Supplemental Material

Hrd1 and ER associated protein degradation are critical elements of the adaptive ER stress response in cardiac myocytes

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METHODS

Cultured Cardiac Myocytes: Cells were isolated by enzymatic digestion of 1-4 day-old neonatal rat hearts and purified by Percoll density gradients, as described [1,2]. Cultures were plated in Dulbecco's modified Eagle's media (DMEM)/F-12 containing 10% fetal bovine serum (FBS) and after 24h the medium was changed to DMEM/F-12 containing 2% FBS. Unless otherwise stated, all experiments were carried out in 2% FBS-containing media.

Hrd1 Antiserum Preparation and Characterization: A custom Hrd1 antiserum was generated in rabbits against a keyhole limpet hemocyanin-conjugated synthetic peptide, PEDGEPDAAELRRRRLQKLE, which is identical to residues 593-612 in the C-terminus of human Hrd1, and is conserved in residues 588-607 of mouse Hrd1, and in residues 584-603 of rat Hrd1. The cross-reactivity of the antibody against Hrd1 was shown by transfecting HeLa cells with an expression plasmid encoding either untagged, or FLAG-tagged mouse Hrd1, then demonstrating, by immunoblotting, that the Hrd1 antibody cross-reacts with endogenous and overexpressed Hrd1 at 68 kD, the predicted molecular mass or Hrd1. Moreover, when extracts from FLAG-Hrd1-transfected cells were examined, FLAG and Hrd1 cross-reactive material co-migrated on SDS gels, again, at about 68 kD. The specificity of the Hrd1 antiserum against endogenous and overexpressed Hrd1 was shown by blocking it with the peptide to which it was raised (see above), and then using immunoblotting to show that the 68 kD form of endogenous and overexpressed Hrd1 observed with the unblocked antiserum was not present when blocked antiserum was used.

siRNA Transfection into Cultured Cardiac Myocytes: Cultured cardiac myocytes were plated on 24 mm plates at 0.5 to 0.8 X 10^6 cells per well, then transfected with small interfering (si) RNA oligoribonucleotides targeted to rat Hrd1 (Life Sciences Technologies, Inc., Stealth siRNAs (set of 3) RSS324147, RSS324148, RSS324149), or control (Life Sciences Technologies, Inc.,12935-300). Each well was transfected with 10 pmoles of each siRNA using TransMessenger™ Transfection Reagent (Qiagen, Valencia, CA). After ~20h, in some cases, cells were treated with adenovirus for 24h, after which they were treated ± CHX (100mM) in 2% FBS containing media for varying times as shown in the figures.

Adenovirus (AdV): AdV-Hrd1- A recombinant Adv encoding full-length mouse Hrd1 (1-612) (Adv-Hrd1) was produced by first generating a PCR product that included the full-

length mouse Hrd1 (NCBI RefSeq NM_028769) and cloning it into pcDNA3.1, from which the Hrd1 coding region was excised and cloned into the adenovirus shuttle vector, pAdTrack-CMV, which was then used to generate the desired Adv strain in 293 cells, as previously described [3]. A recombinant AdV encoding TCR- α-HA was produced by generating a PCR product from the plasmid template that was a generous gift from Dr. Ron Kopito, Stanford University, that included the full-length TCR-α and C-terminal HA epitope tag. All further cloning and AdV preparation was as described above for AdV-Hrd1.

AdV- TCR-α-HA- A recombinant AdV encoding TCR-α-HA was produced by generating a PCR product from the plasmid template that was a generous gift from Dr. Ron Kopito, Stanford University, that included the full-length TCR- α and C-terminal HA epitope tag. The portion of the plasmid containing the TCR-α-HA coding region was excised and cloned into the adenovirus shuttle vector, pAdTrack-CMV, which was then used to generate the desired Adv strain in 293 cells.

Cell Extracts and Immunoprecipitation: Cells were extracted and subjected to immunoprecipitation and immunoblotting, as previously described [4].

Immunoblotting and Antibodies: The following antibodies were used at the following concentrations for immunoblotting: FLAG (Sigma-Aldrich F1804; 1:12,000), CHOP (Cell Signaling, D46F1, 1:1,000), Gapdh (RDI, TRK5G4; 1:150,000), HA-probe F-7 (Santa Cruz, SC-7392; 1:1,000), Hrd1 (see above; 1:20,000), and KDEL (ENZO Life Sciences; ADI-SPA-827; 1:8,000), which detects the C-terminal KDEL on Grp94 and Grp78.

Hrd1 Ubiquitination Assay: To examine the ubiquitin ligase activity of Hrd1, HeLa cells were transfected with a plasmid encoding HA-tagged ubiquitin, and either a control plasmid, or a plasmid encoding mouse FLAG-mouse-Hrd1. Twenty-four hours later, extracts were either fractionated by SDS-PAGE, then examined by immunoblotting for Hrd1 and Gapdh, or they were subjected to FLAG immunoprecipitation, fractionated by SDS-PAGE, and then blotted for FLAG, in order to detect overexpressed FLAG-Hrd1. In some cases, FLAG-immunoprecipitates were blotted for HA in order to detect ubiquitin on immunoprecipitated FLAG-Hrd1. A HA-ubiquitin ladder demonstrates the ability of FLAG-Hrd1 to ubiquitin itself, which serves as an indicator of the ubiquitin ligase activity of Hrd1.

