of interplay between ROS signaling and HDAC5, and suggest the possibility of augmenting HDAC5 catalytic activity as a therapeutic strategy to dampen oxidative stress in the context of cardiac disease.

EXPERIMENTAL PROCEDURES

Reagents

Vendors and catalog numbers for all of the antibodies, chemicals and kits employed in the current study are provided in Table S2. Oligonucleotide sequences for cloning and quantitative PCR analysis are provided in Table S1.

Cell culture

Neonatal rat ventricular myocytes (NRVMs) and fibroblasts (NRVF) were prepared and cultured from hearts of one-day old Sprague Dawley rats as previously described (36). For adenoviral infection, viruses (MOI 10) were added at the time of seeding the cells. After incubation with adenovirial particles overnight, cells were washed and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM; Corning) supplemented with L-glutamine, penicillin-streptomycin, and 0.1% Nutridoma-SP (Roche Applied Sciences). Infected cells were harvested or analyzed 48 hours or 72 hours after infection. NRVFs and HEK293A cells were maintained in high glucose DMEM (Corning) containing 1% L-glutamine, penicillin-streptomycin (PSG; Corning) and 10% fetal bovine serum (FBS; Gemini). Passage one NRVFs were used for experiments. Rat aortic smooth muscle cells (RASMCs; passage eight), were cultured in Minimal Essential Medium (MEM; Corning) supplemented with 1% PSG and 10% FBS. NRVFs and RASMCs were equilibrated in corresponding medium with 0.1% FBS for 24 hours prior to incubation with DMSO, TMP195 or AI-1 for an additional 48 hours.

RNA-sequencing analysis

NRVMs cultured in serum-free, Nutridoma-supplemented medium were treated with either DMSO or 3 μM TMP195 for 48 hours. Total RNA was prepared using the RNeasy Plus Mini Kit (Qiagen). Libraries were prepared using the Ovation® RNA-Seq Systems 1-16 for Model Organisms (NuGEN). Libraries were sequenced by the University of Colorado Denver Genomics and Microarray Core on a HiSeq 2500 (Illumina). All analyses were performed using the rat RN6 genome and RN6 RefSeq gene annotations.

FastQ files were aligned to RN6 using the TOPHAT2/Cufflinks pipeline. Geometric method was used for normalization. Genes that had an expression greater than 1 were selected for further analysis. Significant differences were computed using a Mann-Whitney-U test and corrected for multiple comparisons. Genes that had a P-value less than 0.05 were considered significant.

A heat map of gene expression was generated using the R statistical suite. Pathway analysis was performed using GSEA and databases available through the MSig database via the Broad Institute, and using ConsensusPathDB provided by the Max Plank Institute (37,38). An expression network was created from significantly regulated genes within significantly upregulated gene sets.
using cytoscape (39). The gene sets included to create Fig. 2D were 'NFE2L2.V2', 'NRF2_Q4', 'Oxidative stress induced gene expression via nrf2', 'Transcriptional Activation by NRF2' and 'NRF2 Pathway'. Raw and processed RNA-seq data were deposited to the GEO online database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSExxxxxx.

**qRT-PCR**

RNA was extracted from cells using QIAzol lysis reagent (Qiagen). cDNA was prepared using Verso cDNA synthesis kit (Thermo Fisher Scientific) with 500ng of total RNA. qPCR was performed with PowerUp™ SYBR Green Master Mix (Thermo Fisher Scientific) on a StepOnePlus Real-Time PCR System (Applied Biosystems). Specific primers are listed in Table S1. Relative mRNA expression level was calculated using the 2^−ΔΔCT method and normalized to 18S RNA.

**Immunoblotting**

Total protein was extracted from NRVMs using RIPA buffer supplemented with protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (0.45µm; Life Science Products). Membranes were incubated with primary antibodies overnight at 4°C. Blots were probed with corresponding HRP-conjugated mouse or rabbit secondary antibodies (Southern Biotech) after primary antibody incubation. Enhanced chemiluminescent HRP substrate (SuperSignal West Pico Chemiluminescent Substrate; Thermo Scientific) were used for developing and blots were and bands were visualized using a FluorChem HD2 Imager (Alpha Innotech).

