DUSP8 Regulates Cardiac Ventricular Remodeling by Altering ERK1/2 Signaling

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Rationale: Mitogen-activated protein kinase (MAPK) signaling regulates the growth response of the adult myocardium in response to increased cardiac workload or pathological insults. The dual-specificity phosphatases (DUSPs) are critical effectors, which dephosphorylate the MAPKs to control the basal tone, amplitude, and duration of MAPK signaling.

Objective: To examine DUSP8 as a regulator of MAPK signaling in the heart and its impact on ventricular and cardiac myocyte growth dynamics.

Methods and Results: Dusp8 gene–deleted mice and transgenic mice with inducible expression of DUSP8 in the heart were used here to investigate how this MAPK-phosphatase might regulate intracellular signaling and cardiac growth dynamics in vivo. Dusp8 gene–deleted mice were mildly hypercontractile at baseline with a cardiac phenotype of concentric ventricular remodeling, which protected them from progressing towards heart failure in 2 surgery-induced disease models. Cardiac-specific overexpression of DUSP8 produced spontaneous eccentric remodeling and ventricular dilation with heart failure. At the cellular level, adult cardiac myocytes from Dusp8 gene–deleted mice were thicker and shorter, whereas DUSP8 overexpression promoted cardiac myocyte lengthening with a loss of thickness. Mechanistically, activation of extracellular signal–regulated kinases 1/2 were selectively increased in Dusp8 gene–deleted hearts at baseline and following acute pathological stress stimulation, whereas p38 MAPK and c-Jun N-terminal kinases were mostly unaffected.

Conclusions: These results indicate that DUSP8 controls basal and acute stress-induced extracellular signal–regulated kinases 1/2 signaling in adult cardiac myocytes that then alters the length–width growth dynamics of individual cardiac myocytes, which further alters contractility, ventricular remodeling, and disease susceptibility. (Circ Res. 2016;119:249-260. DOI: 10.1161/CIRCRESAHA.115.308238.)

Key Words: dilated cardiomyopathy ■ disease susceptibility ■ dual-specificity phosphatase ■ heart failure ■ myocardium

In its broadest sense, the mitogen-activated protein kinase (MAPK) signaling cascade consists of a sequence of successively acting kinases that result in dual phosphorylation and activation of 3 main branches identified by the terminal kinases, p38, c-Jun N-terminal kinases 1 and 2 (JNK1/2), and extracellular signal–regulated kinase 1 and 2 (ERK1/2).1,2 Phosphorylation and activation of these 3 terminal MAPks result from the upstream dual-specificity MAPK kinases (MAPKKs, also called MEKs or MKks) that include MEK1/2 for ERK1/2, MKK3/MKK6 for p38, and MKK4/MKK7 for JNK1/2.2 Upstream of MAPKKs, multiple MAPKKKs form a complex network of kinases that either directly sense environmental stimulation or are activated by effectors such as G proteins (Ras, Rac, Rho, and others) and G protein–coupled receptors.3–5 In the heart, MAPks play a complex role in regulating cardiac hypertrophy and heart failure. For instance, cardiac-specific overexpression of dominant-negative mutants of p38α, MKK3, or MKK6 rendered the heart more susceptible to cardiac hypertrophy, and Mapk14 (p38) heart-specific null mice are more prone to heart failure.3,4 Similarly, genetic inhibition of JNK1/2 also rendered the heart more susceptible to cardiac hypertrophy and failure, similar to the phenotype of mice with cardiac-specific deletion of the genes encoding MKK4 or MKK7 protein.9–11 With respect to ERK1/2 signaling, MEK1 heart-specific transgenic mice, which showed constitutive ERK1/2 signaling, were characterized by concentric hypertrophic remodeling with a relatively cardioprotective phenotype.12 By comparison, mice with heart-specific deletion of Mapk1/3 (ERK1/2) presented with extreme dilation and decompensation, a phenotype also observed in transgenic mice overexpressing the ERK1/2 inactivating dual-specificity phosphatase 6 (DUSP6) in cardiac myocytes of the heart.13,14

Original received December 22, 2015; revision received May 10, 2016; accepted May 25, 2016. In April 2016, the average time from submission to first decision for all original research papers submitted to Circulation Research was 15.28 days.

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The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.115.308238/-/DC1.

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Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.115.308238
The magnitude and duration of MAPK phosphorylation are critical in determining the extent of the biological signaling response, which although critically regulated by the upstream MAPKKs is also equally regulated by dephosphorylation and the subsequent recycling of the MAPKs. The MAPKs are phosphorylated at both threonine and tyrosine residues within the activation loop (TXY motif), which can also be directly dephosphorylated by the Ser/Thr phosphatases protein phosphatase 1, protein phosphatase 2, protein tyrosine phosphatases, or more specifically by dedicated DUSPs.15 There are 13 MAPK-specific DUSPs that have been characterized and classified into 3 subfamilies based on sequence homology, subcellular localization, and substrate specificity.15,16 A unique aspect of the DUSPs is that they are rapidly induced by stress stimulation, where each gene is transcribed and translated within 15 to 40 minutes, providing a negative feedback mechanism to dephosphorylate and inactivate the MAPKs to allow for their subsequent recycling.17–20 Although the basic biology of the DUSPs is well understood, the field currently lacks an understanding of how these 13 different genes are integrated into stress responsiveness in vivo and the effects on organ physiology and disease.20–23