ERAD Assay: ER associated degradation (ERAD) was determined using a C-terminally HA-tagged version of the model substrate, TCR-α, essentially as described [5]. Briefly, 24-48h after plating, neonatal rat ventricular myocytes, which in some cases had been treated with siRNA (e.g. si-Con or si-Hrd1) during plating, were infected with AdV-TCR-α-HA. In some cases, cultures were also infected with another AdV (e.g. AdV-Con or AdV-Hrd1). Twenty-four hours later, culture media was replaced with media containing 2% FBS and no AdV. Twenty-four hours later, cultures were treated with CHX (100mM) for the times shown in the figures, after which they were extracted, followed by SDS-PAGE, and then blotted for HA. The immunoblots were quantified by densitometry, and TCR-α-HA/Gapdh ratios were determined. In cases where the effects of Hrd1 overexpression or knockdown, or TM or TG treatment on ERAD were examined, the TCR-α-HA/Gapdh values, usually the average of n = 3 cultures/treatment, obtained with no CHX treatment were set to 1.0, and the relative levels of TCR-α-HA/Gapdh after 30,

or 60 mins of CHX treatment were compared to it to obtain rates of TCR-α-HA degradation for a given treatment.

Quantitative Real Time PCR: Quantitative real time PCR (qRT-PCR) was carried out as previously described using primers for mouse Hrd1, ATF6, atrial natriuretic peptide, B-type natriuretic peptide, GRP78, collagen 1A1, β-myosin heavy chain, β-actin and GAPDH, which have also been previously described[6].

Mouse Heart Extraction: Extracts from left ventricles were prepared by homogenization using a pestle (Wheaton; 358133) and a 1.5ml microcentrifuge tube in RIPA buffer comprising NaCl 150 mM, Tris-HCl (pH 7.5) 20 mM, Triton-X 1%, Na deoxycholate 0.50%, sodium dodecyl sulfate 1%.

Adeno-associated Virus (AAV): To prepare AAV to knockdown Hrd1 in the heart, an shRNA targeted to mouse Hrd1 was prepared using the following oligonucleotides:

sense: GCTAGCGCTTCTGTGCAGCTGGTAGTTTTCAAGAGAAAATACCAGC
TCGACAGAAGCCTTTTGC

antisense: GCGAGCAAAAAGGCTTCTGTGCAGCTGGTATTTTCTCTTGAAAA
CTACCAGCTGCACAGAAGCG,

which were annealed and cloned into the NheI and XhoI sites in pTRUFU6.
AAV9-sh-Con was prepared by cloning into pTRUFU6. An shRNA directed against a portion of firefly luciferase, which is predicted not to target any mouse transcripts, was generated as a control. The luciferase shRNA was prepared beginning with the following oligonucleotides:

sense: CTAGCGCTCAACAGTATGGGCATGTCTTCAAGAGAGAAATGCCC
ATACTGTTGAGCCTTTTTGC

antisense: GCGAGCAAAAAGGCTCAACAGTATGGGCATTTCTCTCTTGAA
GACATGCCCATACTGTTGAGCG,

which were annealed and cloned into the NheI and XhoI sites in pTRUFU6.

To prepare AAV to overexpress Hrd1 in the heart, the vector, pTRUF12, a gift from Dr. Roger Hajjar, was modified by removing the GFP downstream of the IRES, and adding restriction sites into the multiple cloning site to include Nhe1, Pme1, Xho1, and Mlu1. The CMV promoter was replaced with $CMV_{enh}MLC800$ promoter by standard cloning methods, to generate pTRUF-$CMV_{enh}MLC800$, which was used to generate AAV9-Con. The vector used to generate AAV9-Hrd1 was constructed by cloning the cDNA from pcDNA3.1-Hrd1 (mouse) into the Xho1 and HindIII sites in pTRUF- $CMV_{enh}MLC800$. To generate recombinant AAV9, HEK293T cells were transfected with pTRUF helper plasmid and the appropriate pTRUF-$CMV_{enh}MLC800$, or pTRUFU6 plasmids, and standard virus amplification, purification, and plaque assays were performed. For detailed protocol of AAV9 generation, see the Supporting Materials and Methods of [7]. To administer recombinant AAV, mice were anesthetized with 2% isofluorane and 100 ml of 37ºC heated Lactated Ringer's containing 10^{11} genome-containing units per mouse were injected via tail vein.

Immunocytofluorescence of Cultured Cardiac Myocytes: Neonatal rat ventricular myocytes, isolated as described above, were planed onto glass slides (Labl-Tec cat no. 177380 or 154461) that had been pretreated with fibronectin (25 μg/ml in serum free media for 1h). Cells were plated in DMEM containing 10% fetal bovine serum at a density of $2X10^6$ cells/chamber. Twenty-four hours later, cells were treated with the appropriate AdV, described above. Twenty-four hours later, media was changed to DMEM containing 10% fetal bovine serum and, in some cases, cells were treated with TM (10 μg/ml) for 20h. Slides were then washed with PBS then fixed with 4% paraformaldehyde for 20 min, and then washed with PBS, permeabilized with 0.1% triton X-100 in PBS for 10 min, washed with PBS and then blocked for 1h with 10% horse serum in PBS. After removal of the chamber and gasket, slides were incubated with the appropriate primary antibody diluted in 10% horse serum/PBS. The primary antibodies used were anti-Hrd1 at 1:200 or anti-KDEL at 1:200. Twenty-four hours later, slides were washed with PBS, then incubated with the appropriate secondary antibody, i.e. donkey anti-rabbit-FITC 1:300 and donkey-anti-mouse 1:300. Twenty-four hours later, TOPRO was added at 1:10,000 to stain nuclei, after which cells were mounted with Vectashield and images were obtained on a Zeiss 710 laser-scanning confocal microscope with a 63X objective. Shown in each figure are small black and white images of each staining layer adjacent to the image of the merged layers.