**Indirect Immunofluorescence**

NRVMs were fixed with 4% paraformaldehyde at room temperature (RT) for 10 minutes, permeabilized with PBS-T (0.2% Triton X-100) for 15 minutes, and blocked with PBS containing 5% BSA for 30 minutes at RT. Cells were subsequently incubated with anti-α-actinin antibody (Sigma) for 1 hour. Fluorochrome-conjugated secondary antibody (anti-Rabbit Alexa Fluor® 488) was applied for 30 minutes. Nuclei were counterstained using DAPI (Life Technologies). Images were acquired on EVOS FL Cell Imaging System (Life Technologies, AMF4300). NRVM cell area was quantified based on α-actinin staining using Image J software (NIH).

**Plasmids**

The complementary DNA for human KEAP1 (a gift from Qing Zhong, Addgene plasmid #28023) was PCR amplified using PFU Turbo DNA Polymerase (Agilent Technologies) and sub-cloned into pET28a to encode 6xHis-tagged full-length hKEAP1. Site-directed mutagenesis was performed using the QuikChange method (Agilent Technologies). For generation of adenoviral constructs overexpressing FLAG-tagged forms of HDAC4, cDNAs were subcloned into the pENTR/2B entry vector (Invitrogen) and recombined with the Adenovirus constructs for knockdown of HDAC4, HDAC5 and HDAC9, complementary oligonucleotides for shRNAs were designed using BLOCK-i™ RNAi Designer (Thermo Fisher) and annealed, and ligated into the pENTR/U6 vector (Invitrogen). Positive clones were recombined with pAd/BLOCK-i™-DEST vector (Invitrogen) to yield shRNA-expressing adenoviral constructs.

**siRNA knockdown of NRF2**

To knockdown endogenous NRF2 expression, NRVMs were transfected with a non-specific siRNA control or siRNA oligonucleotides targeting Nrf2 (50 nmol/L, Sigma) using Lipofectamine™ RNAiMAX (Thermo Fisher). Twenty-four hours after transfection, cells were treated with DMSO or 3 µM TMP195 for an additional 48 hours.

**Adenovirus preparation**

Adenoviral vectors encoding shRNAs targeting HDAC4, HDAC5 and HDAC9 mRNA transcripts or overexpressing FLAG-HDAC5 (H1006A) were digested by Pacl and transfected into HEK293A cells cultured in antibiotic-free medium at 60-70% confluence using Lipofectamine 3000 (Thermo Fisher). Viruses were amplified and recovered from HEK293A cell lysates, and titers were determined using the Sea-Plaque agarose method (40).

**Recombinant KEAP1 protein purification, in vitro labeling, and mass spectrometry**

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6xHis-hKEAP1 was expressed using pET28a in Tuner™ (DE3) E. coli (Novagen). Cells were lysed by sonication on ice and pelleted by centrifugation at 27,000g in a Sorvall SS-34 fixed angle rotor for 50 minutes. Clarified lysates were incubated with 1 ml of pre-washed TALON® metal affinity resin (Takara) at 4°C for 3 hours. Resin was pelleted at 3000 rpm for 5 minutes and washed 3X with 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM imidazole and 0.1% β-mercaptoethanol. 6xHis-hKEAP1 was eluted by incubating resin with 2 ml of wash buffer containing 500 mM imidazole at 4°C for 30 minutes. Buffer exchange was performed using Amicon Ultra-centrifugal filter units (Millipore) with 25 mM Tris-HCl (pH 8.0).

To determine the half maximal inhibitor concentration (IC_{50}) of TMP195 against class I, IIa and IIb HDACs, HDAC activity assays were performed as previously described (41). Assays were run simultaneously for class I, IIa, and IIb HDAC activities with parallel sets of sample aliquots. Left ventricular (LV) homogenates from Sprague Dawley rats tissue were prepared as a source of endogenous HDACs using PBS (pH 7.4) containing 0.5% Triton X-100, 300 mM NaCl and protease/phosphatase inhibitor cocktail. Data was plotted using GraphPad Prism and IC_{50} of TMP195 was obtained using nonlinear regression fit.

