Supplemental Material

Surgical procedures. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center. Adult age matched HDAC9 knockout (KO), HDAC5 KO mice and wild type (WT) mice of either sex received a MI. Adult age matched HDAC9 knockout (KO), HDAC5 KO mice and wild type (WT) mice of either sex were anesthetized with 2.4% isoflurane and placed in a supine position on a heating pad (37°C). Animals were intubated with a 19G stump needle and ventilated with room air using a MiniVent mouse ventilator (Hugo Sachs Elektronik, Germany; stroke volume 250 µl, respiratory rate 210 breaths per minute). Via left thoracotomy between the fourth and fifth ribs, the left anterior coronary artery (LCA) was visualized under a microscope and ligated using a 6-0 prolene suture. Regional ischemia was confirmed by visual inspection under a dissecting microscope (Leica) by discoloration of the occluded distal myocardium. Sham operated animals underwent the same procedure without occlusion of the LCA.

At 4 weeks of age, mice were either sham operated or ovariectomized (ovex), and either left untreated or treated with 17ß estradiol (0.16 µg/d) for 4 weeks. Anesthesia was induced by intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine, after which the lumbar dorsum is shaved bilaterally and the exposed skin prepared for aseptic surgery (a 10% povidone-iodine scrub followed by a 70% alcohol wipe). For each ovary, a 3/4 cm dorsal flank incision penetrating the abdominal cavity was made. The exposed ovary and associated oviduct were severed and removed, after which the incision was closed by a suture. The animals were treated with saline or 17ß estradiol (0.16 µg/d) for 4 weeks .

Transthoracic echocardiography. Three weeks following MI, two-dimensional echocardiography was performed in conscious mice using the fully digital Vingmed System (GE Vingmed Ultrasound, Horten, Norway) and a 11.5-MHz linear array transducer as previously described. Briefly, cine loops and still images were digitally stored for subsequent analysis using the EchoPac software (GE Vingmed Ultrasound). Two-dimensional short-axis views of the LV at the level of the tip of the papillary muscle were recorded with a typical frame rate of 263/s. Left ventricular (LV) parameters and heart rates were obtained from M-mode interrogation in a short-axis view. M-mode tracings were used to measure posterior wall thicknesses at end-diastole and end-systole (PWthd, PWths, respectively), and LV internal diameter (LVID) was measured as the largest anteroposterior diameter in either diastole (LVIDd) or systole (LVIDs). The data were analyzed by a single observer blinded to the murine genotype. LV fractional shortening (FS) was calculated according to the following formula: FS (%) = [(LVIDd - LVIDs)/LVIDd] x100. All echocardiographical analyses were performed in a blinded fashion for genotype.

Histology and histochemistry. After collecting, heart tissue was either cryo-embedded, or incubated for 30 minutes in Krebs buffer (118mM NaCl, 4.7mM KCL, 1.2mM $KH2PO_4$, 1.2 $MgSO_4$, 25mM $NaHCO_3$, 11mM glucose) to arrest the heart in diastole, fixed in 3.7% paraformaldehyde, and embedded in paraffin. Sections were stained with hematoxylin and eosin to visualize infarcted area (n = 3-6 in each group). To examine the capillary density, all sections were incubated with biotinylated *Griffiona simplicifolia* lectin (Vector Laboratories, UK). Briefly, sections were pretreated with 3% hydrogen peroxide to inhibit endogenous peroxidase activity and incubated with 2% horse serum. Subsequently, they were incubated with biotinylated *Griffonia (Bandeiraea) simplicifolia* lectin I (Vector) (20 µg/ml with 10 mM HEPES, pH 7.5, 0.15 M NaCl) for 30 min at room temperature. After rinsing with PBS, the slides were incubated for 30 min with ABC reagent. Subsequently all sections were stained for immunoperoxidase using a commercially available kit (HRP DakoCytomation, Denmark). Final color products were developed using a solution containing 3,3'-diaminobenzidine (DAB) and the sections were counterstained with eosin. Serial transverse sections were cut at 10 µm using a Frigocut 2800 cryostat (Leica, Milton Keynes, U.K.).

