Science Translational Medicine
AAAS

www.sciencetranslationalmedicine.org/cgi/content/full/10/427/eaao0144/DC1

Supplementary Materials for

Histone deacetylase activity governs diastolic dysfunction through a nongenomic mechanism

Mark Y. Jeong, Ying H. Lin, Sara A. Wennersten, Kimberly M. Demos-Davies, Maria A. Cavasin, Jennifer H. Mahaffey, Valmen Monzani, Chandrasekhar Saripalli, Paolo Mascagni, T. Brett Reece, Amrut V. Ambardekar, Henk L. Granzier, Charles A. Dinarello, Timothy A. McKinsey*

*Corresponding author. Email: timothy.mckinsey@ucdenver.edu

Published 7 February 2018, *Sci. Transl. Med.* **10**, eaao0144 (2018)
DOI: 10.1126/scitranslmed.aao0144

The PDF file includes:

Other Supplementary Material for this manuscript includes the following:
(available at
www.sciencetranslationalmedicine.org/cgi/content/full/10/427/eaao0144/DC1)

SUPPLEMENTARY MATERIALS

Materials and Methods

Hematological analyses

Complete blood count with differential analysis on DSS rat samples was performed by the University Colorado Hospital clinical laboratory using a Sysmex XE-5000 analyzer.

Tissue procurement

After hemodynamic recordings, animals were sacrificed by exsanguination and hearts excised and placed in ice-cold saline. RV was dissected from LV by cutting along the septum and the outer wall of the LV, and all parts weighed. Fifty milligram biopsies of the LV were flash-frozen in liquid nitrogen for subsequent biochemical and myofibril mechanics analyses. A remaining portion of the LV was mixed with optimal cutting temperature (OCT) compound prior to freezing for subsequent histological assessment of cross-sections. Tibias were cleaned and lengths were measured.

Histological analyses

Myocyte cross sectional area was quantified using latitudinal mid-sections of the LV treated with neuraminidase type V (Sigma) and stained with fluorescein labeled peanut agglutinin (10mg/ml; Vector Laboratories). Images were captured with an AxioVert 200 inverted microscope using an AxioCam MRc digital camera, and analyzed with AxioVision Release 4.8.1 imaging software (Zeiss). Approximately 100-125 myocytes were analyzed and averaged for each animal. Analysis focused on the epicardium and endocardium, where cross sections of myocytes were

most clearly visualized. For fibrosis analysis, sections were stained with Picrosirus red dye, and quantification of staining was completed by determining the average stained pixels^2 per total pixels^2 in images of the LV (18 images used per animal).

Myofibril mechanics analyses

To quantify myofibril mechanics, frozen LV sections were skinned in 0.5% Triton-X in rigor solution (132 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM Tris, 5 mM EGTA, pH 7.1) containing protease inhibitors (10 μm leupeptin, 5 μm pepstatin, 200 μm PMSF and 10 μm E64), as well as 500 μm NaN_3 and 500 μm DTT at 4^{o}C overnight. Skinned LVs were washed in fresh rigor solution and homogenized (Tissue-Tearor, Thomas Scientific) in relaxing solution (pCa 9.0) containing protease inhibitors. Myofibril suspensions were transferred to a temperature controlled chamber (15°C) containing relaxing solution. Myofibril bundles were mounted between two micro-tools. One tool was connected to a motor that could produce rapid length changes (Mad City Labs). The second tool was a calibrated cantilevered force probe (6-8 μm/μN; frequency response 2-5 KHz). Myofibrils were set 5-10% above slack myofibril length. Average sarcomere lengths and myofibril diameters were measured using ImageJ software. Mounted myofibrils were activated and relaxed by rapidly translating the interface between two flowing streams of solutions of different pCa. Data were collected and analyzed using customized LabView software. Representative traces were overlaid using OriginLab software. Measured mechanical and kinetic parameters were defined as follows: resting tension (mN/mm^2) = myofibril basal tension in fully relaxing condition; maximal tension (mN/mm^2) = maximal tension generated at full calcium activation (pCa 4.5); submaximal tension generation for force-pCa relationship; the rate constant of tension development following maximal calcium activation

= k_{ACT}; and relaxation parameters were defined as: duration of the linear relaxation = linear duration, normalized linear slope of the linear relaxation = slow k_{REL}, and the rate constant of exponential relaxation = fast k_{REL}. Force-pCa relationship (calcium sensitivity) was determined by normalizing submaximal tension (P) to maximal tension generated at pCa 4.5 (P0). Lines were drawn according to the parameters estimated by fitting to the Hill equation ($P/P0 = 1/(1 + 10^{(-nh(pCa50-pCa))})$).

Quantification of contraction and relaxation of cultured cardiomyocytes

Adult rat ventricular myocytes (ARVMs) from Sprague Dawley rats were obtained using the Langendorff-perfusion method. Mechanical properties of ARVMs were determined using the IonOptix system. In brief, ARVMs were mounted on the stage of an inverted microscope (Olympus IX-70) and superfused (~1 ml/min at 25°C) with Tyrode's solution (140 mM NaCl, 6 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 2.5 mM pyruvate and 5 mM HEPES, pH 7.4). Cardiomyocytes were field stimulated at a frequency of 1 Hz for up to 30 seconds. Each plate of cardiomyoctes was field stimulated for a total of 15 minutes and 8-10 independent traces were obtained. Data were analyzed using IonWizard software (IonOptix). Percent peak height, which is the percentage myocytes shortened on electrical stimulation, is indicative of peak ventricular contractility. Single exponential tau, which is the exponential decay time constant of the relaxation curve, represents cardiomyocyte relaxation rate.