To assess the catalytic activity of ectopic HDAC5, assays were performed with anti-FLAG immunoprecipitates. NRVMs were infected, at the time of plating, with adenoviruses encoding FLAG-HDAC5 or FLAG-HDAC5 (H1006A) at MOI of 10. After 16 hours of infection, cells were washed and incubated for 56 additional hours in serum-free medium. Cells were lysed in buffer containing Tris (50 mM, pH 8), 150 mM NaCl, sodium Deoxycholate (0.5%), NP-40 (1%) and sodium dodecyl sulfate (0.1%), and sonicated (4 seconds at 30% power with the Sonic Dismembrator 500) prior to clarification by centrifugation (12000g, 5 min at 4°C). 500 μg total protein in 500 μl of lysis buffer was immunoprecipitated with 25 μl of prewashed, packed anti-FLAG IP resin (GenScript), overnight at 4°C on a rotator. Beads were washed 3x with 500 μl PBS and resuspended in 200 μl PBS. Class IIa substrate (10 μl, 1 mM) was added and incubated at 37°C for 2.5 hours. Beads were pelleted at 5000g for 30 seconds and supernatant was transferred to a 96-well plate at 100 μl/well. 50 μl developer solution was then added and incubated at 37°C for another 20 minutes. AMC fluorescence signal was measured. Background signals from FLAG only immunoprecipitates were subtracted. Beads were boiled in sample loading buffer and proteins were resolved by SDS-PAGE and immunoblotted with anti-FLAG antibody to normalize for the amount of immunoprecipitated HDAC5.

To measure class IIa HDAC activity in living NRVMs, 3 x 10⁵ cells were used for each experiment. NRVMs were incubated with MitoSOX™ staining

NRVMs were gently rinsed 3x with PBS to remove residual medium and incubated with 3 μM MitoSOX™ in pre-warmed PBS at 37°C for 20 minutes, in the dark. Cells were subsequently washed 3x with PBS and incubated in PBS during imaging. Images were obtained on an EVOS FL Cell Imaging system (Life Technologies, AMF4300). Mean fluorescence intensity quantification based on MitoSOX™ staining within cells was performed using Image J software (NIH).

HDAC activity assays

To determine the half maximal inhibitor concentration (IC_{50}) of TMP195 against class I, IIa and IIb HDACs, HDAC activity assays were performed as previously described (41). Assays were run simultaneously for class I, IIa, and IIb HDAC activities with parallel sets of sample aliquots. Left ventricular (LV) homogenates from Sprague Dawley rats tissue were prepared as a source of endogenous HDACs using PBS (pH 7.4) containing 0.5% Triton X-100, 300 mM NaCl and protease/phosphatase inhibitor cocktail. Data was plotted using GraphPad Prism and IC_{50} of TMP195 was obtained using nonlinear regression fit.
DMSO or 3 μM TMP195 in 100 μl pre-warmed PBS at 37°C for 3 hours. 5 μl of 1 mM class IIa HDAC substrate was then added and incubated at 37°C for 2.5 hours. AMC fluorescence signal was developed and measured as described above.

**Co-immunoprecipitation**

HEK293A cells were transfected with N-terminus FLAG-tagged cDNA expression vector encoding human HDAC5 using polyethylenimine (PEI) for 48 hours. Cells were lysed in buffer containing Tris (50 mM, pH 7.5), 150 mM NaCl and Triton X-100 (0.5 %) using a syringe with a 25-gauge needle. Total protein was collected after centrifugation of lysates at 12000g at 4°C for 20 minutes. 500 μg total protein were diluted in 500 μl equilibration buffer (50 mM Tris, 150 mM NaCl, pH 7.4), and incubated with 25 μl prewashed packed anti-FLAG IP resin overnight at 4°C on a rotator. Beads were then washed (thrice with equilibration buffer; 0.5 mL per wash), boiled in sample loading buffer and then loaded on 10% SDS-PAGE gels. Whole cell lysates were used as input controls.