RNA extraction and RT-PCR analysis. Total RNA from the infarcted area was isolated using Trizol (Invitrogen). A 10 µg aliquot representative of three animals per sample group was then analyzed on Affymetrix U74Av2 microarrays. A subset of differentially expressed RNAs was further characterized by either semi-quantitative PCR or quantitative real time PCR. Briefly, 2 µg RNA from each sample was used to generate cDNA using Super Script II reverse transcriptase per manufacturer's specifications (Invitrogen Life Technologies Inc., Burlington, Ontario, Canada). Real time PCR was cycled between 95 °C/30 s and 60 °C/30 for 40 cycles, following an initial denaturation step at 95°C for 3 min. Amplification products were routinely checked using dissociation curve software (Biorad), and transcript quantities were compared using the relative Ct method, where the amount of target normalised to the amount of endogenous control (18S) and relative to the control sample is given by $2^{-\Delta\Delta Ct}$. Real time PCR results were verified by electrophoresis of the reverse transcribed material in 1.2% agarose gels and visualized under UV illumination after ethidium bromide staining.

In situ hybridization. Section in situ hybridization was performed on fresh-frozen tissue four days after either sham surgery or myocardial infarction. For pre-hybridization, slides were heated to 58°C for 30 min. Utilizing RN'ase-free glass staining dishes and metal racks (Shandon, Pittsburgh, PA), slides were deparaffinized in xylene and hydrated through a series of graded ethanol/DEPC-saline rinses (95%, 85%, 60%, 30%) to DEPC-saline. To accomplish microwave RNA retrieval, the slides were transferred to upright plastic racks and immersed in plastic containers (Miles Tissue-Tek, Elkhart, IN) filled with DEPC-1X Antigen Retrieval Citra pH 6.0 (Biogenex, San Ramon, CA). Empty slots were filled with blank slides, and the plastic slide dish was covered loosely. The slides were heated in a 750 watt microwave at 90% power for 5 min. Any evaporated solution was replaced with DEPC-H_2O, and the container was heated at 60% power for an additional 5 min. The slides were cooled for 20 min, then returned to their metal racks and washed twice in DEPC-PBS, pH 7.4, for 5 min each. Subsequently, the slides were fixed for 20 min in 4% paraformaldehyde/DEPC-PBS, pH 7.4, and washed twice in DEPC-PBS, pH 7.4, for 5 min each. To further unmask RNA, the slides were permeabilized for 7.5 min with 20 µg/ml pronase-E in 50 mM Tris-HCl, pH 8.0/5 mM EDTA, pH 8.0/DEPC-H_2O. Excess pronase-E was removed by a 5-min DEPC-PBS, pH 7.4, wash before re-fixing in 4% paraformaldehyde/ DEPC-PBS, pH 7.4, for 5 min. Slides were washed in DEPC-PBS, pH 7.4, for 3 min. The slides were then acetylated in 0.25% acetic anhydride/0.1 M triethanolamine;–HCl, pH 7.5, twice for 5-min. Next, the slides were equilibrated in 1 x SSC, pH 7.0, for 5 min followed by incubation in 50 mM n-ethylmaleimide/1 x SSC, pH 7.0, for 20 min. Five-min washes in DEPC-PBS, pH 7.4, and DEPC-saline followed and then the slides were dehydrated through graded ethanol/DEPC-saline rinses (30%, 60%, 85%, 95%) to absolute ethanol, and dried under vacuum for 2 h.

The coding regions of *VEGFa,* and *VEGFr2* were subcloned into pCDNA, linearized and transcribed as follows: antisense VEGFa*, BamHI* and T3; antisense Flk-1, *NotI* and T7, generously provided by Dr. T. Sato, at UT Southwestern. Riboprobes and hybridization mixture containing 50% formamide, 0.3 M NaCl, 20 mM Tris-HCl, pH 8.0, 5 mM EDTA, pH 8.0, 10 mM $NaPO_4$, pH 8.0, 10% dextran sulfate, 1 x Denhardt's, and 0.5 mg/ml tRNA were thawed from -80°C storage. Probes were diluted in aliquots of hybridization mixture sufficient to achieve 7.5 x 10^3 cpm/µl and the mixture heated to 95°C for 5 min. Diluted probes were then cooled to 37°C and 1 M DTT was added to achieve a final concentration of 10 mM DTT. Riboprobe was applied directly over the section, and slides were placed in a Nalgene utility box lined with 5 x SSC/50% formamide-saturated gel blot paper. Each slide was coverslipped with parafilm.