Immunocytofluorescence of Mouse Heart Sections: Hearts were cleared by retroperfusion in situ with PBS at 70 mmHg, arrested in diastole with 60 mM KCl, fixed by perfusion for 15 min with 10% formalin (Sigma; HT501128), excised, fixed in formalin for 24 hours at room temperature, and embedded in paraffin. Paraffin-embedded hearts were sectioned and placed on slides, which were then deparaffinized, then rehydrated. Antigen retrieval was achieved by boiling the slides in 10 mM citrate pH 6.0 for 12 min, after which slides were washed several times with distilled water, and once with Tris/NaCl, or TN buffer (100 mM Tris, and 150 mM NaCl). Affinity-purified Hrd1 antiserum was diluted with TNB and added to slides which were incubated at 4 °C for approximately 12-16h. Samples were then washed with TN buffer and incubated with secondary antibodies at room temperature in the dark for 2 h. Images were obtained using a Leica TCS SP2 laser-scanning confocal microscope. Images were obtained with a 63X objective. Shown in each figure are small black and white images of each staining layer adjacent to the image of the merged layers.

TUNEL Assay: Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining was performed using the In Situ Cell Death Detection Kit, TMR red (Roche Applied Science). After deparaffinization, antigen retrieval was performed by boiling in 10mmol/L citrate at 50% microwave power. Sections were incubated for 1 hour at 37°C with 0.5ul Enzyme diluted in 29.5ul Dilution Buffer (Roche; 11966006001) and 25ul Labeling Solution. Sections were then washed and incubated with Alexa fluor 488–conjugated wheat germ agglutinin (Life Technologies) for 1.5 hours, and with TOPRO for 15 min. TUNEL staining was quantified in 3,000 nuclei from each of 5 or more randomly selected fields per heart. At least 4 hearts from each group were examined. Matched areas from the LV, IVS, and RV were sampled in each heart. Operators were blinded to treatments.

Trans-aortic Constriction: Trans-aortic constriction (TAC) was carried out essentially as described [8]. Briefly, adult male C57BL/6 **(Fig. 4)** or FVB **(Fig. 6)** mice were anesthetized with isofluorane, intubated, and a trans-sternal thoracotomy performed. A constriction of the aorta at the arch was performed by tying a 7.0 suture against a 27-gauge needle.

The needle was removed leaving a calibrated stenosis of the aorta. Sham-operated mice were exposed to the same procedure, except that the aorta was not constricted. The chest was closed and the animals were allowed to recover.

Echocardiography: Echocardiography was carried out on anesthetized mice using a Visualsonics Vevo 770, or a Visualsonics Vevo 2100 high-resolution echocardiograph, as previously described [9].

Calcium Handling: Eight week-old FVB mice were treated with $1x10^{11}$ genome containing units of AAV9-Con or AAV9-Hrd1. Cardiac myocytes were isolated 3 weeks after AAV9 administration and used for Ca^{2+} handling experiments, as described [10].

Statistics: Cell culture experiments were performed at least 3 times with triplicate cultures for each treatment, unless otherwise stated, or shown. Values for data are mean ± standard error of the mean (SEM). Unless otherwise indicated, statistical treatments were by ANOVA followed by Newman-Keuls post hoc analysis.