For immunoprecipitation with anti-KEAP1 antibody, HEK293A cells treated with MG132 (20 μM) for 4 hours were lysed in IP lysis buffer as above. 500 μg total protein homogenate was incubated with 10 μg anti-KEAP1 antibody (Proteintech) or normal rabbit IgG (Cell Signaling Technologies) overnight at 4°C on a rotator. The protein complexes were immunoprecipitated using 20 μl prewashed packed anti-FLAG IP resin overnight at 4°C on a rotator. Beads were then washed (thrice with equilibration buffer; 0.5 mL per wash), boiled in sample loading buffer and then loaded on 10% SDS-PAGE gels. In both experiments, proteins were transferred to nitrocellulose membranes (0.45 μm; Life Science Products) and immunobotted using anti-NRF2, anti-HDAC5 (Cell Signaling Technologies), anti-MEF2 (Santa Cruz Biotechnology), anti-KEAP1 (Proteintech) or anti-FLAG M2-HRP (Sigma) antibodies. For both Co-IPs, whole cell lysates were used as input controls.

**Luciferase reporter assays**

HEK293A cells (1x10^5) were seeded on 24-well plates and co-transfected with pGB5-Luc, pcDNA3.1-FLAG or pcDNA3.1-FLAG-HDAC5, and pcDNA3 vectors expressing GAL4, GAL4-NRF2, GAL4-MEF2A or GAL4-MEF2D using PEI. A Renilla luciferase expression vector (hRLuc/TK) was used as an internal control. Cells were lysed in passive lysis buffer provided with the Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was measured using a BioTek Synergy 2 plate reader.

**Statistical analysis**

Statistical significance (P<0.05) was determined using unpaired t-test (two groups) or one-way ANOVA for multiple comparisons via a Tukey post hoc test (GraphPad Prism 7.02).

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**CONFLICT OF INTEREST**

The authors declare that they have no conflicts of interest with the contents of this article.

**AUTHOR CONTRIBUTIONS**

T.H., F.C.S. and R.A.B. performed experiments and analyzed results. P.D.T. analyzed RNA-seq data. M.H. provided reagents and guidance on studying NRF2. T.A.M. contributed to study design and data interpretation. All authors participated in writing the paper and approved the final version of the manuscript.
FIGURE LEGENDS

Figure 1. Class IIa HDAC catalytic domain inhibition does not suppress agonist-dependent hypertrophy of cardiomyocytes. (A) Neonatal rat ventricular myocytes (NRVMs) were treated with vehicle control (Veh; DMSO, 0.1% final concentration) or TMP195 (3 μM) in the absence or presence of phenylephrine (10 μM) for 48 hours. Cells were fixed and analyzed by indirect immunofluorescence with an antibody against sarcomeric α-actinin. Scale bar = 50 μm. (B) Cell area was quantified. (C - E) Quantitative RT-PCR analysis of Nppa, Myh7 and Xirp2 mRNA expression with RNA prepared from parallel plates of NRVMs. For B-E, values represent means +SEM; P<0.05 vs. corresponding conditions but without PE. For B, n = the number of cells quantified; for C-E, n = plates of NRVMs per condition.

Figure 2. Class IIa HDAC catalytic domain inhibition alters gene expression in cardiomyocytes. (A) NRVMs were treated with vehicle control (DMSO; 0.1% final concentration) or TMP195 (3 μM) for 48 hours. A heat map representing significantly upregulated or downregulated transcripts determined by RNA-seq analysis. (B) Pathway enrichments were computed using Gene Set Enrichment Analysis (GSEA) and databases available through the Molecular Signatures Database (MsigDB). The top twenty increased and decreased pathways in the Oncogenic Signature database are displayed as a waterfall plot; UNKN, unknown. (C) Conserved cis-regulatory elements for genes enriched (positive log P-value and normalized enrichment score [NES]) or decreased (negative log P-value and NES) in TMP195-treated NRVMs. (D) A network interaction model highlights the impact of TMP195 treatment on NRF2 target gene expression in NRVMs. The color of the node indicates direction of the expression change with TMP195 treatment (green = downregulation and red = upregulation). The magnitude of change is indicated by the size of the node.