DUSP8, also known as M3/6, belongs to a group of DUSPs that also includes DUSP10 and DUSP16, all 3 of which have a more complicated domain structure compared with the other DUSPs, and all 3 are localized to both the cytoplasm and the nucleus.24 Although all Dusp genes seem to be transcriptionally inducible, DUSP8 also has basal levels of expression in the heart and brain.25 DUSP8 was reported to prefer p38 and JNK based on overexpression studies, although it can also affect ERK1/2.26–28 In this study, we generated mice lacking the Dusp8 gene and transgenic mice with cardiac myocyte–specific overexpression of DUSP8 to further examine how it might regulate cardiac MAPK signaling and disease responsiveness. We observed that Dusp8 gene–deleted (knockout) mice presented with baseline concentric cardiac remodeling that was enhanced on stress stimulation. This concentric ventricular remodeling was associated with increased cardiac contractility at baseline and protection from dilation and heart failure in 2 different surgical models of induced pathology.
At the end of each surgical protocol, mice were euthanized and hearts were harvested for analysis of heart weight/body weight ratios. For histological analysis, adult hearts were fixed overnight in 10% formalin-containing phosphate-buffered saline and dehydrated for paraffin embedding. Serial 5-μm heart sections were stained with Masson trichrome to detect interstitial fibrosis as blue.

**Cell Analysis**

Neonatal rat cardiac myocytes were generated as previously described from 1- to 2-day old newborns and then infected with a recombinant adenoviruses expressing either β-galactosidase or DUSP8 for 36 hours. Cells were serum starved for 1 hour, stimulated with 10 μmol/L of phenylephrine (phenylephrine, Sigma, P6126) for 5 or 15 minutes, and harvested for Western blot analysis. Adult mouse ventricular myocytes were isolated from whole hearts on a hanging apparatus with a solution containing liberase blendzyme (Roche, 05401151001) as previously described. After isolation, cardiac myocytes were plated on laminin-coated dishes and cultured in medium 199 (Corning, 10-060-CV). Cardiac myocyte length and width were measured with NIH ImageJ software on phase contrast images of fixed cells. Alternatively, adult ventricular myocytes were serum starved for 1 hour and then stimulated with 10 μmol/L of phenylephrine or 0.1 mmol/L of Ang II (Sigma, A9525) and harvested for RNA and protein analysis. Adult rat cardiac myocytes were isolated using a solution containing 0.5 mg/mL type II collagenase (Worthington, LS004176) and 0.24 mg/mL hyaluronidase (Sigma, H3506), cultured in DMEM (Dulbecco’s Modified Eagle’s Medium; Hyclone, SH30022.01) containing 1% fetal bovine serum (Sigma, F2442). Rat cardiac myocytes were then infected with recombinant adenoviruses expressing either β-galactosidase. MEK1, or DUSP8 for 48 hours. Alternatively, rat cardiac myocytes were transfected with 50 nmol/L of control (Drumhac, D-001810-10-05) or Dusp8 siRNA (Drumhac, L-084829-02-0005) for 48 hours. Myocytes were either fixed for subsequent cell size analysis or stimulated with 10 μmol/L of phenylephrine for protein analysis.

Mouse embryonic fibroblasts (MEFs) were isolated from embryonic day 12.5 Dusp8 WT and knockout embryos. Briefly, embryos were removed from the uterus and washed in phosphate-buffered saline, the heads and other visceral organs were removed, then the remaining tissues were minced with a sterile razor blade and digested with 0.25% trypsin for 30 to 45 minutes at 37°C before the trypsin was inactivated with DMEM media containing 10% fetal bovine serum. The MEFs were collected as the cells growing out from the debris and cultured for 2 passages and harvested for Western blot analysis in the following buffer: 10 mmol/L HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 1 mmol/L DTT (dithiothreitol), 0.4% IGEPAL (octylphenoxypolyethoxyethanol), and a protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific, 78440). Protein samples were centrifuged at 15000g for 5 minutes to collect the supernatant as a cytoplasmic fraction, and the pellet as a nuclear fraction, which was then eluted into buffer containing 20 mmol/L HEPES pH 7.9, 0.4 mol/L NaCl, 1 mmol/L EDTA, 10% glycerol, 1 mmol/L DTT, and protease/phosphatase inhibitors. Equal amount of cytoplasmic and nuclear proteins were loaded for Western blot analysis. For analysis of interaction between DUSP8 and MAPKs, HEK293 cells were transfected with Flag-DUSP8 for 36 hours, lysed into buffer containing 20 mmol/L HEPES, 0.5% Triton X-100, 150 mmol/L NaCl, 1 mmol/L EDTA, and a protease/phosphatase inhibitor cocktail. Protein samples were incubated with anti-Flag M2 magnetic beads (Sigma, M8823) for 1 hour at room temperature and eluted into 2× laemmli sample buffer.