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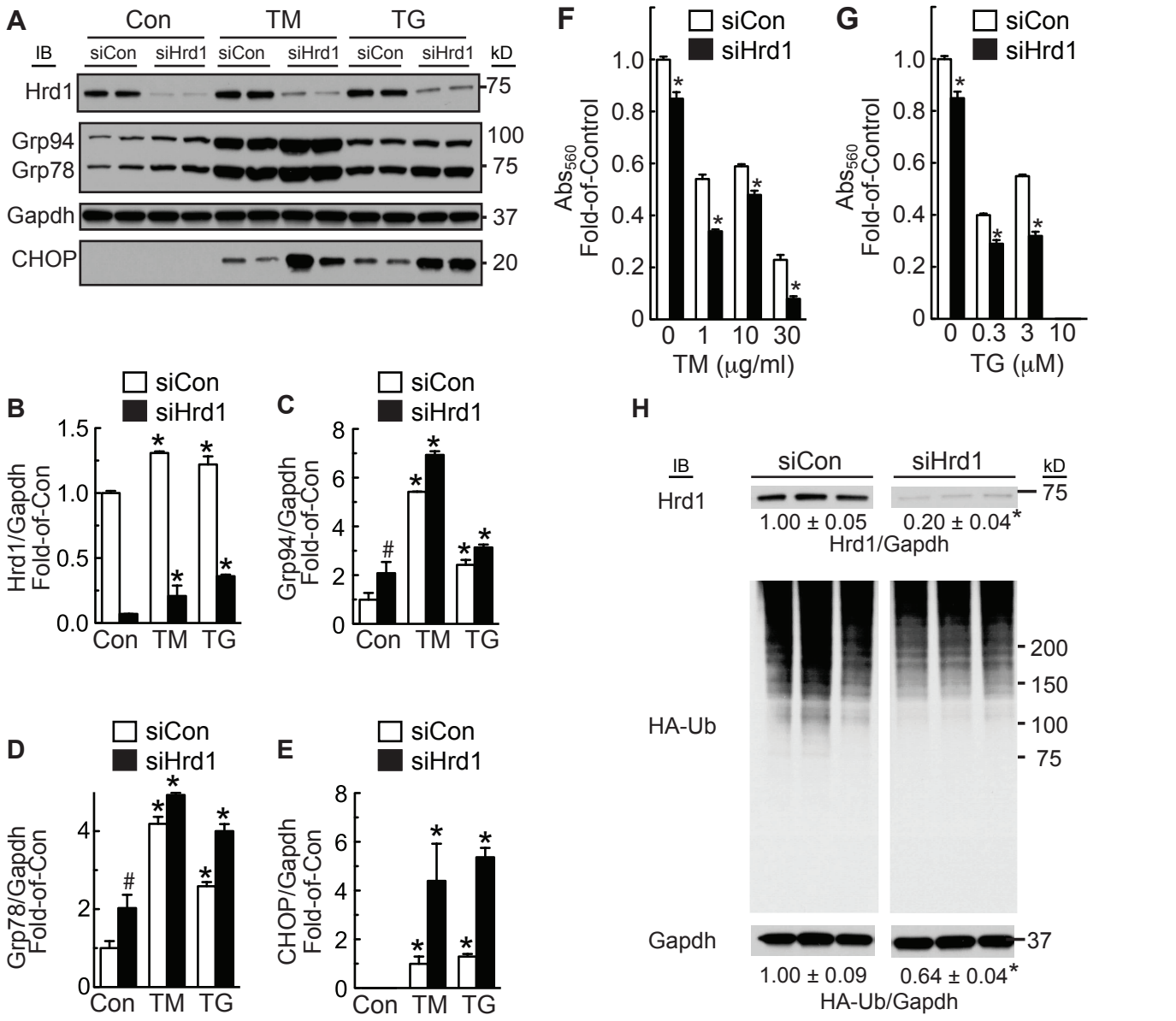
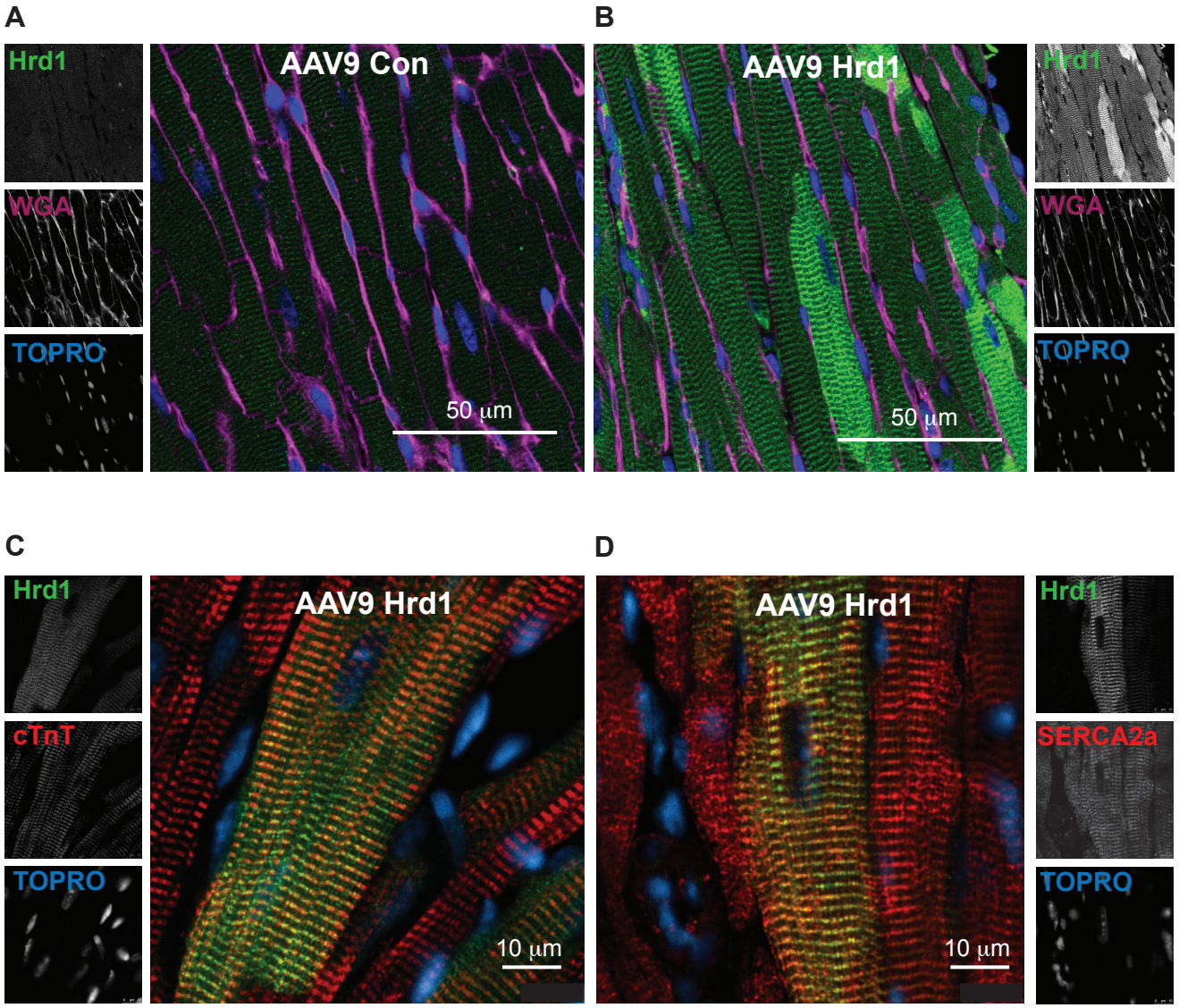