Figure 3. Class IIa HDAC catalytic domain inhibition stimulates NRF2 target gene expression in cardiomyocytes. NRVMs were treated with vehicle control (DMSO; 0.1% final concentration) or TMP195 (3 μM) for 48 hours. Hmox1 mRNA expression was assessed by qRT-PCR (A), and HMOX1 protein levels were determined by immunoblotting (B and C); calnexin served as a loading control. (D and E) NRVMs were treated as described in A, and Txnrd1 and Srxn1 mRNA was quantified by qRT-PCR. (F and G) NRVMs were transfected with control siRNA or two distinct siRNAs targeting rat Nrf2, and 24 hours post-transfection cells were treated with TMP195 for X48 additional hours. Nrf2 and Srxn1 mRNA was quantified by qRT-PCR. (H) Structures of HDAC inhibitors. (I and J) NRVMs were treated with TMP269 (3 μM) or DMSO control for 36 hours prior to qRT-PCR analysis. (K and L) NRVMs were treated with TSA (200 nM) or DMSO for 36 hours prior to qRT-PCR analysis. For all graphs, values represent means +SEM. For C, D, E and I-L, *P < 0.05 vs. vehicle. For F, *P < 0.05 vs. siControl from the same treatment group. For G, *P < 0.05 vs. siControl + vehicle.

Figure 4. HDAC5 knockdown stimulates NRF2 target gene expression in cardiomyocytes. NRVMs were infected with adenoviruses (MOI = 10) encoding non-targeting control shRNA (shCtrl) or shRNAs targeting HDAC4 (shHD4), HDAC5 (shHD5), HDAC9 (shHD9). After 72 hours of infection, total RNA was isolated, and Hdac4 (A), Hdac5 (B), Hdac9 (C) and Hmox1 (D) mRNA levels were determined by qRT-PCR. (E) Protein homogenates were prepared from NRVMs infected with adenoviruses encoding shCtrl or shHD5 for 72 hours. Immunoblotting was performed with antibodies against the indicated proteins. (F) NRVMs were infected with adenoviruses encoding shCtrl or shRNAs targeting two distinct regions of the Hdac5 mRNA transcript (shHD5#1 and shHD5#2) for 72 hours prior to preparing total RNA for qRT-PCR analysis of Srxn1 mRNA expression. For all graphs, values represent means +SEM; *P < 0.05 vs. shCtrl.

Figure 5. Class IIa HDAC catalytic domain inhibition does not stimulate NRF2 through KEAP1 inactivation. NRVMs were treated with vehicle control (DMSO; 0.1% final concentration), TMP195 (3 μM)
μM) or AI-1 (10 μM) for the indicated times. *P < 0.05 vs. DMSO treated peptide. (G) Schematic depiction of the in vitro assay to determine if TMP195 covalently couples to KEAP1. (H) The signals of unmodified tryptic peptides that contain KEAP1 Cys-151, which is a common site of electrophilic addition, were measured on an LTQ IonTrap following in vitro incubation of KEAP1 with vehicle control, TMP195 or AI-1; AI-1 served as a positive control. The liquid chromatography (LC) peaks for the Cys-151-containing peptide is indicated with a black arrow; reduced signal indicates that a compound conjugated to the peptide. The inset shows a side-by-side comparison of the peak for the Cys-151-containing peptide in the different treatment groups. (I) The mass spectrometry experiment in (H) was repeated to include three additional samples per treatment group. Relative abundance reflects area under the curve normalized to the DMSO group. Values represent means + SEM; *P < 0.05 vs. DMSO treated peptide.