**Results**

**DUSP8 Is Induced by Cardiac Stress to Regulate MAPK Signaling**

DUSP8 is predominantly expressed in the brain and heart, where it is localized to both the cytoplasm and the nucleus to inactivate MAPK signaling by direct dephosphorylation of the end effector kinases. To understand whether DUSP8 is functionally associated with cardiac diseases, we first analyzed DUSP8 expression from microarray data deposited in the public National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) database. DUSP8 was up-regulated in hearts from patients with dilated cardiomyopathy compared with nondisease control hearts (Online Figure I). We then investigated Dusp8 mRNA expression in myocytes and nonmyocytes isolated from neonatal or adult rodent hearts. Expression of Dusp8 mRNA was predominantly detected in myocytes (Online Figure II), which was further confirmed by protein analysis (Figure 1A; Online Figure IIB). We also assessed DUSP8 induction in cultured cardiac myocytes after...
Ang II or phenylephrine stimulation, 2 agonists known to induce G protein–coupled receptor signaling and expression of other DUSP genes.\(^3\) Both stimuli significantly enhanced the levels of DUSP8 protein and increased Dusp8 mRNA in cardiac myocytes preferentially over nonmyocytes (Figure 1B; Online Figure IIC and IID). DUSP8 expression was also significantly increased in the hearts of mice subjected to transverse aortic constriction (TAC) or myocardial infarction (MI), harvested at the times shown in hours after the procedure.

A recombinant adenovirus was also generated to overexpress DUSP8 in cultured neonatal rat cardiac myocytes to more carefully evaluate the effect on MAPK phosphorylation status at baseline and with 5 and 15 minutes of phenylephrine stimulation. Compared with Ad\(\beta\)gal–infected cardiac myocytes, AdDUSP8 infection led to a significant reduction of phenylephrine-simulated phosphorylation of ERK1/2 and JNK1/2, but not p38 (Figure 1D; Online Figure II, panel I). Interestingly, among the MAPKKs, phosphorylation of MEK1/2 was also mildly diminished upon DUSP8 overexpression (Figure 1D). In contrast, knockdown of DUSP8 with small interfering RNA in cultured cardiac myocytes selectively increased the phosphorylation of ERK1/2 at baseline and with phenylephrine stimulation, without influencing JNK1/2 or p38 phosphorylation (Figure 1E; Online Figure IIJ).

These changes in ERK1/2 phosphorylation associated with manipulation in DUSP8 expression also resulted in a profound effect on myocyte morphology in culture. Adult rat cardiac myocytes infected with AdDUSP8 were thinner compared with control myocytes infected with Ad\(\beta\)gal, whereas cardiac myocytes transfected with Dusp8 small interfering RNA for 48 h had increased width and reduced length, a phenomena similar to that of AdMEK1 overexpressing cells (Figure 1F). Indeed, analysis of length/width ratios revealed that loss of DUSP8 by small interfering RNA led to a type of concentric growth of adult myocytes in culture, whereas overexpression of DUSP8 promoted a type of dilated or eccentric growth in culture (Figure 1G). Thus, DUSP8 primarily regulates ERK1/2 MAPK signaling.
in cardiac myocytes, which influences the growth patterning characteristics of these cells.

**Dusp8 Null Mice Have Increased ERK1/2 Phosphorylation and Concentric Remodeling of the Heart**

To assess the physiological function of DUSP8 in the heart, we generated *Dusp8* gene-deleted embryonic stem cells, then mice. Restriction enzyme sites and exons and the neomycin (Neo) resistance cassette in the T.V. are shown. A, Schematic representation of the *Dusp8* genetic locus and the targeting vector (T.V.) used to create *Dusp8* gene-deleted embryonic stem cells, then mice. Restriction enzyme sites and exons and the neomycin (Neo) resistance cassette in the T.V. are shown. B, Real-time polymerase chain reaction (RT-PCR) analysis of *Dusp8* mRNA in the brain, heart, and lung of 2-mo-old wild-type (WT) vs *Dusp8* knockout (KO) mice. Gapdh was used as PCR control. C, RT-PCR analysis of expression of multiple *Dusp* mRNAs in hearts of 2-mo-old *Dusp8* WT or KO mice; n=3 individual samples. D, Heart weight (HW) normalized to body weight (BW) in *Dusp8* WT and KO mice at the indicated ages. Number of mice used is shown in the bars. E and F, Echocardiographic assessment of fractional shortening (FS) and interventricular septal thickness in diastole (IVSd) in *Dusp8* WT and KO mice at the indicated ages. *P<0.05 vs WT. Number of mice used is shown in the bars. G, Representative Masson trichrome–stained histological sections from the hearts of *Dusp8* WT and KO mice at 6 mo of age. Magnification is ×40 total. H, Representative microscopic phase–contrast images of adult cardiac myocytes isolated from 6-mo-old *Dusp8* WT and KO mice. Magnification is ×200 total. I, Quantification of length/width ratio of adult cardiac myocytes isolated from *Dusp8* WT and KO mice at 6 mo of age as shown in H. A total of 248 myocytes were analyzed for each group. *P<0.05 vs WT. J, Analysis of the surface area of adult myocytes isolated from 6-mo-old *Dusp8* WT and KO mice as shown in H. A total of 248 myocytes were analyzed for each group. K and L, Invasive hemodynamic measurement of (K) cardiac contractility at baseline (load dependent) as maximum rate of pressure change in the left ventricle over time (dP/dt max) or (L) time constant for isovolumic relaxation (τ) showing the exponential decay of ventricular pressure during isovolumic relaxation in *Dusp8* WT and KO mice; n=5 for each group. *P<0.05 vs WT. M, RT-PCR analysis for mRNA levels of atrial natriuretic factor (ANF), B-type natriuretic peptide (BNP), β-myosin heavy chain (β-MHC), and α-myosin heavy chain (α-MHC) in 2-mo-old *Dusp8* WT or KO mice; n=4 for each group. *P<0.05 vs WT.
protection as shown previously in select mouse models.\textsuperscript{12,13} Using invasive hemodynamic measurements in vivo, \textit{Dusp8} null mice showed a small, albeit significant increase in cardiac contractility and faster relaxation times compared with WT controls (Figure 2K and 2L). Interestingly, investigation of gene expression profiles in adult hearts from \textit{Dusp8} null mice showed a significant and consistent reduction in \(\beta\)-MHC mRNA levels compared with WT mice, but not other hypertrophic markers (Figure 2M). The \(\beta\)-MHC gene encodes a slower ATPase isofrom of MHC compared with the \(\alpha\)-MHC isoform that is more highly expressed in the rodent heart.\textsuperscript{34}