The box was sealed and slides were hybridized for 14 h at 55°C.
After hybridization, parafilm coverslips were removed and the slides were placed in upright plastic racks and immersed in a 5 x SSC/10 mM DTT wash at 55°C for 40 min. Subsequently, the slides were washed for 30 min at 65°C in HS (2 x SSC/50% formamide/100 mM DTT), followed by three 10-min washes in NTE (0.5 M NaCl/10 mM Tris-HCl, pH 8.0/5 mM EDTA, pH 8.0) at 37°C. Slides were transferred to a fourth NTE wash containing RN'ase-A (2 µg/ml) and incubated 30 min at 37°C. Excess RN'ase-A was

removed in a fifth NTE wash for 15 min at 37°C, before the slides were returned to HS for another 30 min at 65°C. After this second HS, the slides were washed for 15 min at 37°C each in 2 x SSC and 0.1 x SSC. Finally, the slides were dehydrated in graded ethanol rinses (30%, 60%, 85%, 95%) to absolute ethanol, and dried under vacuum.

Electrophoretic mobility shift assays. Oligonucleotides corresponding to the conserved MEF2-binding site in the ERα regulatory region, and the mutated MEF2-binding sites were synthesized (Integrated DNA Technology) as follows (+ strand sequences are shown with the MEF2 site in bold and the mutation underlined):

ERα MEF2 site 1:	5' GGCTTCTCGATAC**TTATTTATAT**ATATTAGATATT-3'
ERα MEF2mut site 1:	5' GGCTTCTCGATAC**TTA<u>CCCG</u>TAT**ATATTAGATATT-3'
ERα MEF2 site 2 oligo:	5' GGCCACTGGTG**CTAAATATAG**CTGTCGGTGG -3'
ERα MEF2mut site 2 oligo:	5' GGCCACTGGTG**CTA<u>GGCG</u>TAG**CTGTCGGTGG -3'
ERα MEF2 site 3 oligo:	5' GGCTCTTTCCAGAT**GTATTTATAG**TAGAAG -3'
ERα MEF2mut site 3 oligo:	5' GGCTCTTTCCAGAT**GTA<u>CCCG</u>TAG**TAGAAG -3'

Annealed oligonucleotides were radiolabeled with [^{32}P]dCTP using the Klenow fragment of DNA polymerase and purified using G50 spin columns (Roche). Nuclear cell extracts were isolated from Cos-1 cells that were transfected with pcDNAMYC-MEF2C. Unlabeled oligonucleotides used as competitors were annealed as described above and added to the reactions at the indicated concentrations. DNA-protein complexes were resolved on 5% polyacrylamide native gels and the gels were exposed to BioMax X-ray film (Kodak).

GST pulldown. GST-ERα was generated by subcloning PCR amplified fragment into EcoRI and XhoI sites of the pGEX-KG vector, while GST-dHDAC9 (aa131-586) was subcloned in the pGEX-KG vector as a XbaI-XhoI fragment. Plasmids encoding fusion proteins were transformed into BL21-codon plus cells (Stratagene). After growing up the cells and inducing protein expression, the culture was incubated at room temperature for 4 to 6 h, after which the cells were harvested and GST protein was purified with glutathione beads in accordance with the Amersham procedure.

Proteins translated in vitro were labeled with [35S]methionine in a coupled transcription-translation T7 reticulocyte lysate system (Promega). Glutathione beads conjugated with 1 ug of protein were incubated with 10 ul of TNT product at 4˚C for 2 h in 500 ul of GST-binding buffer (20 mM Tris [pH 7.3], 150 mM NaCl, 0.5% NP-40, protease inhibitor cocktail from Roche, 1 mM phenylmethylsulfonyl fluoride [PMSF]). The beads were washed three times with GST-binding buffer. Fifty microliters of sodium dodecyl sulfate (SDS) loading buffer was then added to the beads. After boiling, 20 ul was loaded onto an SDS-PAGE gel and analyzed by autoradiography.

Supplemental Figures

Supplemental Figure I. Representative M-mode images of sham or infarcted WT and HDAC9 KO females 3 weeks after permanent LCA ligation.

Supplemental Figure II. Representative M-mode images of sham or infarcted HDAC9 KO females that received ovariectomy or ovariectomy and estrogen in addition to MI, compared to WT females post-MI.

Supplemental Figure III. Class II HDAC mutant male animals develop age dependent hypertrophy, while this remodeling is absent in female mutant mice.