**Figure 6. Class IIa HDAC catalytic domain inhibition and HDAC5 knockdown elevated cardiomyocyte ROS levels and trigger NRF2 target gene expression.** NRVMs were treated with vehicle control (DMSO; 0.1% final concentration) or TMP195 (3 μM) for 48 hours. The cells were subsequently washed with PBS and incubated with MitoSOX™ (3 μM) in PBS for 20 minutes at 37°C. (A) Mitochondrial ROS levels were determined by live cell imaging and quantifying fluorescence intensity of MitoSOX™ in single cells. Values represent means +SEM, with n = the number of cells quantified; *P < 0.05 vs. vehicle. (B) Bright-field images of representative cells with the MitoSOX™ signal overlaid; scale bar = 50 μm. (C) NRVMs were infected with adenoviruses encoding shCtrl or shHD5 for 72 hours, and mitochondrial ROS was quantified as in A. NRVMs were pretreated with N-acetylcysteine (NAC; 5 mM) for 1 hour, and were subsequently incubated with DMSO vehicle or TMP195 (3 μM) for 48 hours. Hmox1 (D) and Srxn1 (E) mRNA levels were determined by qRT-PCR. Values represent means +SEM, with n = plates of cells per condition; *P < 0.05 vs. vehicle.

**Figure 7. HDAC5 gain-of-function reduces cardiomyocyte oxidative stress and NRF2 target gene expression.** (A) Schematic representation of the HDAC5 activity assay. (B and C) Seventy-two hours after infection, NRVM protein homogenates were immunoprecipitated (IP) with anti-FLAG antibody and immunoprecipitates were incorporated into in vitro HDAC activity assays employing a class IIa HDAC-specific substrate, as described in the Experimental Procedures. A portion of each immunoprecipitate was subjected to immunoblotting with anti-FLAG antibody. Values represent means +SEM, with n = plates of cells per condition. Signal from immunoprecipitates of lysates from Ad-β-Gal-infected NRVMs was subtracted as background, and the values were normalized to the amount of IPed WT or H1006A determined in (C); *P < 0.05 vs. WT. (D) Class IIa HDAC activity was quantified in living NRVMs using a cell-permeable substrate. Values were obtained 5.5 hours after treatment with TMP195 (3 μM) or DMSO vehicle control. Values represent means +SEM, with n = the number of independent wells of NRVMs quantified; *P < 0.05 vs. vehicle control. (E) NRVMs were infected with adenovirus encoding β-galactosidase (β-Gal), WT HDAC5 or HDAC5 (H1006A) for 72 hours. The cells were subsequently washed with PBS and incubated with MitoSOX™ (3 μM) in PBS for 20 minutes at 37°C. Mitochondrial ROS levels were determined by live cell imaging and quantifying fluorescence intensity of MitoSOX™ in single cells. Values represent means +SEM, with n = the number of cells quantified; *P < 0.05 vs. β-Gal and *P < 0.5 vs. WT. (F) NRVMs were infected with adenoviruses, as described above, Hmox-1 mRNA...
expression was determined by qRT-PCR. Values represent means +SEM, with n = the number of plates of cells per condition; *$P < 0.05$ vs. $\beta$-Gal.
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Figure 1

A

|                | Unstimulated | Phenylephrine | Unstimulated | Phenylephrine |
|----------------|--------------|---------------|--------------|---------------|
| PE: +          | Vehicle      | TMP195        | Vehicle      | TMP195        |
| n=4 n=4 n=4 n=4 | n=4 n=4 n=4 n=4 |

B

Myocyte Hypertrophy

C

Nppa

D

Myh7

E

Xirp2

Cell Area (μm²)

Relative mRNA Expression

Myocyte Hypertrophy

n=39 n=26 n=37 n=29

n=4 n=4 n=4 n=4

n=4 n=4 n=4 n=4

n=4 n=4 n=4 n=4

n=4 n=4 n=4 n=4

* * *
Figure 3

A  

Hmox1

Relative mRNA Expression

Veh  TMP195

n=3  n=3

B

Vehicle  TMP195

HMOX1

Calnexin

Relative Protein Expression

Veh  TMP195

n=3  n=3

C

HMOX1

Relative mRNA Expression

Veh  TMP195

n=3  n=3

D

Txnrd1

Relative mRNA Expression

Veh  TMP195

n=3  n=3

E

Srxn1

Relative mRNA Expression

Veh  TMP195

n=3  n=3

F

Nrf2

Relative mRNA Expression

siCtrl  siNrf2(#1)  siNrf2(#2)