To investigate the underlying signaling mechanism for the concentric remodeling observed in \textit{Dusp8} knockout hearts, we analyzed total MAPK phosphorylation at 2 months of age. We have previously shown that \textit{Dusp1/4} double-null mice had a baseline augmentation in total phospho-p38 levels in the heart, whereas \textit{Dusp6} null mice had a selective increase in total baseline ERK1/2 phosphorylation in the heart.\textsuperscript{20,21} MEFs from WT and \textit{Dusp8} gene–deleted mice were first analyzed because these cells typically have less sporadic MAPK activation at baseline compared with cardiac myocytes (R. Liu and J.D. Molkentin, unpublished data, 2015). Indeed, WT MEFs showed low levels of ERK1/2, JNK1/2, and p38 activation at baseline, yet \textit{Dusp8} null MEFs showed a dramatic increase in baseline and phenylephrine-stimulated ERK1/2 phosphorylation, with no changes in JNK1/2 or p38 (Figure 3A; Online Figure IIIA). These results suggest that DUSP8 has a specific function in dephosphorylating ERK1/2 in fibroblasts, but not other MAPKs.

Because DUSP8 is most highly expressed in heart and brain, we also investigated baseline MAPK phosphorylation levels in these whole tissues in young adult mice. Consistent with the data in MEFs, ERK1/2 but not JNK1/2 or p38, phosphorylation levels were significantly elevated at baseline in both heart and brain tissue (Figure 3B; Online Figure IIIB). To analyze ERK1/2 phosphorylation in more depth, \textit{Dusp8} null mice and WT controls were subjected to either acute phenylephrine injection or a TAC surgical procedure, and thereafter hearts were harvested 10, 60, and 120 minutes for analysis of MAPK phosphorylation levels (Figure 3C and 3D; Online Figure IIIC through IIIE). Here, the data show a significant increase only in ERK1/2 phosphorylation at 10 minutes, whereas by 60 or 120 minutes after stimulation, ERK1/2 phosphorylation was equally downregulated between null and WT mice (see Discussion section of this article). There were no phosphorylation changes in p38 or JNK1/2 between WT and null hearts (Figure 3C and 3D; Online Figure IIIC through IIIE). The acute increase in ERK1/2 phosphorylation suggested that the effect of DUSP8 might be restricted to shorter durations; hence, we repeated the analysis over a more refined acute time course of 10, 20, 30, and 40 minutes after TAC stimulation. The data showed significantly greater ERK1/2 phosphorylation at all 4 time points in \textit{Dusp8} null hearts compared with WT controls (Figure 3E; Online Figure IIIF). Taken together, these results suggest that DUSP8 regulates both baseline and short-term ERK1/2 signaling, but by 60 minutes and beyond other DUSPs are likely induced where they contribute enough to diminish this effect.

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\caption{Loss of \textit{Dusp8} leads to increased extracellular signal–regulated kinases 1/2 (ERK1/2) phosphorylation. A, Western blot assessment of mitogen-activated protein kinase (MAPK) phosphorylation and total MAPK levels in \textit{Dusp8} wild-type (WT) and knockout (KO) mouse embryonic fibroblasts (MEFs) in culture with or without phenylephrine (PE) stimulation at 10 \textmu{mol}/L for the indicated times (0 time point is no PE stimulation). Of note, there is an open lane between the 6 WT and KO protein samples on the Western blot, given the variable bleed over from the adjacent lanes. B, Quantitative analysis of phosphorylated MAPKs relative to total MAPKs in 2-mo-old \textit{Dusp8} WT and KO hearts and brains shown in Online Figure IIIB; \(n=4\) for each group. *\(P<0.05\) vs WT. C to E, Western blot analysis of cardiac MAPK phosphorylation and total MAPK levels in \textit{Dusp8} WT or KO mice after (C) PE or (D and E) transverse aortic constriction (TAC) stimulation as indicated in the panel, for the indicated period of time in minutes; \(n=4\) or greater for each. F, Western blot analysis of interaction between endogenous MAPKs (ERK1/2, p38, and c-Jun N-terminal kinase 1/2 [JNK1/2]) and exogenously expressed Flag-dual-specificity phosphatase 8 (DUSP8) in HEK293 cells. This experiment was repeated 3x with similar results.}
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DUSP6 can also affect baseline ERK1/2 phosphorylation status in the heart and other tissues, as shown previously, which is likely because of a known direct interaction between this phosphatase and ERK2. A Flag-DUSP6 encoding plasmid was transfected into HEK293 cells followed by pulldown of DUSP6 with a Flag resin, which identified both ERK1 and ERK2 bands, and the 54-kDa isoform of JNK1/2 (Figure 3F). Hence, DUSP8 is part of a complex with ERK1/2 and a specific isoform of JNK1/2, which could explain how it might regulate basal levels of phosphorylation of at least ERK1/2 in the heart and other tissues (MEFs).