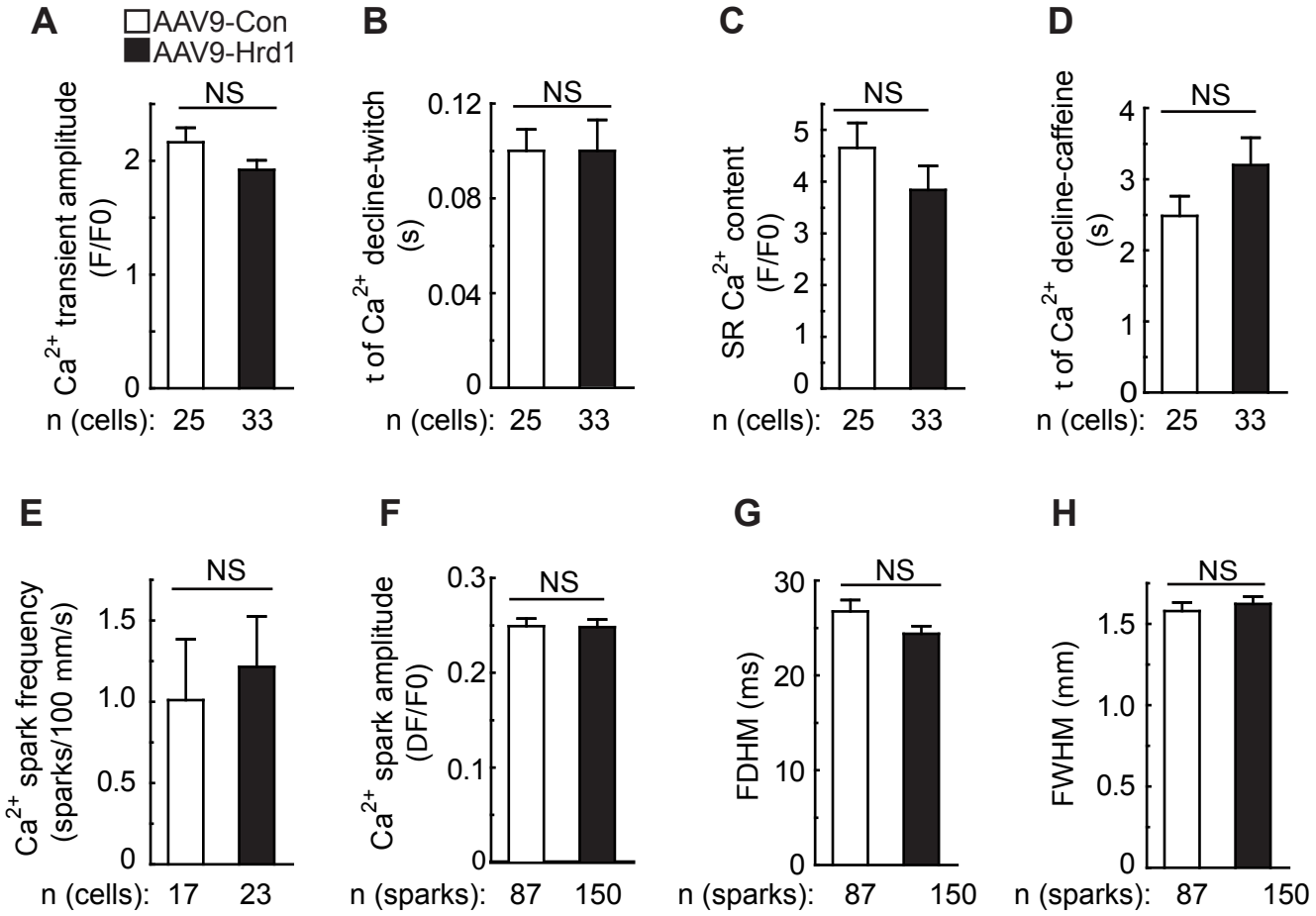
Online Figure I- Effects of Hrd1 Knockdown on Markers of ER Stress and Myocyte Viability
Cultured cardiac myocytes were treated with siCon or siHrd1 for 48h, then vehicle, TM (10 μg/ml) or TG (1 μM) for 72h. **A,** Hrd1, Grp94, Grp78, Gapdh, and CHOP were measured by immunoblotting. **B-E,** Densitometry of the blots shown in (a) normalized to vehicle-treated siCon, except for CHOP, which was normalized to TM-treated siCon. * = $p \leq 0.05$ different from Con; # = $p \leq 0.05$ different from Con/siCon by t-test. **F and G,** Cultured cardiac myocytes were treated as described in **A**, after which cell viability was determined by MTT assay. * = $p \leq 0.05$ different from siCon at the same dose and time of TM or TG treatment, as determined by t-test. **H,** Cultured cardiac myocytes were infected with AdV-HA-Ubiquitin and treated with either siCon or siHrd1. After 48h, extracts were analyzed for ubiquitylation by anti-HA immunoblotting. Densitometry was used to determine the relative levels of Hrd1 **(top)** and HA-Ub **(bottom)**. * = $p \leq 0.05$ different from siCon by t-test.