Myofibrillar protein analyses

To determine the amount of phosphorylation of myofilament proteins, myofibril slurries were centrifuged at 1500 G for 5 min, and protein pellets were resuspended in isoelectric focusing

(IEF) buffer (8 M Urea, 2 M Thiourea, 4% Chaps). Protein (20 μg) was resolved by SDS-PAGE and stained with ProQ Diamond (Molecular Probes P33301). Gels were washed in methanol and restained with Commassie Brilliant Blue (G-250) to determine total protein abundance. To determine the abundance of β-MyHC within myofibrils, immunoblotting was performed with an anti-β-MyHC antibody (Sigma, m8421) prior to densitometry analysis (Alpha Innotech). For assessment of HDAC isoforms, whole LV lysates were homogenized in IEF buffer and were resolved by SDS-PAGE alongside solubilized myofibrillar protein pellets. Immunoblotting was performed with antibodies specific for HDAC2 (Cell Signaling Technology, mAb #5113), HDAC3 (Cell Signaling Technology, mAb #3949), and HDAC4 (Cell Signaling Technology, mAb #5392). Acetylation analysis was performed by immunoblotting myofibrillar proteins with anti-acetyl-lysine antibodies (Cell Signaling Technology, #9441 and #9681 combined), Immunoblotting was also performed to assess expression of total (Santa Cruz Biotechnology, sc-137237) and phospho-MyBP-C (Ser-282; Enzo Life Sciences, ALX-215-057-R050) and total (Santa Cruz Biotechnology, sc-52266) and phospho-TnI (Ser-24/25; Cell Signaling Technology, #4004) in myofibrillar preparations, as well as total (Badrilla, A010-14) and phospho-PLN (Ser-16; Badrilla, A010-12) in whole LV homogenates. For titin, MyBP-C and TnI, membranes were labeled with secondary antibodies conjugated with fluorescent dyes with infrared excitation spectra (CF680, goat anti-rabbit, Biotium, #20067-1). Blots were scanned using Odyssey Infrared Imaging System (LI-COR Biosciences) and the images analyzed using LI-COR software.

Titin isoforms were analyzed using the vertical agarose gel electrophoresis (VAGE) system. Briefly, frozen LV tissue was homogenized in VAGE buffer (8 M Urea, 2M Thiourea, 3% SDS, 75 mM DTT, 0.03% Bromophenol Blue, 0.05 M Tris pH 6.8) at 20:1, 40:1, or 60:1

v/w, vortexed for 30 seconds vigorously, warmed to 65°C for 15 minutes, vortexed again, and loaded on a 1% VAGE gel with 10 mM β-mercaptoethanol in the upper chamber, run at 15 mA for 5 h, and silver stained. Titin PEVK and N2B element phosphorylation was determined by immunoblotting with phospho-specific antibodies.

Ex vivo acetylation/deacetylation of myofibrillar proteins

Myofibril pellets were washed twice with p300 reaction buffer (25 mM Tris HCl pH 7.9, 50 mM KCl, 6.25 mM $MgCl_2$, 10% glycerol, 1 mM DTT) and divided into two aliquots. One aliquot was resuspended in 250 μL reaction buffer containing 25 μM recombinant p300 and acetyl-CoA (10 μM). The second aliquot was resuspended in reaction buffer only. Both aliquots were incubated in a water bath (30°C) for one hour. Myofibrils were subsequently washed once with relaxing solution containing protease inhibitors and mechanics studies were performed. Parallel samples were resolved by SDS-PAGE and immunoblotted with anti-acetyl-lysine antibodies (Cell Signaling Technology; mixture of #9441 and #9681). For deacetylation reactions, myofibrils were treated as described above and washed with HDAC2 reaction buffer (25 mM of Tris-HCl, pH 8.0, 137 mM of NaCl, 2.7 mM of KCl, 1 mM of $MgCl_2$, 0.1 mg/ml BSA), followed by incubation with recombinant HDAC2 (100 ng/μL BPS Bioscience) for 30 minutes at 15°C.

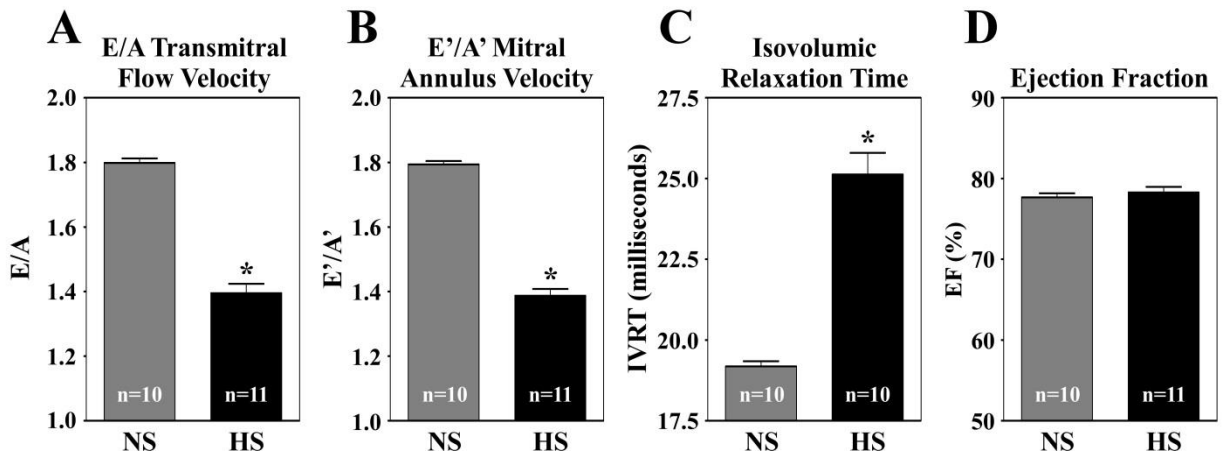


fig. S1. DSS rats fed a 4% NaCl–containing diet develop diastolic dysfunction with preserved EF. DSS rats were fed normal salt (NS) diet or a 4% NaCl containing diet (high salt [HS]) for 9 weeks. **(A)** Doppler measurement of mitral inflow velocity (E/A). **(B** and **C)** Measurements of septal mitral annulus velocities (E'/A') and isovolumic relaxation time (IVRT). **(D)** Ejection fraction (EF) determination of systolic function. Data are presented as mean +SEM, and were compared by Student's t-test. **P*<0.05 vs NS-fed controls.