**Assessment of Stress-Induced Hypertrophy in Dusp8 Null Mice**

Heart-specific MEK1 transgenic mice, which have constitutively augmented ERK1/2 phosphorylation in the heart, have mild concentric hypertrophic remodeling, are hypercontractile, and are partially protected from heart failure–inducing stimuli. Thus, the mild concentric heart remodeling and increase in cardiac contractility we observed in Dusp8 null mice was hypothesized to positively affect cardiac health after disease stimulation. First, we subjected WT and Dusp8 null mice to infusion of Ang II/phenylephrine or a TAC surgical procedure for 2 weeks, which induced an identical increase in heart weights in both the groups (Figure 4A and 4D). However, Dusp8 null mice maintained cardiac functional performance significantly better than WT controls subjected to either stimulus and they continued to show concentric ventricular remodeling (Figure 4B and 4E; Online Table II). Moreover, isolation of individual adult cardiac myocytes from these hearts after 2 weeks of stimulation showed the same propensity toward myocyte thickening without a matching increase in length, compared with WT controls, although total cellular areas were the same (Figure 4C, 4F, and 4G). There was still a significant reduction in β-MHC mRNA levels in the heart after 2 weeks of Ang II/phenylephrine stimulation or TAC in null versus WT control mice. However, the induction of ANF and B-type natriuretic peptide, and decrease in α-MHC, after either stimulation were not statistically different between Dusp8 null and WT mice (Figure 4H and 4I).

To investigate the effect of Dusp8 gene deletion on the propensity for heart failure progression in the setting of more severe disease, we subjected Dusp8 null and WT control mice to 12 weeks of TAC stimulation. The data show that Dusp8 null mice maintained cardiac function, whereas the WT controls significantly decompensated in a progressive manner during the 12 weeks of TAC, although total increases in heart weight normalized to body weights were not different between the WT and null groups (Figure 4J and 4K). These results indicate that the mild baseline concentric remodeling response and increased contractile performance characteristic of Dusp8 null mice does not predispose to maladaptation, but instead provides prolonged protection.

Compensatory remodeling and heart failure after MI injury in Dusp8 null and WT mice were investigated as well. Importantly, loss of Dusp8 from the heart did not affect the degree of myocyte loss and total area of infarction after ischemia/reperfusion injury to the heart, compared with WT hearts (Figure 5A and 5B). With respect to post-MI ventricular remodeling and heart failure, WT mice showed a significant reduction in cardiac ventricular performance as measured by echocardiography at 1, 2, and 3 weeks after injury, whereas Dusp8 null mice were protected and maintained performance similar to sham surgical controls (Figure 5C). Further echocardiographic analysis also suggested protection as indicated by less ventricular dilation in Dusp8 null hearts 3 weeks after MI injury, as well as a maintained thickness in the septal wall and a trend toward greater thickness in the LV free wall in null compared with WT mice (Figure 5D). Indeed, histological analysis of these hearts 3 weeks after MI showed greater ventricular dilation in WT mice, whereas the null mice had noticeably thicker septa and remaining LV free walls (Figure 5E). Thus, Dusp8 null mice were partially protected from cardiac insults that would otherwise cause failure (see Discussion section of this article).

**Cardiac-Specific Overexpression of Dusp8 Causes Cardiomyopathy**

To further investigate the manner in which DUSP8 might alter MAPK signaling and ventricular remodeling, cardiac-specific inducible transgenic mice to overexpress this phosphatase in the heart were also generated (Figure 6A). The DUSP8 cDNA was cloned into the α-MHC promoter-containing responder vector to generate transgenic mice. When bred with transgenic mice containing the α-MHC–detranscribed cyclase transactivator protein, and doxycycline is absent, this line now produced expression in the heart (Figure 6A). Using this bitransgenic strategy in the absence of doxycycline, virtually all double-transgenic (DTG) mice died within 2 weeks of birth (data not shown), indicating that overexpression of DUSP8 is detrimental to the heart during development. However, treatment of pregnant mothers with doxycycline-containing chow to shut down the inducible system during development and up to the first 3 weeks after birth resulted in viability of all DTG mice. On weaning, mice were removed from the doxycycline-dependent transgene repression, whereby DUSP8 mRNA and protein expression were strongly induced in the heart during the next 6 weeks (Figure 6B and 6C).