A. Histological data indicate that male mutant HDAC5 and HDAC9 mice develop age dependent cardiac hypertrophy while this remodeling is absent in the female mutant littermates (Scale bars: 40 μm).
B. Heart weight versus tibia length ratio (HW /TL) indicates an age-dependent increase in class II HDAC mutant males while this increase is absent in females. * $p<0.05$ compared to male mice of the same genotype.
C. Realtime PCR analysis indicates that there is a strong induction of stress responsive genes in male mutant animals, while this increase is severely blunted in female mice mutant for either HDAC5 or -9. The data are expressed as fold expression compared to WT males.

Supplemental Table I. Primer sequences for RT-PCR analysis of angiogenic genes

Target gene	Reverse primer 5-3'	Forward primer 5-3'
Cyclophilin	AGCTAGACTTGAAGGGGAATG	ATTTCTTTTGACTTGCGGGC
Angiopoietin-1	GCAAGGCTGATAAGGTTATGA	AGCTACCAACAAACAACAGCA
Angiopoietin-2	TTCTTCTTTACGGATAGCAAC	AGCCACGGTCAACAACTGC
Angiopoietin-3	GCAGTTGTTCCCTCTTCTCTT	AACAGGGCCCTGGAGACC
VEGFa	CTCCAGGGCTTCATCGTTA	CAGAAGGAGAGCAGAAGTCC
VEGFb	TGCCCATGAGTTCCATGC	CCCAGTTTGATGGCCCCA
VEGFt	TTTAAGGAAGCACTTCTGTGTGT	GTAAAAACAAACTTTTCCCTAATTC
VEGFd	GGTGCTGAATGAGATCTCCC	GCAAGACGAGACTCCACTGC
VEGFr1	GCTGCTTGGAGATCTCACTG	TCAGCAGCTCAAGTGTCACC
VEGFr2	TTCCAGATGCTGGGCAAGTC	ATGACATCTTGATTGTGGCAT
VEGFr3	TGCATGCTGGGTGGACTATCA	GCAGGAGGAGGAAGAGGAGC
Tie1	AATGGCAGACCAGGCAATC	CCCCACTGGTCTCCTTTAG
Tie2	GTTGACTCTAGCTCGGACTGT	GAAGTCGAGAGGCGATCCC
bFGF	CACATTTAGAAGCCAGTAATCT	CCCGACGGCCGCGTGGAT

Supplemental Table II. Primer sequences for electrophoric mobility shift assay for MEF2 sites upstream of the ERα gene (+ strand sequences are shown with the MEF2 site in bold and the mutation underlined).

MEF2 site	Primer sequence
ERα MEF2 site 1	5' GGCTTCTCGATAC**TTATTTATAT**ATATTAGATATT-3'
ERα MEF2mut site 1	5' GGCTTCTCGATAC**TTA<u>CCCG</u>TAT**ATATTAGATATT-3'
ERα MEF2 site 2	5' GGCCACTGGTG**CTAAATATAG**CTGTCGGTGG-3'
ERα MEF2mut site 2	5' GGCCACTGGTG**CTA<u>GGCG</u>TAG**CTGTCGGTGG -3'

Supplemental Table III. Echocardiographic analysis of either HDAC5 KO females and HDAC9 KO females versus WT females indicates less dilation and a better maintenance of fractional shortening in class II HDAC mutant females in response to MI. * $p<0.05$ compared to corresponding WT females.

	wild type		HDAC5 KO		HDAC9 KO	
	sham	*3 weeks post-MI*	*sham*	*3 weeks post-MI*	*sham*	*3 weeks post-MI*
n	5	9	5	10	5	10
HR	630 ± 25	581 ± 18	594 ± 27	585 ± 19	644 ± 29	607 ± 43
PWthd, mm	0.81 ± 0.04	0.90 ± 0.08	0.86 ± 0.07	0.93 ± 0.14	0.83 ± 0.04	0.92 ± 0.03
PWths, mm	1.39 ± 0.09	1.33 ± 0.08	1.62 ± 0.11 *	1.5 ± 0.07	1.60 ± 0.09 *	1.46 ± 0.08
LVIDd, mm	2.62 ± 0.07	3.31 ± 0.31	2.3 ± 0.15	3.23 ± 0.25	2.4 ± 0.25	3.57 ± 0.17
LVIDs, mm	0.71 ± 0.04	2.41 ± 0.25	0.65 ± 0.09	1.95 ± 0.17 *	0.68 ± 0.16	2.01 ± 0.19 *
% FS	73.3 ± 1.5	27.8 ± 1.8	73.8 ± 3.9	39.8 ± 1.4 *	76.7 ± 2.8	43.5 ± 5.6 *