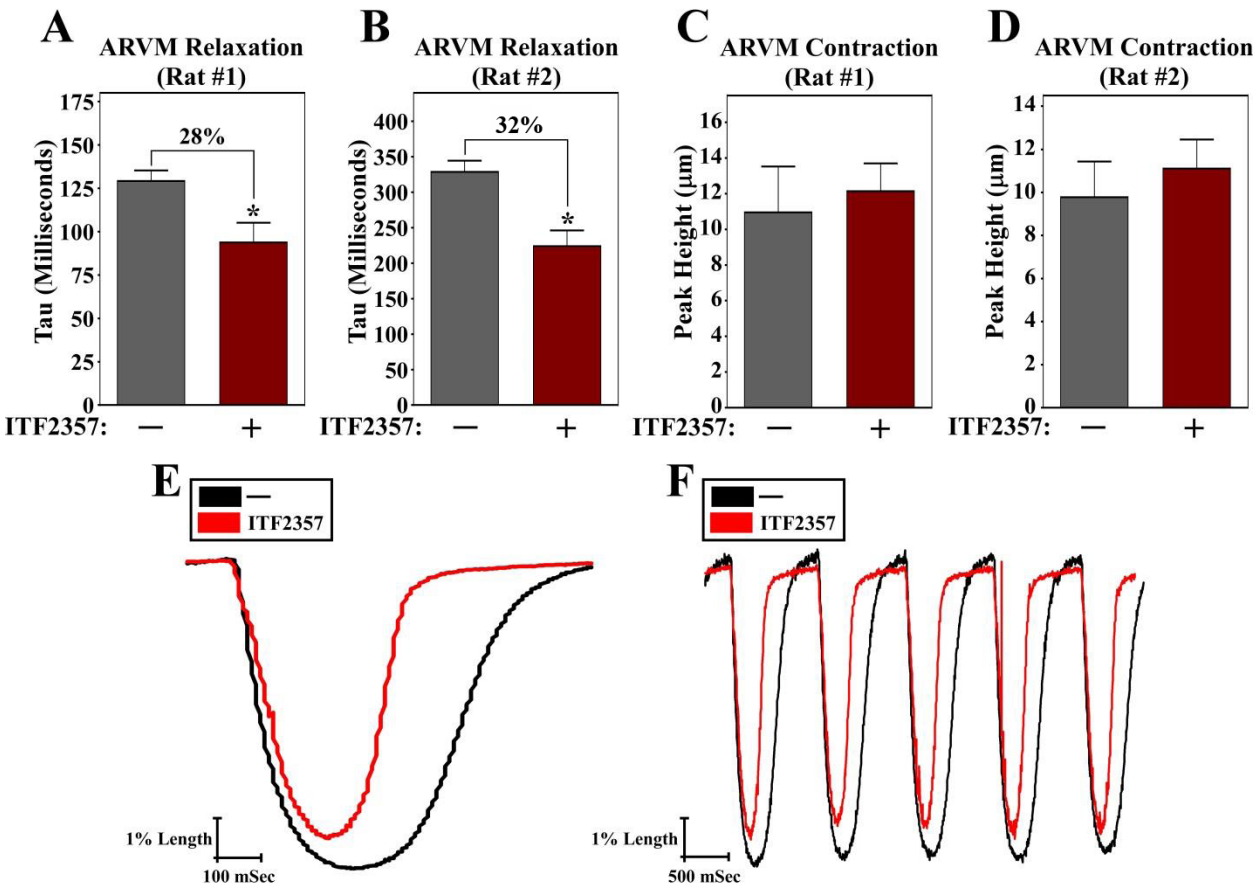


fig. S2. Treatment of ARVMs in culture with ITF2357 led to acceleration of myocyte relaxation kinetics without affecting contractility. ARVMs were cultured for 24 hours in the absence or presence of ITF2357 (1 µM) or DMSO control and electrically paced for cell shortening /re-lengthening recordings. **(A and B)** Assessment of relaxation by quantification of exponential tau in ARVMs from two independent rats. **(C and D)** Peak height determination of myocyte contraction. Data are presented as mean +SEM, and were compared by Student's t-test; n = 7 – 11 cardiomyocytes per condition. **(E and F)** Representative traces of percentile length of ARVMs treated with or without ITF2357.

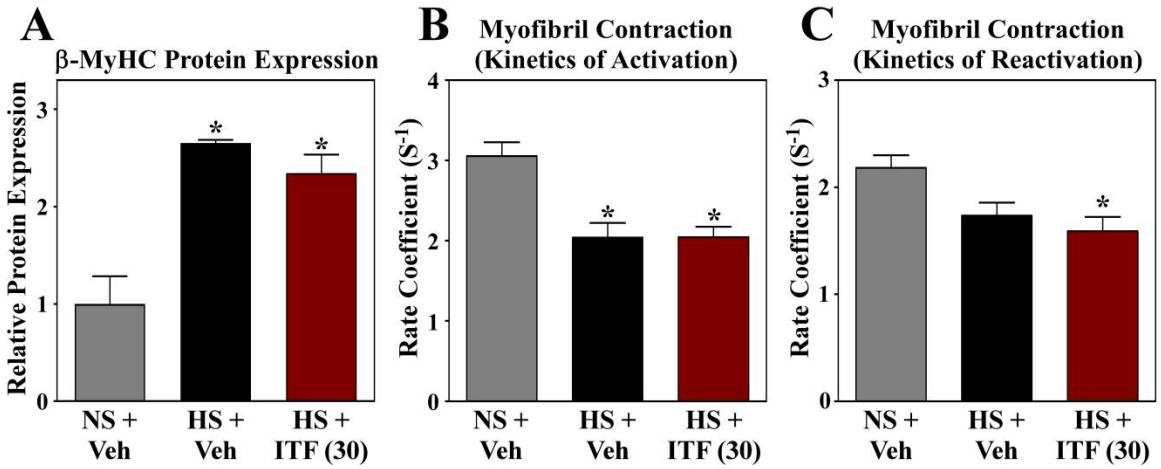
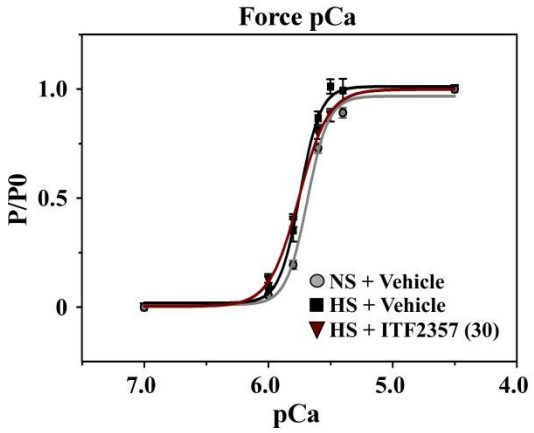