Online Figure I



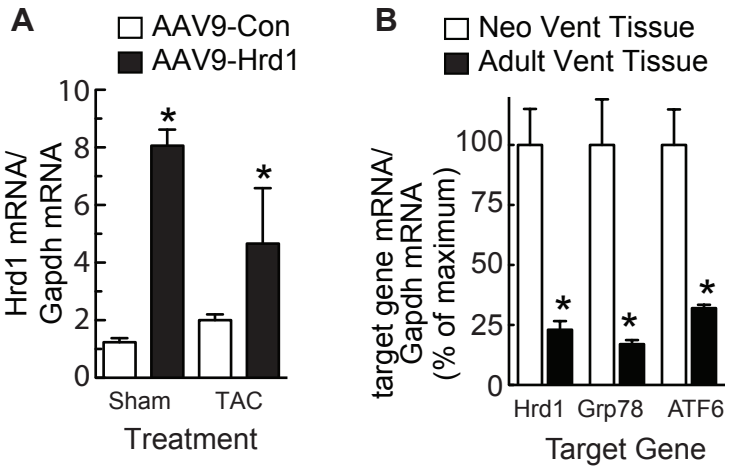
Online Figure II- Immunocytofluorescence Analaysis of Hrd1 Overexpression in Mouse Hearts
A and B, Confocal immunocytofluorescence microscopy analysis of mouse heart sections for Hrd1 (green), wheat germ agglutinin (WGA), to outline cells (magenta), and TOPRO for DNA (blue), in sections of hearts from mice treated for 6 weeks with AAV9-Con A, or AAV9-Hrd1 B. Bar = 50 μm.
C and D, Confocal immunocytofluorescence microscopy analysis of mouse heart sections for Hrd1 (green), cardiac troponin T (red) **C**, SERCA2a (red) **D**, and TOPRO for DNA (blue) in sections of hearts from mice treated for 6 weeks with AAV9-Hrd1. Bar = 10 μm.

Online Figure II



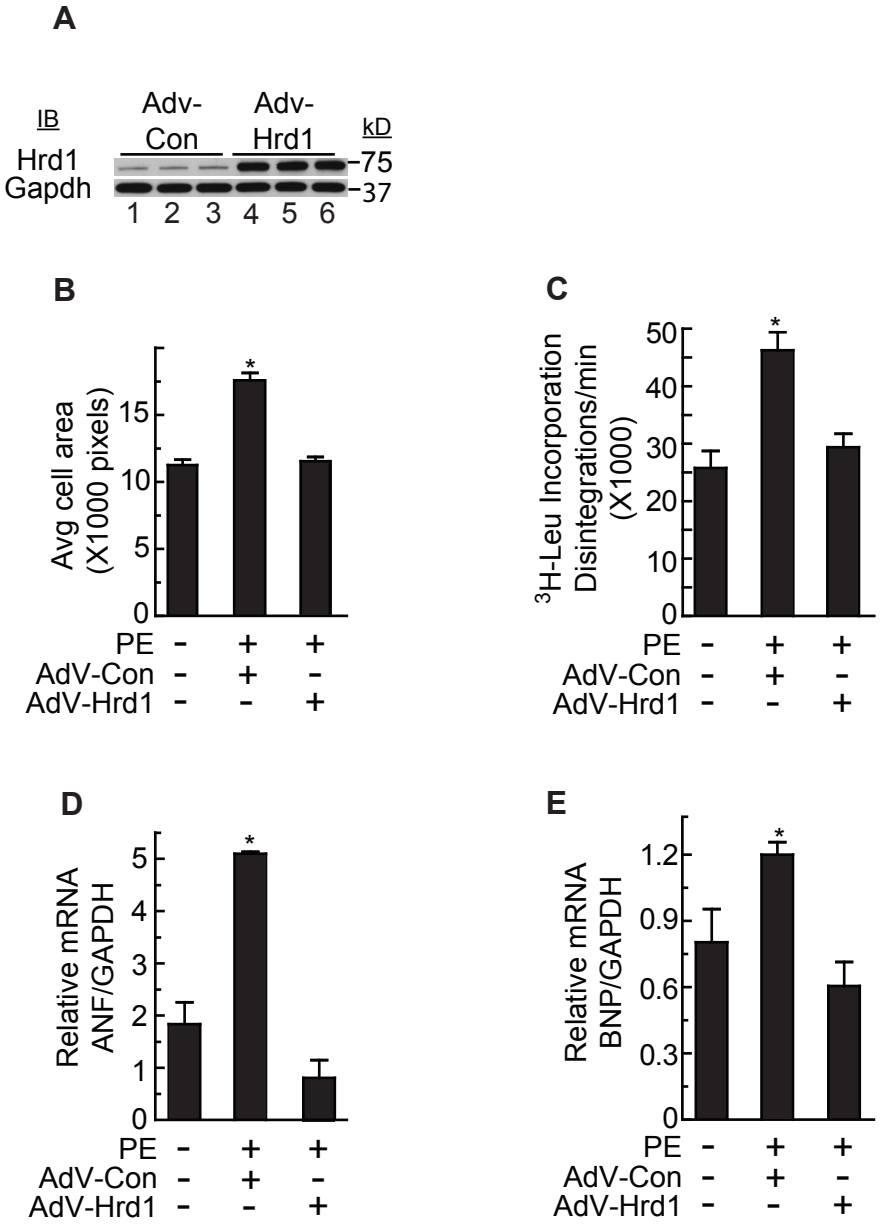
Online Figure III- Effect of Hrd1 overexpression on calcium handling in the heart
Cardiac myocytes were isolated from 11 week-old FVB mice 3 weeks after injection of $1x10^{11}$ genome containing units of AAV9-Con, or AAV9-Hrd1. **A,** Ca^{2+} transient amplitude; **B,** Half-life of Ca^{2+} decline (t); **C,** SR Ca^{2+} content after caffeine treatment; **D,** Half-life of Ca^{2+} decline (t) after caffeine treatment; **E,** Ca^{2+} spark frequency; **F,** Ca^{2+} spark amplitude; **G,** Ca^{2+} spark full duration at half-maximum (FDHM); **H,** Ca^{2+} spark full width at half-maximum (FWHM). NS = no significant differences.