Data are expressed as means ± SEM. *AWthd*, anterior wall thickness in diastole; *AWths*, anterior wall in systole;
PWthd, posterior wall thickness in diastole; *PWths*, posterior wall thickness in systole;
LVIDd, left ventricular internal diameter in diastole; *LVIDs*, left ventricular internal diameter in systole;
FS, left ventricular fractional shortening calculated as (LVIDd-LVIDs)/LVIDd

Supplemental Table IV. Morphometric analysis of either WT or HDAC9 KO females 3 weeks post-MI indicates signs of hypertrophy and failure in the WT females, which are severely blunted in the HDAC9 KO females. * p<0.05 compared to corresponding WT female.

	wildtype		**HDAC9 -/-**	
	sham	*3 weeks post MI*	*sham*	*3 weeks post MI*
n	5	9	5	11
BW operation, g	26.0 ± 0.9	26.2 ± 0.7	25.1 ± 1.7	26.6 ± 0.7
BW, g	26.3 ± 1.0	24.0 ± 0,6	25.3 ± 1.6	24.4 ± 0,9
HW, mg	134.6± 3	196.1 ± 13	141.8± 6	158.8 ± 8 *
LVW, mg	90.5 ± 2.8	111.5 ± 4.7	88.0 ± 4.3	99 ± 1.8 *
RVW, mg	**24.5 ± 0.9**	**35.0 ± 3.8**	**22.5 ± 0.3**	**23.0 ± 0.8** *
Atria weight, mg	**12.0 ± 0.6**	**26.8 ± 4.5**	**11.5 ± 0.9**	**13.2 ± 0.7** *
Lung weight, mg	**173.8 ± 9.0**	**256.3 ± 36.5**	**157.4 ± 8.6**	**153 ± 16.5** *
Liver weight, mg	1286 ± 49	1161 ± 41	1123 ± 54	1034 ± 50
TL, mm	17.4 ± 0.1	17.5 ± 0.1	16.9 ± 0.3	17.5 ± 0.1
HW/BW, mg/g	5.1 ± 0.2	8.1 ± 0.4	5.6 ± 0.1	6.5 ± 0.3 *
HW/TL, mg/mm	**7.8 ± 0.2**	**11.2 ± 0.7**	**8.4 ± 0.7**	**9.0 ± 0.4** *
LVW/BW, mg/g	3.3 ± 0.1	4.7 ± 0.2	4.0 ± 0.2	3.9 ± 0.1 *
LVW/TL, mg/mm	**5.2 ± 0.2**	**6.4 ± 0.3**	**5.3 ± 0.2**	**5.6 ± 0.1** *

Data are expressed as means ± SEM. *HW*, heart weight; *LVW*, left ventricular weight; RVW, right ventricular weight; *TL*, tibial length; *BW*, body weight.

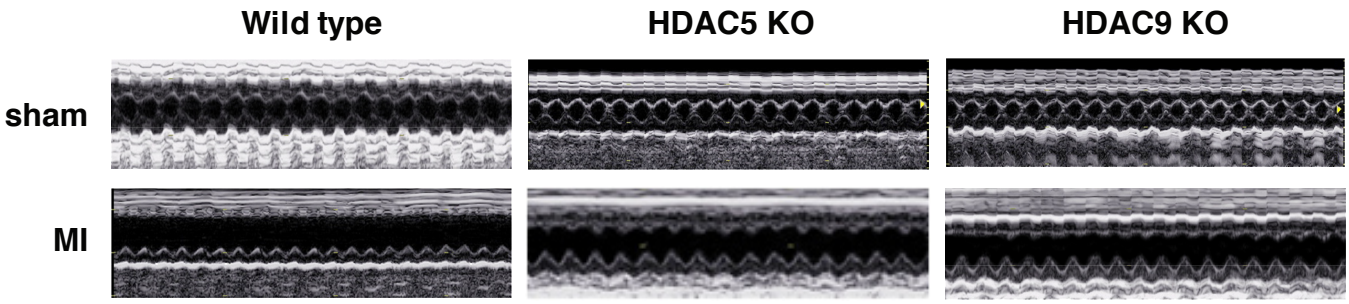
Supplemental Table V. Gene expression analysis by microarray on the infarcted region HDAC9 KO females versus WT females indicates upregulation of ER signaling related genes.