fig. S3. HDAC inhibition does not affect HS diet–induced increases in β-MyHC expression or function. (A) Immunoblot analysis of beta-myosin heavy chain (β-MyHC) isoforms in purified LV myofibrils from DSS rats fed normal salt (NS) diet or a 4% NaCl containing diet (high salt [HS]) for 10 weeks. Rats were gavaged daily with either vehicle control of ITF2357 (30 mg/kg). Band intensity was quantified by densitometry. **(B** and **C)** Ex vivo analysis of kinetics of myofibril activation and reactivation. Data are presented as mean +SEM, and were compared by one-way ANOVA with Newman-Keuls post-test. **P*<0.05 vs NS + Vehicle. n = 6 rats for NS + vehicle and HS + ITF2357, and n = 5 for HS + vehicle; 7-12 myofibrils per animal were analyzed.



	NS + Vehicle	HS + Vehicle	HS + ITF (30)
Log EC_{50}	5.686	5.746	5.754
Hill Slope	-5.099	-5.242	-3.634

fig. S4. Myofibril calcium sensitivity is unaffected by HS feeding or ITF2357 treatment of DSS rats. DSS rats were fed normal salt (NS) diet or a 4% NaCl containing diet (high salt [HS]) for 10 weeks and gavaged daily with either vehicle control of ITF2357 (30 mg/kg). Ex vivo myofibril mechanics analysis was employed to determine force-pCa relationships. Each myofibril tension point was normalized to tension generated at pCa 4.5 (P0). Data points are mean ± SEM. Lines are drawn according to the parameters estimated by fitting to the Hill equation ($P/P0 = 1/(1 + 10^{(-nh(pCa50-pCa))}$). n = 4 rats per group, 16-20 myofibrils myofibrils per animal were analyzed.