Hearts from DUSP8 DTG mice were collected at 9 weeks of age for analysis of MAPK phosphorylation status. The data showed downregulation in baseline ERK1/2, JNK1/2, and p38 phosphorylation in the heart because of DUSP8 overexpression, as well as a significant increase in MEK1 phosphorylation, similar to the effect observed in Mapk1/3 (ERK1/2) knockout hearts through a feedback mechanism as previously observed (Figure 6D; Online Figure IVA). Associated with this global reduction in total MAPK phosphorylation in the heart was an increase in baseline dilation in LV chamber dimension and a loss in cardiac ventricular performance (Figure 6E and 6F; Online Figure IVB). Heart weight normalized to body weight was increased as was the length of individual adult myocytes isolated from DUSP8 DTG hearts, indicating dilated cardiac remodeling (Figure 6G and 6H). DUSP8 overexpression also induced cardiac hypertrophic marker gene expression with a dramatic increase in β-MHC mRNA and a decrease in α-MHC mRNA, as well as generalized cardiac fibrosis (Figure 6I and 6J). Overexpression of DUSP8 reduced the inducible phosphorylation of ERK1/2, p38, and...
Figure 4. Analysis of cardiac hypertrophy and remodeling in Dusp8 null (KO) mice after stress stimulation. A, Heart weight (HW)/body weight (BW) ratio in Dusp8 wild-type (WT) and KO mice 2 wk after angiotensin II (Ang II)/phenylephrine (PE) infusion or PBS control. Number of mice used is shown within the bars. *P<0.05 vs PBS. B, Echocardiographic assessment of fractional shortening (FS) in Dusp8 WT and KO mice after 2 wk of Ang II/PE infusion. *P<0.05 vs WT Ang II/PE. Number of mice analyzed is shown in the bars. C, Analysis of length/width ratio of adult cardiac myocytes isolated from Dusp8 WT and KO mice after Ang II/PE infusion. Approximately 250 myocytes were analyzed for each group. *P<0.05 vs WT. D, HW/BW ratio in Dusp8 WT and KO mice 2 wk after transverse aortic constriction (TAC) or a sham procedure. Number of mice analyzed is shown in the bars. *P<0.05 vs WT. E, Echocardiographic analysis of FS in Dusp8 WT or KO mice after 2 wk of TAC or a sham procedure. *P<0.05 vs WT TAC. Number of mice analyzed is shown in the bars. F, Analysis of length/width ratio of adult cardiac myocytes isolated from Dusp8 WT and KO mice after 2 weeks of TAC. Approximately 250 myocytes were analyzed for each group. *P<0.05 vs WT TAC. G, Analysis of adult cardiac myocyte area from dissociated hearts from Dusp8 WT and KO mice after Ang II/PE, TAC or a sham procedure. Approximately 250 myocytes were analyzed for each group. H and I, Real-time polymerase chain reaction analysis of mRNA levels of atrial natriuretic factor (ANF), B-type natriuretic peptide (BNP), α-myrin heavy chain (α-MHC), and α-myosin heavy chain (α-MHC) from hearts of Dusp8 WT and KO mice after Ang II/PE (H) or TAC (I) stimulation for 2 wk compared with sham-operated groups. At least 4 mice (hearts) were analyzed in each group. *P<0.05 vs WT or KO sham; #P<0.05 vs WT sham. J, Echocardiographic analysis of FS in Dusp8 WT and KO mice after TAC or a sham procedure for the indicated time in weeks. *P<0.05 vs KO TAC. Number of mice analyzed is shown. K, HW/BW ratio in Dusp8 WT and KO mice 12 wk after a TAC or a sham procedure. Number of mice analyzed is shown in the bars. *P<0.05 vs Sham.

JNK1/2 after TAC stimulation for 10 minutes, compared with control mice (Figure 6K; Online Figure IVC). TAC stimulation for 2 weeks also induced greater decompensation and loss of ventricular performance in DUSP8 DTG mice compared with tetracycline transactivator littermate controls (Online Figure IVD). Although overexpression of DUSP8 models the known increase in this gene product in disease hearts, the levels achieved here in the transgenic approach were higher and might be responsible for some loss of specificity in also dephosphorylating JNK1/2 and p38. However, the transgenic approach still provides insight into how regulation of ERK1/2 by this DUSP family member can underlie cardiac remodeling and disease responsiveness (see Discussion section of this article).

Discussion

Loss of Dusp8 resulted in a profile of elevated ERK1/2 phosphorylation at baseline and with acute stimulation, resulting in a cardiac phenotype of concentric growth because of an increase in cardiac myocyte width, as well as a mild increase in contractility. This profile in Dusp8 null mice conferred protection to the heart in response to challenges that would otherwise lead to pronounced ventricular dilation and heart failure. Conversely, overexpression of DUSP8 decreased phosphorylation of ERK1/2, as well as p38 and JNK, resulting in the opposite phenotype of eccentric and dilatory ventricular remodeling that was associated with lengthening of individual cardiac myocytes. That ERK1/2 are the primary mechanistic explanation for eccentric versus concentric remodeling of the heart because of altered DUSP8 signaling is consistent with past data in genetically altered mice with specific manipulation of MEK1 or ERK1/2. 12-14,21