ER coactivators/ repressors	**Fold upregulation**
-PBP	69 x
-Ah receptor repressor	19.7 x
-pCAF	16 x
-Interferon activated gene	14.9 x
-PKC	8.0 x
-Rho GTPase	3.2 x
-**ER targets**	
-GABA-A receptor	10.6 x
-17-β hydroxysteroid	9.8 x
-ATP binding cassette	8.0 x
-Progesteron receptor	5.6 x
-Muscle creatine kinase	4.0 x
Angiogenic factors	
-angiopoetin	19.7 x
-FGF related gene	3.0 x
-VEGF-B precursor	3.0 x
-VEGF-A	1.7 x
-ERRβ	2.3 x
-ERRα	2.0 x
-ERα	1.6 x

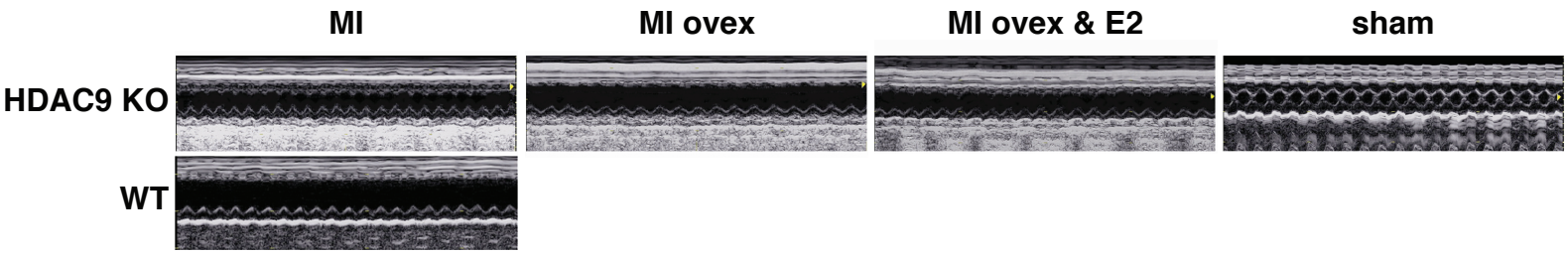
Supplemental Table VI. Echocardiographic analysis of HDAC9 KO females after ovariectomy and subsequent supplementation of estrogen indicates a decrease in fractional shortening in ovariectomized HDAC9 mutant females post-MI while the protective effect is restored by estrogen treatment. * $p<0.05$ compared to HDAC9 mutant females post-MI.

	HDAC9 KO			
	sham	*MI*	*MI ovex*	*MI ovex E2*
n	5	10	8	7
HR	644 ± 29	607 ± 43	630 ± 19	595 ± 17
PWthd, mm	0.83 ± 0.04	0.92 ± 0.03	0.78 ± 0.05	0.72 ± 0.02
PWths, mm	1.80 ± 0.09	1.46 ± 0.08	1.67 ± 0.08	1.54 ± 0.08
LVIDd, mm	2.4 ± 0.25	3.50 ± 0.18	3.14 ± 0.23	3.34 ± 0.32
LVIDs, mm	0.71 ± 0.04	2.03 ± 0.15	2.30 ± 0.11 *	1.99 ± 0.16
% FS	73.3 ± 1.5	42.1 ± 1.8	26.8 ± 1.4	40.9 ± 2.8