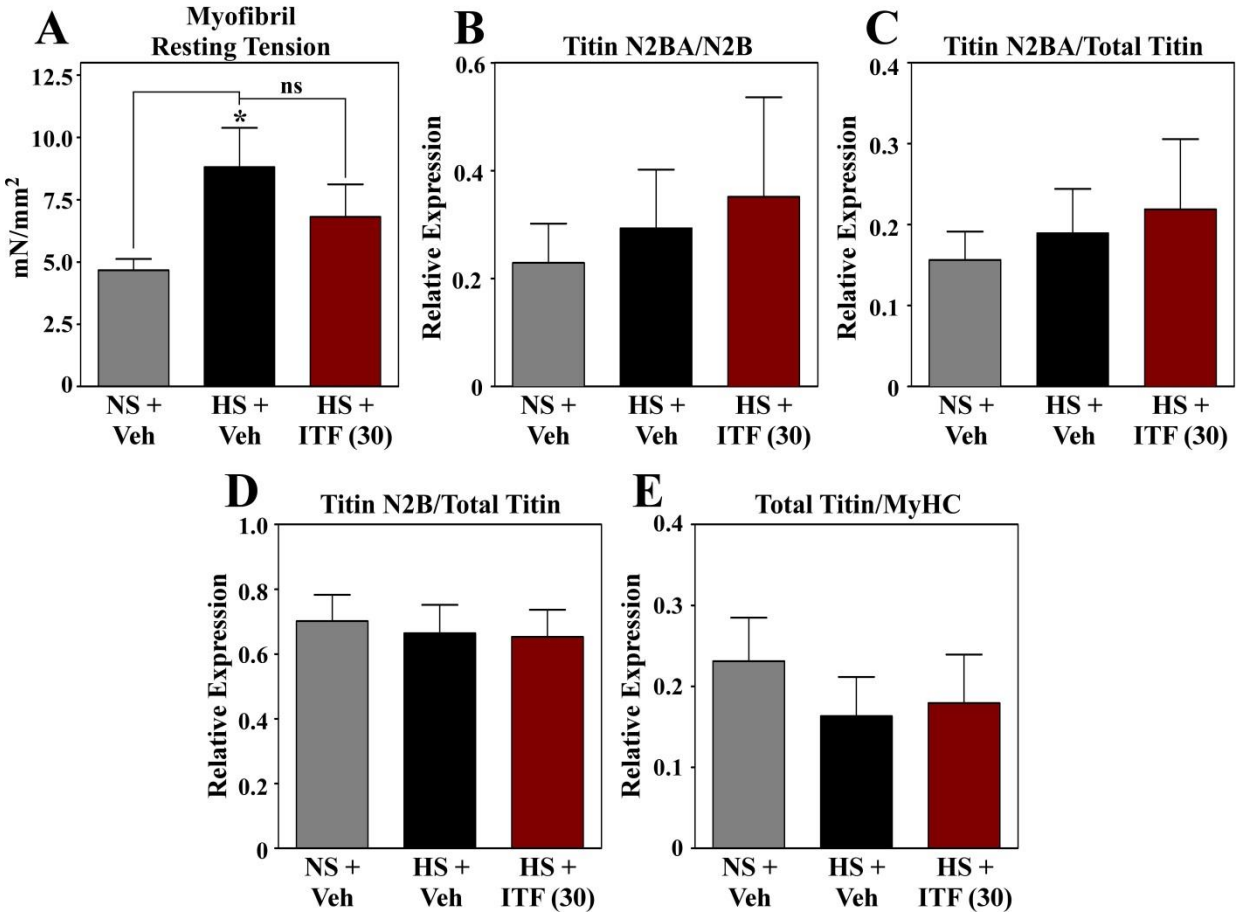


fig. S5. Titin expression and function are unaffected by HS feeding or ITF2357 treatment of DSS rats. DSS rats were fed normal salt (NS) diet or a 4% NaCl containing diet (high salt [HS]) for 10 weeks and gavaged daily with either vehicle control of ITF2357 (30 mg/kg). **(A)** Ex vivo mechanics analyses of myofibril resting tension. **(B-E)** Expression of the large titin isoform, N2BA, and the short titin isoform, N2B, were quantified. Data are presented as mean +SEM, and were compared by one-way ANOVA with Newman-Keuls post-test. **P*<0.05 vs NS-fed controls. For A, n = 6 rats for NS + vehicle and HS + ITF2357, and n = 5 for HS + vehicle; 7-12 myofibrils per animal were analyzed. For B-E, n = 6 per group.

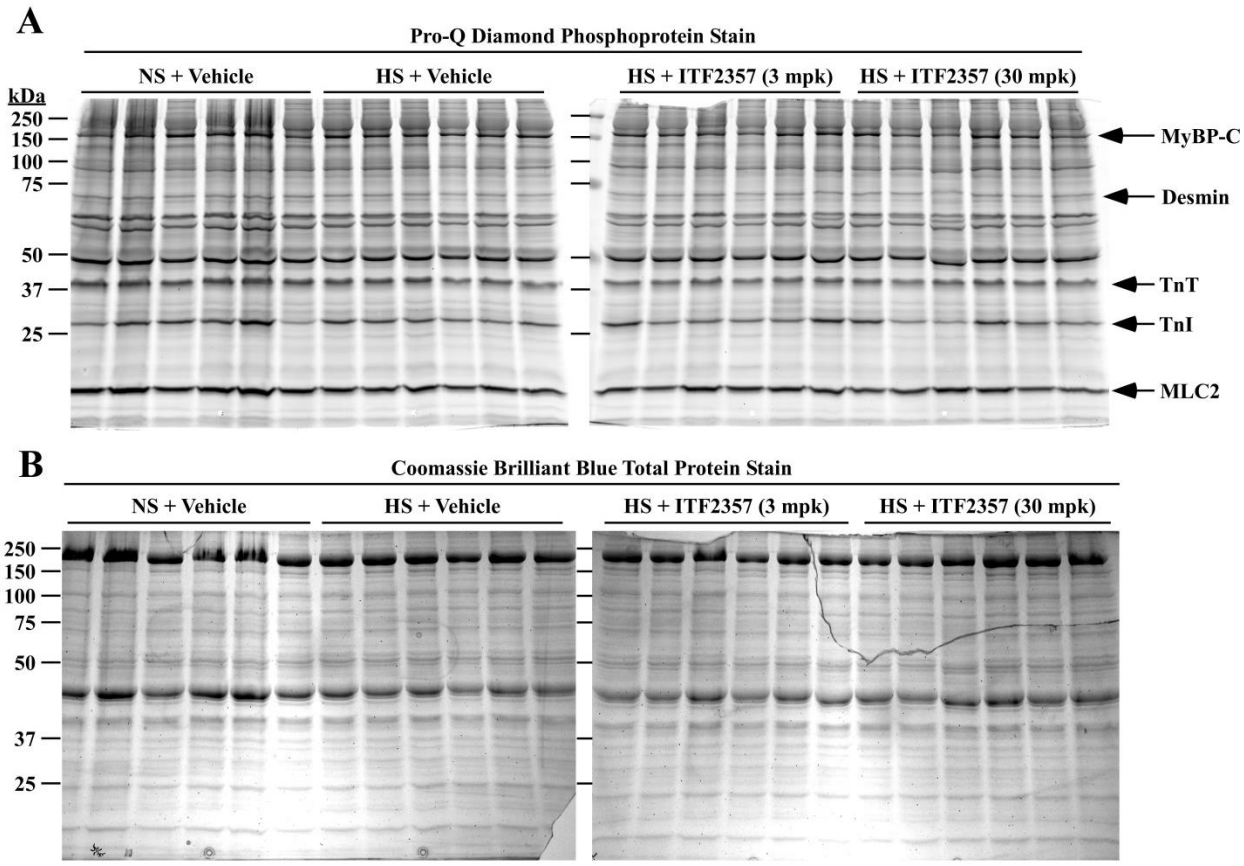


fig. S6. Myofibrillar protein phosphorylation and expression are unaffected by HS feeding or ITF2357 treatment of DSS rats. (A) Myofibrillar proteins were prepared from LV homogenates from the DSS rat groups shown in Fig. 1. Proteins were resolved by SDS-PAGE and ProQ Diamond® stain was used to determine the global phosphorylation status of myofilament proteins. The positions of myosin binding protein C (MyBP-C), desmin, troponin T (TnT), troponin I (TnI) and myosin light chain 2 (MLC2) are indicated with arrows. Molecular weight markers are shown in kilodaltons (kDa). **(B)** The same gels were stained Coomassie Brilliant Blue dye to determine the relative abundance of myofibrillar proteins. Each lane represents protein from an independent rat.