One of the interesting aspects of our study is the relationship between DUSP8 and MAPK signaling dynamics in the heart, which is also dependent on the backdrop of other available or coinduced Dusp genes in cardiac myocytes. We observed that loss of Dusp8 produced both an increase in baseline ERK1/2 phosphorylation and greater net phosphorylation levels in the first 40 minutes of stress to the heart. However, overexpression of DUSP8 in the heart or cultured
cardiac myocytes caused dephosphorylation of all 3 major MAPK terminal effectors. Past literature has suggested that cardiac myocytes caused dephosphorylation of all 3 major MAPK terminal effectors. Past literature has suggested that DUSP8 preferentially regulates p38 and JNK1/2 in select cell lines, although cardiac myocytes were not investigated, and these past approaches were also based on overexpression strategies. Thus, results from knockout mice or knockout cell lines are not in agreement with overexpression approaches in attributing the potential physiological targets of DUSP8. This same general paradigm is reminiscent of data obtained with Dusp1 gene–targeted mice. Dusp1 null mice showed no effect on ERK1/2 phosphorylation in the heart, yet cardiac overexpression of DUSP1 led to the equal inactivation of ERK1/2, JNK1/2, and p38. Thus, loss-of-function approaches are likely of greater biological relevance in ascertaining the true regulatory targets of various DUSP family members compared with overexpression approaches. In this case, our results suggest that DUSP8 is a more dedicated regulator of ERK1/2 in the heart, brain, and also fibroblasts.

Biochemical characterization studies revealed that MAPK family members have evolutionarily conserved interaction domain with 2 aspartic acids that are essential for binding to MAPKKs, phosphatases, and substrates. Similar to other DUSP proteins, DUSP8 possesses a docking site (56KRR58) in the N terminus for possible ERK1/2 recognition by DUSP8 by analyzing how amino acid residues within this domain might specify the type of MAPKs that can be bound. It is also likely that other regions outside of the docking domain could further specify ERK1/2 binding, as suggested by in depth biochemical analysis of the DUSP6–ERK2 interaction. The profile of greater ERK1/2 phosphorylation in vivo in the absence of DUSP8 is intimately tied to the expression and induction of other DUSP family members that have activity toward ERK1/2. We previously showed that DUSP6 serves as a major DUSP for regulating baseline ERK1/2 dephosphorylation status in cardiac myocytes, but not in response to stress induction. Thus, although both DUSP6 and DUSP8 seem to help establish a set point in basal ERK1/2 phosphorylation status in the heart, DUSP8 can also regulate ERK1/2 dephosphorylation with acute stress stimulation over at least 40 minutes of time. After 60 minutes, ERK1/2 now starts to show dephosphorylation, likely because of the effects of other DUSP family members who are induced and that possibly play a greater role in long-term dampening of this signaling pathway, such as DUSP2, 5, and 7 that also have reported specificity for ERK1/2. Thus, DUSP8 seems to have a unique regulatory role in the heart by controlling both the baseline and the acute amplitude of ERK1/2 phosphorylation during a stress or injury response.

Dusp8 null mice also displayed an unexpected cardiac phenotype at baseline. Hearts from these mice were significantly remodeled with a profile of concentric ventricular hypertrophy and a mild but significant increase in baseline contractility, although absolute heart weights were not changed. Isolated adult cardiac myocytes from hearts of Dusp8 null mice were significantly thicker and shorter, a phenotype that is reminiscent of transgenic mice expressing activated MEK1 in the heart (constitutive ERK1/2 activity). Indeed, both MEK1 transgenic and Dusp8 knockout mice were significantly protected from pathological insults that would otherwise cause heart failure. This profile of concentric remodeling with thicker cardiac myocytes seems to be protective, possibly by simply maintaining...
lower ventricular wall stress because of LaPlace’s law. With respect to loss-of-function, mice with heart-specific deletion of \(\text{Mapk1/3} \) \((\text{ERK1/2})\) showed extreme cardiac dilation characterized by longer cardiac myocytes that lacked thickness. By comparison, inhibiting for ERK1/2 phosphorylation. Thus, the dynamic equilibrium of ERK1/2 phosphorylation is intimately tied to an ill-defined molecular mechanism whereby cardiac myocyte length/width ratio is encoded, either developmentally or in response to new hypertrophic stimuli to the adult heart.

We believe that ERK1/2 are unique in their ability to promote cardiac myocyte thickening growth compared with the other MAPKs. Indeed, cardiac-specific MEK3 or MEK6 transgenic mice, which have greater p38 MAPK signaling, showed cardiomyopathy and ventricular dilation, similar to MKK7 TG mice with greater JNK1/2 activity. By comparison, inhibition of p38 or JNK1/2 in the heart seemed to promote hypertrophic thickening of the heart. Thus, p38 and JNK1/2 signaling have the opposite effect of ERK1/2 in affecting myocyte and ventricular growth dynamics, although we do not think that as ERK1/2. Thus, although DUSP8 overexpression in the heart also promoted p38 and JNK1/2 inactivation in addition to ERK1/2, the hearts of these mice still dilated instead of becoming thicker, likely because of the more central role of ERK1/2 signaling. Indeed, we previously published that DUSP8 KO heart samples are shown as a control. D, Western blot analysis for mitogen-activated protein kinase (MAPK) phosphorylation and total MAPKs in tTA controls and DTG mice at 9 wk of age. At least 6 separate heart samples were analyzed for each group with similar results. E and F, Echocardiographic assessment of fractional shortening [FS] and left ventricle [LV] internal chamber dimension at end diastole [LVIDd] in tTA controls and DTG mice at 9 wk of age with 6 wk of transgene induction. Thus, although there are 13 DUSP proteins that are dedicated to regulating and recycling the MAPKs, each seems to have a highly specialized regulatory role.