Online Figure III



Online Figure IV- ER Stress Response Gene Levels in the Heart
A, The hearts from mice treated with either AAV9-Con or AAV9-Hrd1, then subjected to either sham or TAC surgery were extracted and analyzed for Hrd1 and Gapdh mRNA levels by qRT-PCR. n = 3 mice per treatment. * = $p \leq 0.05$ different from the AAV9-Con as determined by t-test. **B,** Hrd1, Grp78, ATF6 and Gapdh mRNA levels in neonatal (Neo) or adult rat ventricle (Vent) tissue extracts were measured by qRT-PCR. n = 5 samples of each. * = $p \leq 0.05$ different from neonatal ventricle tissue, as determined by t-test.

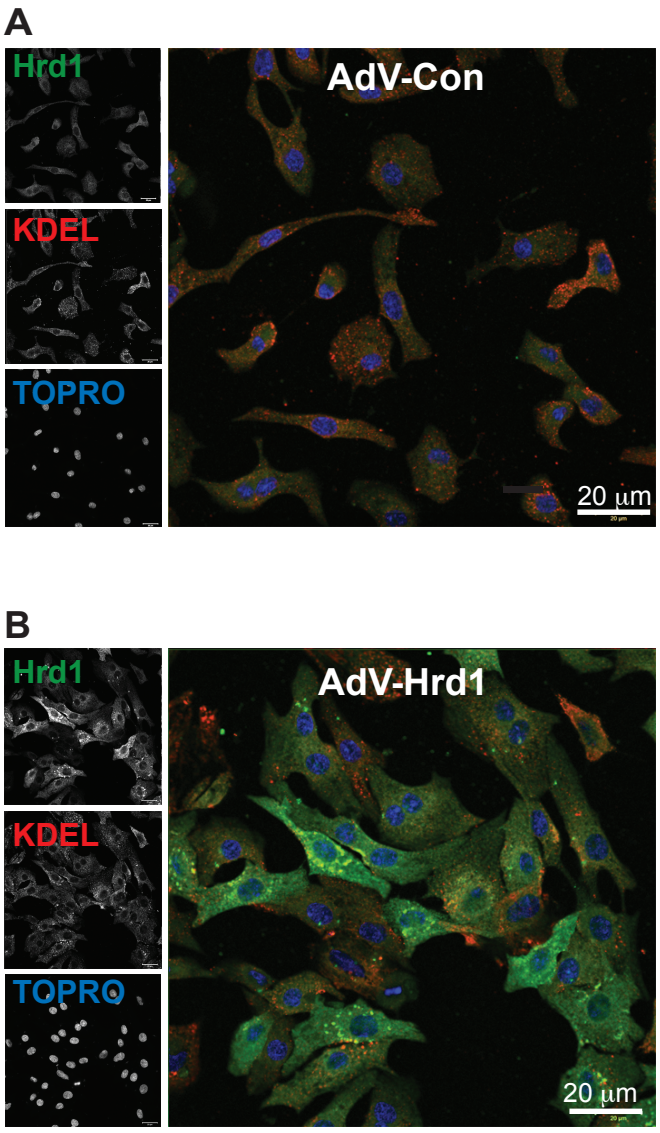
Online Figure IV



Online Figure V- Hrd1-dependent effects on cardiac myocyte hypertrophy

A, Representative immunoblot of protein extracts from Adv-Con and Adv-Hrd1 infected cultured cardiac myocytes. B-E, Myocytes were infected with AdV-Con or AdV-Hrd1 then treated with or without phenylephrine (PE), as shown. **B,** Cell size was determined by photomicroscopy, and morphometry, then expressed as mean ± SEM from from 3 independent experiments analyzing at least 300 cells per treatment per experiment. **C,** Incorporation of ^{3}H-leucine into TCA-precipitable protein in cardiac myocyte culture extracts. **D and E,** qRT-PCR examination of atrial natriuretic factor (ANF) **D,** and brain natriuretic peptide (BNP) **E,** mRNA levels in cardiac myocytes. *$p < 0.05$ different, as determined by t-test.