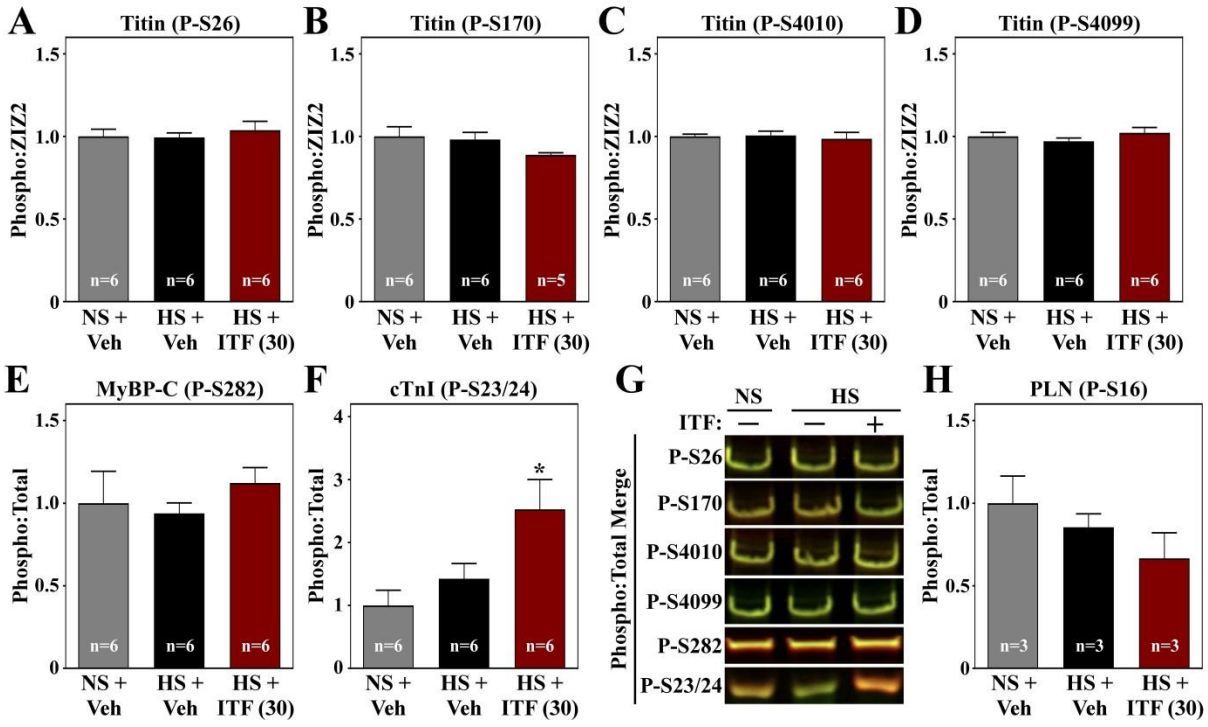


fig. S7. Site-specific phosphorylation of myofibrillar protein and myofibrillar protein expression are unaffected by HS feeding or ITF2357 treatment of DSS rats. (A-D) Phosphorylation of titin at the indicated sites was determined by immunoblotting and normalized to total abundance of the titin Z1Z2 element. **(E-F)** Phosphorylation of MyBP-C and TnI was determined by immunoblotting and normalized to the total amount of each protein. **(G)** Representative images of the overlays of the phospho- and total signals for each protein; different fluorescent secondary antibodies were employed to visualize total and phospho-proteins. **(H)** Densitometry of immunoblots was used to quantify phosphorylation of PLN at serine-16, which is a PKA target site.

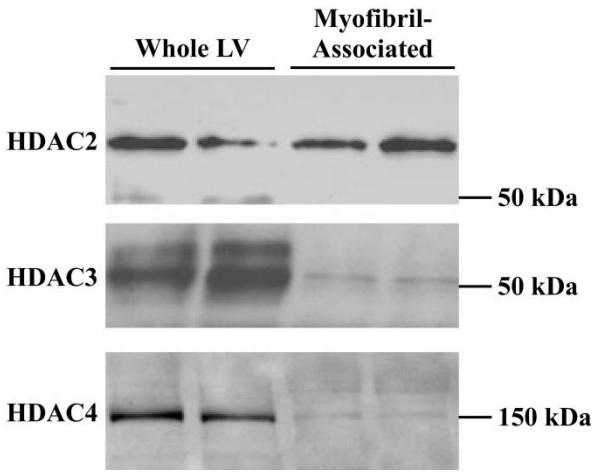


fig. S8. HDAC2 co-purifies with cardiac myofibrils. Whole LV homogenates and myofibril enriched protein preparations from DSS rat LVs were resolved by SDS-PAGE and immunoblotted with antibodies specific for the indicated HDAC isoforms.

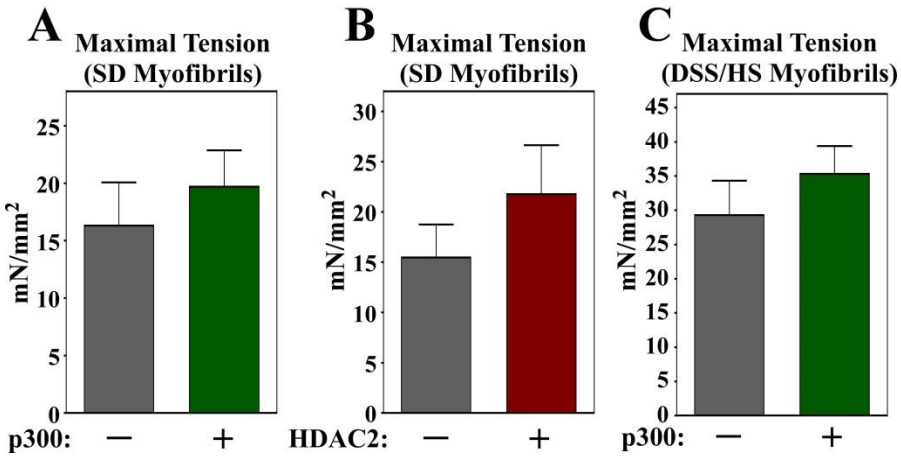


fig. S9. Ex vivo acetylation/deacetylation does not alter myofibril contraction. Myofibril maximal tension generation data associated with the relaxation data shown in Fig. 4.