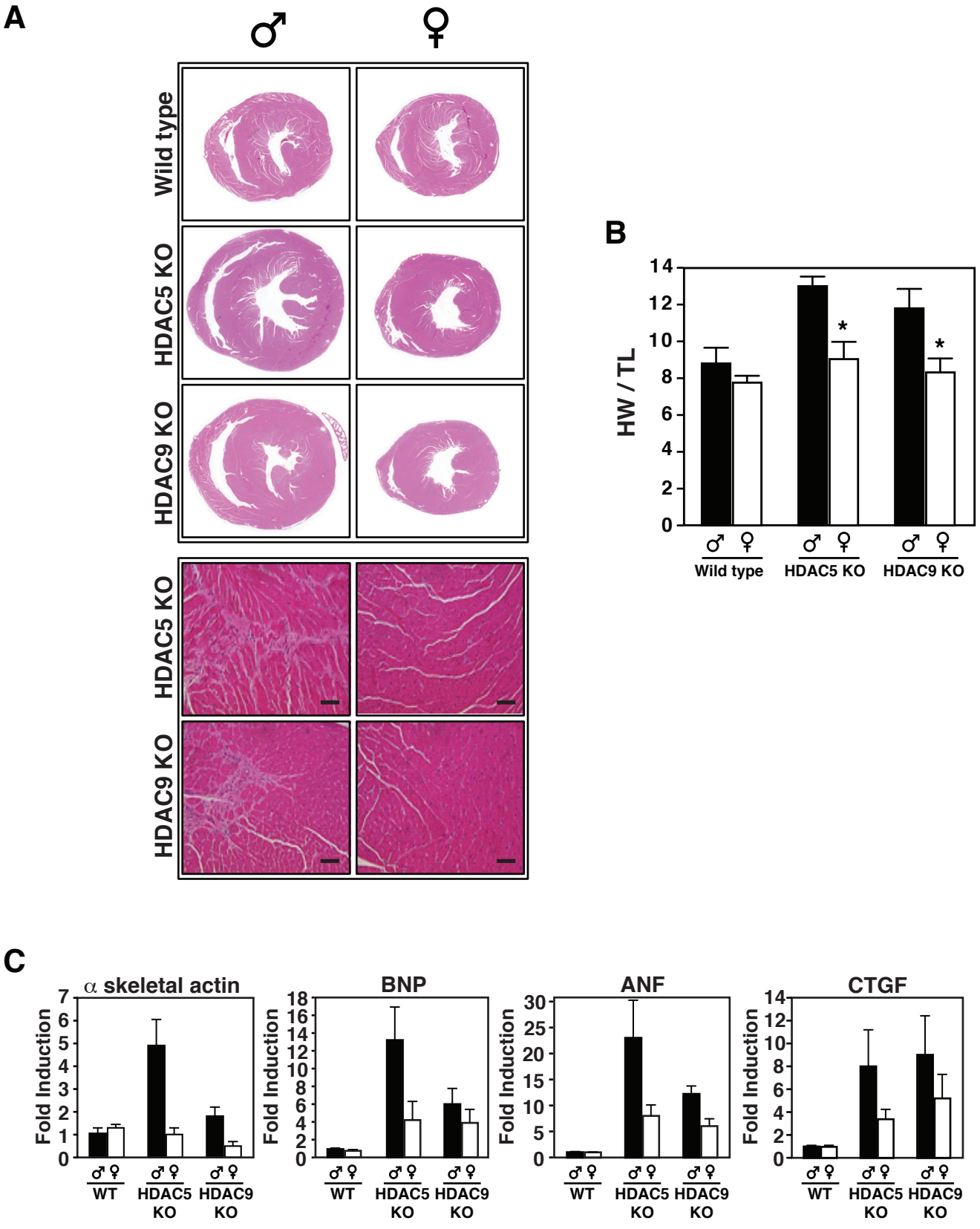
Data are expressed as means ± SEM. *AWthd*, anterior wall thickness in diastole; *AWths*, anterior wall in systole; *PWthd*, posterior wall thickness in diastole; *PWths*, posterior wall thickness in systole; *LVIDd*, left ventricular internal diameter in diastole; *LVIDs*, left ventricular internal diameter in systole; *FS*, left ventricular fractional shortening calculated as (LVIDd-LVIDs)/LVIDd



Supplemental Figure 1. Representative M-mode images of sham or infarcted WT and HDAC9 KO females 3 weeks after permanent LCA ligation.



Supplemental Figure 2. Representative M-mode images of sham or infarcted HDAC9 KO females that received ovariectomy or ovariectomy and estrogen in addition to MI, compared to WT females post-MI.



Supplemental Figure 3. Class II HDAC mutant male animals develop age dependent hypertrophy, while this remodeling is absent in female mutant mice.

A. Histological data for 5-6 month old animals indicate that male mutant HDAC5 and HDAC9 mice develop age dependent cardiac hypertrophy while this remodeling is absent in the female mutant littermates (Scale bars: 40 μm).

B. Heart weight versus tibia length ratio (HW /TL) indicates an age-dependent increase in class II HDAC mutant males while this increase is absent in females. * $p<0.05$ compared to male mice of the same genotype.

C. Realtime PCR analysis indicates that there is a strong induction of stress responsive genes in male mutant animals, while this increase is severely blunted in female mice mutant for either HDAC5 or -9. The data are expressed as fold expression compared to WT males.