SUPPLEMENTAL MATERIAL
Online Materials and Methods Supplement

Mice

A targeting vector that replaces exon 2 and exon 3 of the *Dusp4* gene with the neomycin cassette was generated and electroporated into mouse embryonic stem cells. Arms for the targeting vector were generated by PCR from SV129j genomic DNA to match the SV129j-based embryonic stem cells that were used. Correctly targeted embryonic stem cells were identified by Southern blotting and subsequently injected into C57Bl/6 blastocysts to generate chimeric mice, which were bred with C57Bl/6 mice to obtain germ line and homozygous null *Dusp4* mice with a final hybrid background of C57Bl/6-SV129j. The generation of *Dusp1* gene-deleted mice (*Dusp1*$^{-/-}$) was previously described [1]. *Pln* null mice were also previously described [2]. All experiments involving animals were approved by the Institutional Animal Care and Use Committee at Cincinnati Children's Hospital Medical Center.

Echocardiography, pressure overload, invasive hemodynamics and drug treatment

All mice were anesthetized with 2% Isoflurane by inhalation. Echocardiography was performed in M-mode using a Hewlett Packard SONOS 5500 instrument equipped with a 15 MHz transducer as described previously [3]. Cardiac hypertrophy was induced by transverse aortic constriction (TAC) to produce pressure overload as previously described [4]. Doppler echocardiography was performed on all mice subjected to TAC to ensure equal pressure gradients across the aortic constriction between the groups. Invasive hemodynamics was performed using the closed chest approach by cannulating the right carotid artery with a Millar pressure-transducing catheter placed through the aorta and into the left ventricle. Recordings were made using a Millar MPVS-400 integrated with ADinstruments Powerlab technology and further analyzed using Labchart software. The mice were treated intraperitoneally with 10 mg/kg anisomycin for the indicated times. When indicated, the mice were treated with 50 mg/kg/day of the p38-specific inhibitor SB731445 [5], which was formulated in mouse chow. Blood and heart tissue levels of SB731445 were assessed by mass spectrometry.

Cell culture

Adult cardiomyocytes were isolated as previously published [6]. Following isolation, cardiomyocytes were attached to laminin-coated dishes and cultured in serum free media. Measurements were performed using ImageJ software (NIH) of phase contrast images. Mouse embryonic fibroblasts were isolated at approximately embryonic day 12.5 from *Dusp1/4* deficient and wild-type mice in a cell culture hood under aseptic conditions. Freshly isolated embryos were carefully dissected from the uterus and placed on 10 cm dish containing PBS to remove blood clots, placental, and other maternal tissues. Heads and visceral organs were removed from each embryo body, then the body was placed onto a fresh 6 cm dish containing 2 ml of trypsin, and minced with a sterile razor blade. Plates containing minced tissue with trypsin were placed in a 37°C incubator for 30-45min. After incubation, trypsin activity was quenched by adding 4 ml of MEF media (DMEM, 1:100 Gentamicin (Gibco 15710-064), 1:100 Glutamine (Gibco 25030-149), 1:100 MEM non-essential amino acids (Gibco 11140-050), 1:1000 2-mercaptoethanol (Gibco 21985-023) and 10% fetal calf serum) and tissues were further dissociated by pipetting 10-20 times until a single cell suspension was formed. The cell suspension was then transferred to a

10 cm plate and MEF media was added to a final volume of 10 ml. Plates were incubated in 37°C incubator and cells were allowed to grow to confluency (3-4 days approximately). Passage 2 cells were harvested via trypsin and frozen down at $2x10^6$ cells per vial. Cells were split every 4 days until they reached senescence crisis (at 3 months approximately). When indicated, the MEFs were stimulated with 1 μg/ml of anisomycin for the indicated amount of time.