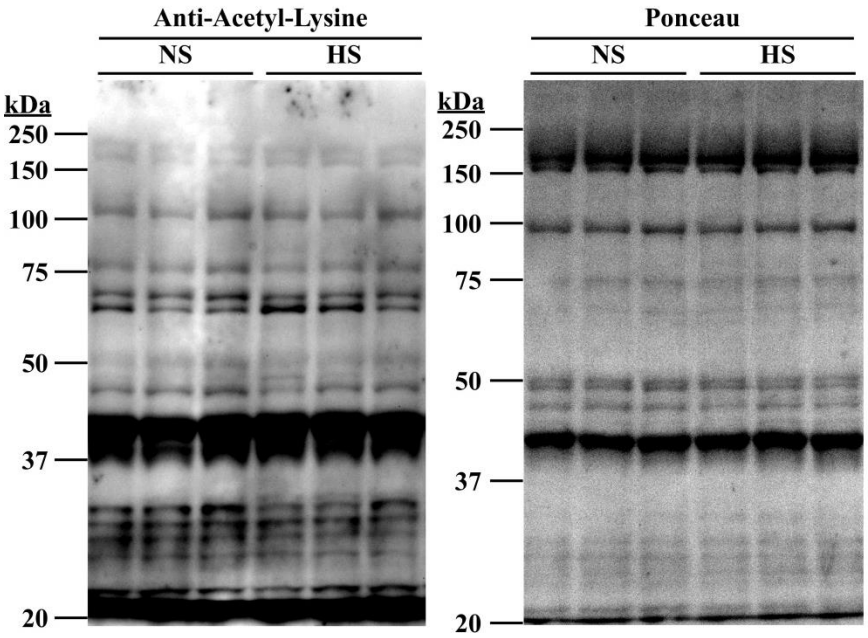


fig. S10. Anti–acetyl-lysine immunoblotting of cardiac myofibrils from DSS rats. (A) Myofibrillar proteins from LVs of DSS rats fed normal salt (NS) or high salt (HS) were resolved by SDS-PAGE and immunoblotted with anti-acetyl-lysine antibodies. **(B)** The membrane was subsequently stained with Ponceau-S dye to control for protein loading.

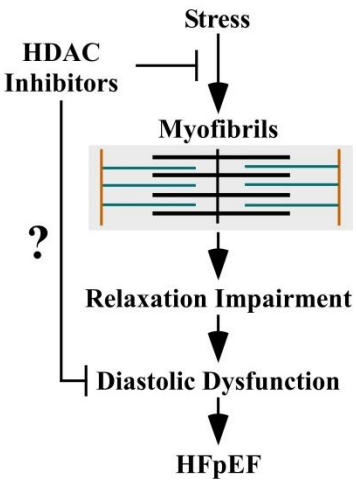


fig. S11. A model for HDAC inhibitor–mediated improvement in diastolic function and treatment of HFpEF. Stress signaling in the heart leads to impaired myofibril relaxation, and HDAC inhibition can block this negative action, likely by stimulating myofibrillar protein acetylation. HDAC inhibitors may also improve diastolic function of the heart through myofibril-independent mechanisms (depicted as a question mark), such as by blocking cardiac fibrosis.

table S1. Hematological profiles of DSS rats. Complete blood count with differential was obtained for each group of DSS rats shown in Fig. 1. **P*<0.05 vs NS-fed controls.

	NS + Vehicle	**HS + Vehicle**	**HS + ITF2357 3 mpk**	**HS + ITF2357 30 mpk**
Hematocrit (%)	46.0 ± 0.47	42.7 ± 0.87	42.3 ± 0.50	42.2 ± 1.55
Red Blood Cells (10^6/μl)	8.92 ± 0.09	7.57 ± 0.38*	7.86 ± 0.17*	7.66 ± 0.39*
Mean corpuscular volume (MCV)	51.6 ± 0.40	56.5 ± 1.15*	54.7 ± 0.66	55.9 ± 1.15
Nucleated Red Blood Cells (10^3/μl)	0.01 ± 0.01	0.11 ± 0.04	0.10 ± 0.03	0.10 ± 0.03
White Cells (10^3/μl)	7.95 ± 0.27	8.99 ± 0.72	10.0 ± 0.82	9.03 ± 0.93
Platelets (10^3/μl)	655 ± 32.3	704 ± 35.8	625 ± 26.2	644 ± 33.9
Neutrophils (10^3/μl)	0.93 ± 0.13	1.51 ± 0.28	1.42 ± 0.18	1.28 ± 0.20
Lymphocytes (10^3/μl)	6.83 ± 0.26	7.10 ± 0.67	8.19 ± 0.65	7.14 ± 0.68
Monocytes (10^3/μl)	0.18 ± 0.06	0.32 ± 0.16	0.38 ± 0.12	0.35 ± 0.09
Mean ± SEM; **P*<0.05 vs NS + Vehicle				

table S2. LV hemodynamic and echocardiographic parameters of DSS rats. A repeat study was performed with DSS rats fed NS, HS or HS + 30 mg/kg.