Veh  TMP195

n=4  n=4  n=4

G

Srxn1

Relative mRNA Expression

siCtrl  siNrf2(#1)  siNrf2(#2)

Veh  TMP195

n=4  n=4  n=4

H

TMP195

TMP269

Trichostatin A

Relative mRNA Expression

Veh  TMP269

n=3  n=3

I

Hmox1

Relative mRNA Expression

Veh  TMP269

n=3  n=3

J

Srxn1

Relative mRNA Expression

Veh  TMP269

n=3  n=3

K

Hmox1

Relative mRNA Expression

Veh  TSA

n=4  n=4

L

Srxn1

Relative mRNA Expression

Veh  TSA

n=4  n=4
Figure 4

(A) **Hdac4**, (B) **Hdac5**, (C) **Hdac9**, (D) **Hmox1**

![Graphs showing relative mRNA expression for various conditions.](image)

(E) Western blots for **HDAC5**, **HMOX1**, **NRF2**, and **α-Tub**

(F) Relative mRNA expression for **Srxn1**

* by guest on April 26, 2019
Figure 5

**A** Relative mRNA Expression

**B** Relative mRNA Expression

**C** Relative mRNA Expression

**D** Relative mRNA Expression

**E** Relative mRNA Expression

**F** Relative mRNA Expression

**G** Relative mRNA Expression

**H** Relative mRNA Expression

**I** Relative mRNA Expression
Figure 6

A. Mitochondrial ROS

Mitochondrial ROS Fluorescence (AU)

Vehicle TMP195

n=172 n=209

B. Brightfield

MitoSOX™

C. Mitochondrial ROS

MitoSOX™ Fluorescence (AU)

shCtrl shHD5

n=145 n=133

D. Hmox1

Relative mRNA Expression

Vehicle TMP195

n=3 n=3

E. Srxn1

Relative mRNA Expression

Vehicle TMP195

n=3 n=3
Figure 7

A
Infect
Ad-FLAG-HDAC5
WT or H1006A or
Ad-β-Gal
NRVMs
IP FLAG
HDAC Activity
Assay
IB
FLAG

B
HDAC5 Activity

C
Uninf
WT
H1006A

D
Class IIa Activity

E
Mitochondrial ROS

F
Hmox1

### Key Points

- **Figure 7A**: Diagram showing the experimental setup for infecting NRVMs with Ad-FLAG-HDAC5, Ad-β-Gal, or a control, followed by HDAC activity assay and IB analysis.

- **Figure 7B**: Bar graph showing relative catalytic activity of HDAC5 with WT and H1006A variants. 

- **Figure 7C**: Western blot for HDAC5 activity with Uninf, WT, and H1006A conditions.

- **Figure 7D**: Bar graph showing relative catalytic activity of Class IIa HDAC5 with Veh and TMP195 treatment.

- **Figure 7E**: Bar graph showing mitochondrial ROS fluorescence (AU) for β-Gal, WT, and H1006A.

- **Figure 7F**: Bar graph showing relative mRNA expression for Hmox1 for β-Gal, WT, and H1006A.

### Legends

- **β-Gal**: Ad-β-Gal
- **WT**: Ad-FLAG-HDAC5 WT
- **H1006A**: Ad-FLAG-HDAC5 H1006A
- **NRVMs**: Neonatal rat ventricular myocytes
- **IB**: Immunoblotting
- **HDAC Activity Assay**: HDAC activity assay
- **Uninf**: Uninfected control
- **Veh**: Vehicle
- **TMP195**: TMP195 treatment
HDAC5 Catalytic Activity Suppresses Cardiomyocyte Oxidative Stress and NRF2 Target Gene Expression
Tianjing Hu, Friederike C. Schreiter, Rushita A. Bagchi, Philip D. Tatman, Mark Hannink and Timothy A McKinsey

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