Online Figure V



Online Figure VI- Immunocytofluorescence Characterization of Hrd1 Overexpression
A, Cultured cardiac myocytes were infected with AdV-Con or **B,** AdV-Hrd1 and 48h later, they were subjected to staining with TOPRO (nuclei; blue), anti-KDEL (ER; red), and anti-Hrd1 (green), followed by confocal microscopy. Bars = 20 μm

Online Figure VI

Online Table I: Echocardiographic parameters of mice treated with AAV9-sh-Con or AAV9-sh-Hrd1 and subjected to either sham or TAC surgery.

	AAV9-sh-Con sham (n = 8)	AAV9-sh-Hrd1 sham (n = 6)	AAV9-sh-Con TAC (n = 9)	AAV9-sh-Hrd1 TAC (n = 9)
FS (%)	24.0±0.76	26.2±1.50	16.5±1.23[1]	12.8±0.74[1,2]
EF (%)	48.3±1.28	51.7±2.36	34.7±2.38[1]	27.5±1.45[1,2]
LVEDV (μl)	69.6±1.86	70.0±1.73	78.5±3.65	105.0±36.08[1,2]
LVESV (μl)	37.1±1.78	35.0±2.53	53.9±4.74[1]	75.8±4.50[1,2]
LVIDD (mm)	4.03±0.04	4.04±0.05	4.25±0.09	4.73±0.10[1,2]
LVIDS (mm)	3.07±0.06	2.99±0.10	3.56±0.13[1]	4.12±0.11[1,2]
PWTD (mm)	0.85±0.03	0.87±0.02	1.04±0.04[1]	1.08±0.06[1]
PWTS (mm)	1.10±0.02	1.15±0.03	1.27±0.04[1]	1.26±0.06[1]
AWTD (mm)	0.81±0.03	0.86±0.04	1.13±0.05[1]	1.13±0.04[1]
AWTS (mm)	1.10±0.02	1.24±0.04	1.37±0.07[1]	1.39±0.05[1]
LV mass (mg)	95.0±3.50	106.4±4.36	152.8±10.50[1]	183.8±14.42[1,2]
HR (bpm)	503±16	538±10	458±16	448±17

FS = fractional shortening
EF = ejection fraction
LVEDV = left ventricular end diastolic volume
LVESV = left ventricular end systolic volume
LVIDD = left ventricular inner diameter in diastole
LVIDS = left ventricular inner diameter in systole
PWTD = left ventricular posterior wall thickness in diastole
PWTS = left ventricular posterior wall thickness in systole
AWTD = left ventricular anterior wall thickness in diastole
AWTS = left ventricular anterior wall thickness in systole
LV mass = left ventricular mass
HR = heart rate in beats per minute

Statistical analyses used a one way ANOVA with a Newman-Keuls post-hoc analysis.

[1] = $p \leq 0.05$ different from AAV9-sh-Con sham
[2] = $p \leq 0.05$ different from AAV9-sh-Con TAC

Online Table II: Echocardiographic parameters of mice treated with AAV9-Con or AAV9-Hrd1 and subjected to either sham or TAC surgery.

	AAV9-Con sham (n = 5)	AAV9-Hrd1 sham (n = 4)	AAV9-Con TAC (n = 11)	AAV9-Hrd1 TAC (n = 10)
FS (%)	43.2±3.20	40.1±2.55	30.1±1.70[1]	40.0±3.45
EF (%)	75.0±3.43	71.2±3.04	57.4±266[1]	70.1±4.30
LVEDV (μl)	39.80±3.40	52.7±6.23	70.23±5.15[1]	52.93±3.85
LVESV (μl)	10.03±1.85	17.33±2.75	30.82±3.96[1]	16.41±375
LVIDD (mm)	3.15±0.11	3.74±0.23	3.98±0.12[1]	3.54±0.11
LVIDS (mm)	1.79±0.13	2.24±0.15	2.80±0.14[1]	2.13±0.17
PWTD (mm)	1.30±0.13	0.89±0.04	1.08±0.07[1]	1.20±0.06
PWTS (mm)	1.74±0.05	1.46±0.07	1.39±0.06	1.66±0.08
AWTD (mm)	1.07±0.04	0.98±0.06	1.07±0.04	1.11±0.03
AWTS (mm)	1.40±0.07	1.39±0.06	1.54±0.04	1.57±0.03
LV mass (mg)	116.9±12.69	105.0±12.52	175.5±8.57[1]	159.5±5.75
HR (bpm)	417±21.6	427±15.3	497±27.8	443±27.3

FS = fractional shortening
EF = ejection fraction
LVEDV = left ventricular end diastolic volume
LVESV = left ventricular end systolic volume
LVIDD = left ventricular inner diameter in diastole
LVIDS = left ventricular inner diameter in systole
PWTD = left ventricular posterior wall thickness in diastole
PWTS = left ventricular posterior wall thickness in systole
AWTD = left ventricular anterior wall thickness in diastole
AWTS = left ventricular anterior wall thickness in systole
LV mass = left ventricular mass
HR = heart rate in beats per minute

Statistical analyses used a one way ANOVA with a Newman-Keuls post-hoc analysis.

[1] = $p \leq 0.05$ different from AAV9-Con sham
[2] = $p \leq 0.05$ different from AAV9-Con TAC