Pressure-Volume Measurements	**NS + Vehicle**	**HS + Vehicle**	**HS + ITF2357 30mpk**
LVEDP (mmHg)	6.3 ± 0.6	13.6 ± 0.5*	10.7 ± 0.6*#
Isovolumic relaxation constant (Tau, Weiss)	10.5 ± 0.3	12.8 ± 0.6*	11.3 ± 0.5
EDPVR	0.0015 ± 0.0002	0.0029 ± 0.0006	0.0013 ± 0.0002#
Arterial Elastance	0.42 ± 0.03	0.68 ± 0.04*	0.58 ± 0.04*
LVESP (mmHg)	126.6 ± 4.2	171.5 ± 5.4*	157.1 ± 7.2*
dP/dT min	-6640 ± 308	-7964 ± 311	-8216 ± 482*
dP/dT max	6286 ± 244	7807 ± 303*	8314 ± 479*
SV (µl)	298.2 ± 13.1	257.0 ± 12.4	279.8 ± 12.5
HR (BPM)	327.7 ± 6.5	355.1 ± 11.5	346.3 ± 8.0
LVESV (µl)	227.6 ± 10.1	168.0 ± 8.1*	160.5 ± 10.8*
LVEDV (µl)	525.8 ± 12.7	425.0 ± 17.1*	440.2 ± 16.7*
	(n = 8)	(n = 9)	(n = 11)
Echocardiography			
E/A	1.79 ± 0.02	1.40 ± 0.03*	1.78 ± 0.02#
E'/A'	1.79 ± 0.01	1.39 ± 0.02*	1.77 ± 0.02#
Deceleration Time (ms)	13.7 ± 0.2	16.5 ± 0.4*	13.5 ± 0.3#
IVRT (ms)	19.2 ± 0.2	25.1 ± 0.7*	21.7 ± 0.4*#
EF (%)	77.7 ± 0.5	78.3 ± 0.7	78.9 ± 0.5
HR (BPM)	388 ± 8	380 ± 12	370 ± 7
	(n = 10)	(n = 10)	(n = 11)
Mean ± SEM; *P<0.05 vs NS + Vehicle, #P<0.05 vs HS + Vehicle			

table S3. Quantification of myocyte cross-sectional area and interstitial fibrosis in DSS rats.

Subject level data are shown. Numbers represent averages from 100-125 myocytes per animal, with four animals per group.

Myocyte cross-sectional area (μm^2)	**NS + Vehicle**	**HS + Vehicle**	**HS + ITF2357 30mpk**
	163.5	203.4	218.8
	135.4	253.7	200.1
	155	196.5	215.5
	136.7	207	221.7
Interstitial fibrosis (collagen fraction %)	**NS + Vehicle**	**HS + Vehicle**	**HS + ITF2357 30mpk**
	1.41	2.96	4.2
	2.12	2.32	2.82
	4.4	4.76	2.29
	2.77	4.41	2.95

table S4. Echocardiographic parameters of aging mice. Values are associated with the study shown in Fig. 5.

Echocardiography	**Normal Chow (20 Months)**	**ITF2357 Chow (20 Months)**
E/A	1.05 ± 0.03	1.30 ± 0.06*
E'/A'	1.10 ± 0.02	1.36 ± 0.04*
E/E'	23.4 ± 2.2	20.4 ± 2.4
Deceleration Time (ms)	26.2 ± 1.8	19.5 ± 1.7*
IVRT (ms)	18.6 ± 0.5	15.2 ± 0.6*
EF (%)	61.3 ± 1.28	62.6 ± 1.81
HR (BPM)	533 ± 17.1	491 ± 18.1
Mean ± SEM; *P<0.05 vs Normal Chow		

table S5. Echocardiographic, hemodynamic, and hypertrophy parameters of aging mice.

See attached Excel spreadsheet for data for individual animals.

table S6. Characteristics of the RCM patients and nonfailing donor controls. Left ventricular tissue from these individuals was employed for myofibril mechanics analyses.

	Age	Gender	Ejection Fraction	Hypertension	Diabetes	Renal failure
RCM 1	66	F	65	Yes	Yes	No
RCM 2	21	M	65	No	No	No
Donor 1	66	F	55	Yes	No	No
Donor 2	52	F	55	No	No	No
Donor 3	48	F	55	Yes	No	No
Donor 4	43	M	65	No	Yes	No
Donor 5	19	M	65	No	No	No

table S7. Group sizes of animal models. The number of animals analyzed at specific times for each endpoint is listed.

Figure 1	**NS + Vehicle**	**HS + Vehicle**	**HS + ITF2357 3 mpk**	**HS + ITF2357 30 mpk**
(B) E/A, number of rats for 0, 6, 8 and 10 weeks	8, 9, 7, 9	9, 10, 9, 10	8, 10, 9, 10	8, 9, 8, 10
(D) E'/A', number of rats for 0, 6, 8 and 10 weeks	8, 9, 7, 9	9, 11, 10, 10	8, 10, 10, 10	8, 10, 9, 9
(E) IVRT, number of rats for 0, 6, 8 and 10 weeks	8, 9, 9, 9	9, 10, 9, 10	8, 10, 10, 10	8, 9, 9, 9
(F) EF, number of rats for 0, 6, 8 and 10 weeks	8, 8, 9, 9	9, 11, 10, 10	8, 10, 10, 10	8, 10, 9, 9
Figure 2	**NS + Vehicle**	**HS + Vehicle**	**HS + ITF2357 3 mpk**	**HS + ITF2357 30 mpk**
(A) LVPW;d, number of rats for 0, 6, 8 and 10 weeks	8, 9, 9, 9	9, 11, 10, 10	8, 10, 10, 10	8, 10, 9, 9
(B) LVAW;d, number of rats for 0, 6, 8 and 10 weeks	8, 9, 9, 9	9, 11, 10, 10	8, 10, 10, 10	8, 10, 9, 9

Figure 5	**Normal Chow**	**ITF2357 Chow**
(B) E/A, number of mice at 15, 17 and 20 weeks	4, 5, 6	7, 5, 6
(C) E'/A', number of mice at 15, 17 and 20 weeks	5, 4, 5	6, 4, 4
(D) IVRT, number of mice at 15, 17 and 20 weeks	4, 5, 6	5, 4, 4