Isolation of adult cardiomyocytes for contractility and Ca^{2+} measurements.
Adult cardiomyocytes were isolated from Wt and DKO mice using a standard isolation procedure via perfusing whole hearts with a Tyrode's solution containing liberase blendzyme (Roche) at 37°C (10-14 minutes total) as previously described [7]. After perfusion, the ventricles were disassociated into individual myocytes, filtered and Ca^{2+} was reintroduced in incremental steps. Myocytes were then incubated with 2 μM Fura-2 acetoxymethyl ester (Invitrogen) and pluronic acid for 15 minutes in M199 media with 2,3-butanedione monoxime (BDM) at room temperature. After loading, the cells were washed and resuspended in Ringer's solution. Electrical and caffeine (10 mM) stimulated Ca^{2+} transients were measured using a DeltaRam spectrofluorophotometer (Photon Technology International), operated at an emission wavelength of 510 nm, with excitation wavelengths of 340 and 380 nm. The stimulating frequency for Ca^{2+} transient measurements was 0.5 Hz. Baseline amplitude in the presence and absence of 50 μM SB239063 compound (calculated by change in base to peak in 340 nm/380 nm ratio) of the Ca^{2+} signal was acquired, and data were analyzed using Felix and Clampfit software. Contractility measurements were acquired using video edge detection as previously described [8]. Myocytes were incubated for a minimum of 10 minutes in vehicle or SB compound before measurements were made.

Western blotting
Western blot analysis of mouse heart homogenates and cell cultures was performed as previously described [9]. Antibodies used were phospho-ERK1/2 (Cell Signaling; 9101), ERK1/2 (Cell Signaling; 9102), phospho-p38 (Cell Signaling; 9211), p38 (Cell Signaling; 9212), phospho T222-MK2 (Cell Signaling; 3316), phospho T334-MK2 (Cell Signaling; 3007), MK2 (Cell Signaling; 3042), MEK1/2 (Cell Signaling; 9122), MKK4 (Cell Signaling; 9152), phospho-JNK1/2 (Promega; V7932), JNK1/2 (Cell Signaling; 9252), GAPDH (Fitzgerald; RDI-TRK5G4-6C5), phospho-MKK6 (Cell Signaling; 9231), MKK6 (Cell Signaling; 9264), α-actinin (Cell Signaling; 4051).

mRNA expression analysis and histology
RNA was extracted from ventricles using the RNeasy Kit according to manufacturer's instructions (Qiagen). Reverse transcription was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression was analyzed by real-time qPCR using SYBR green (Applied Biosystems). The expression of DUSP1 and DUSP4 was analyzed by PCR using the primers 5'-ctccaaggaggatatgaagcg-3' (DUSP1-for), 5'-ctccagcatccttgatggagtc-3' (DUSP1-rev), 5'-gtggaaatcctccctttcctctac-3' (DUSP4-for), and 5'-gatgtcggccttgtggttgtcttc-3' (DUSP4-rev). Standard histological methods were employed with H&E staining to show gross anatomical features of the heart, as well as without staining for assessment of TUNEL according to the CardioTACS (Trevigen).

Statistical analysis

Data are represented as means ± SEM. A two-sample Student *t* test was used to compare means between 2 groups. 1-way ANOVA with Bonferroni correction was used for groups of 3 or more. *P* values less than 0.05 were considered significant.

References

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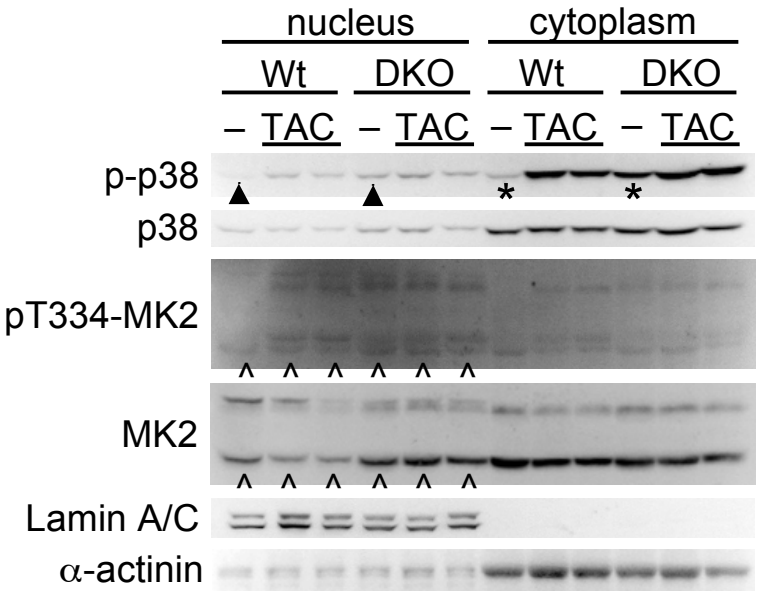
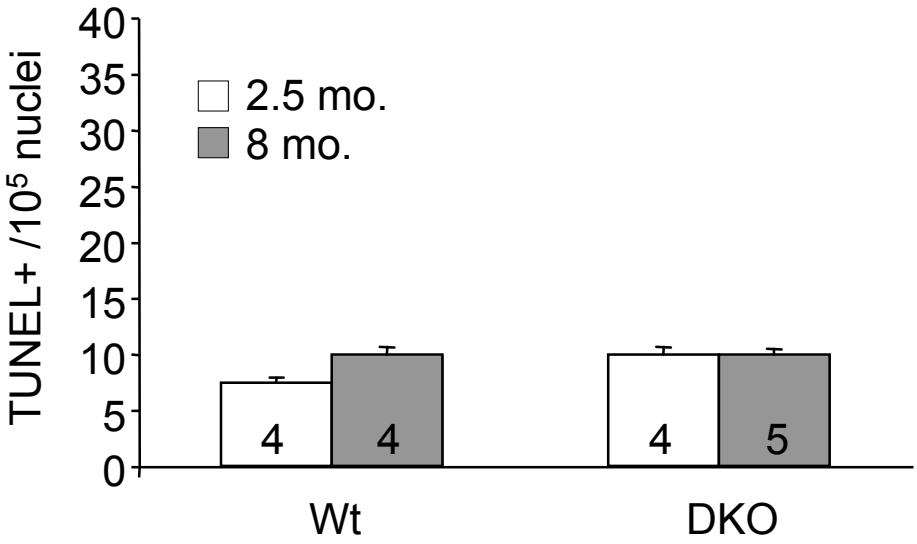
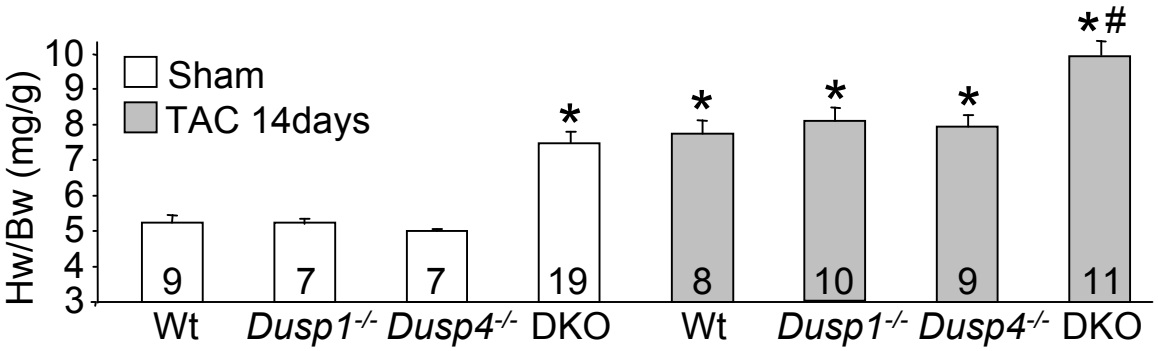


Online Figure I. Western blot analysis of the indicated proteins and phospho-proteins from hearts of adult mice subjected to sham or transverse aortic constriction (TAC, 15 minutes of stimulation) in Wt or *Dusp1/4*$^{-/-}$ animals. Asterisks and arrow heads in the blots show bands that are differentially regulated by loss of *Dusp1/4*, while the open up-arrows show regulation and greater nuclear occupancy of MK2 associated with deletion of *Dusp1/4*. Lamin A/C western shows purity of the nuclear extract, while α-actinin shows purity of the cytoplasmic extract. The results again suggest that loss of *Dusp1/4* upregulates p38 activity in the heart at baseline, and even greater activation with TAC stimulation for 15 minutes.



Online Figure II. Quantitation of TUNEL levels in histological sections from hearts of Wt and *Dusp1/4* DKO mice at 2.5 and 8 months of age. At least 4 full sections were analyzed for each heart, and the total number of hearts analyzed is shown in the bars for each genotype or time point. No differences were observed, as the *Dusp1/4*$^{-/-}$ hearts were largely devoid of significant ongoing cell death.



Online Figure III. Quantitation of heart weight to body weight (Hw/Bw) ratios in 2 month-old Wt, *Dusp1*$^{-/-}$, *Dusp4*$^{-/-}$, and *Dusp1/4*$^{-/-}$ (DKO) mice subjected to Sham or TAC surgery for 14 days (*p<0.01 vs. Sham; #p<0.01 vs. other genotypes with same procedure). The number of animals is indicated in the bars. Double null mice (DKO) that survived the TAC procedure for 14 days showed much greater increases in heart weights.