DUSP8 is 1 of only 3 family members that is present in both the cytoplasm and the nucleus, and DUSP8 has a more complicated multidomain configuration with a unique C-terminal PEST motif rich in proline [P], glutamic acid [E], serine [S], and threonine [T] that mediates its rapid degradation. Our loss-of-function study performed here now suggests for the first time that DUSP8 is a dedicated regulator of ERK1/2 in vivo. This specificity is likely tied to specific complexes that are formed among the MAPKs and the DUSP proteins, consistent with our observation that DUSP8 can form a complex with ERK1/2 in vivo. Indeed, our results suggest that...
DUSP8 is a dedicated regulator of ERK1/2 signaling in the heart, which affects cardiac ventricular geometry, contractility, and disease responsiveness.

Sources of Funding
This work was supported by grants from the National Institutes of Health (to J.D. Molkentin). J.D. Molkentin was also supported by the Howard Hughes Medical Institute. R. Liu and R. Vagnozzi were supported by a training grant from the National Heart Lung and Blood Institute of the National Institutes of Health (NIH; T32HL125204, and F32HL128083, respectively).

Disclosures
None.

References

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This study was designed to examine the involvement of DUSP8 as a regulator of mitogen-activated protein kinase signaling in the heart and the impact on ventricular and cardiac myocyte growth dynamics. We demonstrate that DUSP8 functions primarily through extracellular signal–regulated kinases ½ signaling in cardiac remodeling and restrictive cardiomyopathy. Our results suggest that DUSP8 regulates the dynamics of mitogen-activated protein kinase signaling to influence cardiac remodeling and disease propensity in vivo.
DUSP8 Regulates Cardiac Ventricular Remodeling by Altering ERK1/2 Signaling
Ruijie Liu, Jop H. van Berlo, Allen J. York, Ronald J. Vagnozzi, Marjorie Maillet and Jeffery D. Molkentin

_Circ Res._ 2016;119:249-260; originally published online May 25, 2016; doi: 10.1161/CIRCRESAHA.115.308238

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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DUSP8 regulates cardiac ventricular remodeling by altering ERK1/2 signaling

Ruijie Liu, Jop H. van Berlo, Allen J. York, Marjorie Maillet, Ronald J. Vagnozzi, Jeffery D. Molkentin
Online Figure I. *Dusp8* mRNA is highly expressed in human dilated cardiomyopathy patients. Data in the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) database contained an elaborate microarray experiment from human heart biopsies (GEO accession GDS2206, reference #32) that showed an increase in *Dusp8* mRNA in patients with dilated cardiomyopathy (DCM). Available data included 15 normal hearts samples and 13 dilated cardiomyopathy samples examined with custom made microarrays to assess mRNA levels. *p*<0.05 vs normal.
Online Figure II. Expression and localization of DUSP8, and its effect on MAPK signaling.  

A, Real-time PCR analysis of *Dusp8* mRNA expression in myocytes and nonmyocytes isolated from neonatal or adult hearts. * p<0.05 vs myocytes. N=4 for each group.  

B, Quantification of DUSP8 protein expression from experiments as shown in Figure 1A.  

C, Real-time PCR analysis of *Dusp8* expression in myocytes and nonmyocytes following AngII (0.1 µmol/L) or PE (10 µmol/L) stimulation for 4 hours. *p<0.05 vs control (Con) myocytes.  

D, Quantification of DUSP8 protein induction in response to AngII or PE as shown in Figure. 1B.  

E, Real-time PCR analysis of *Dusp8* mRNA expression in the hearts of mice following TAC or MI for the indicated time periods. *p<0.05 vs 0 min sham control for TAC or MI.  

F, Quantification of DUSP8 protein induction in response to TAC or MI as shown in Figure 1C. * p<0.05 vs 0 min sham control for TAC or MI.  

G, H, Western blot and quantitation of subcellular protein fractions for localization of Flag-DUSP8 in cytoplasm versus nucleus of HEK293 cells transfected with a Flag-*Dusp8* DNA expression plasmid. Lamin A/C and α-tubulin were used as loading controls for nucleus and cytoplasm, respectively. Cyto., cytoplasm; Nuc., nucleus.  

I, Quantification of the indicated phospho-MAPK and total MAPK levels from at least 3 independent experiments as shown in Figure 1D. *p<0.05 vs control ERK (Adβgal infected); #p<0.05 versus control JNK.  

J, Quantification of the indicated phospho-MAPK levels from at least 3 independent experiments as shown in Figure 1E. *p<0.05 vs scramble-ERK.
Online Figure III. DUSP8 preferentially dephosphorylates ERK1/2 in vivo. A, Quantitative analysis of phosphorylated MAPKs relative to total MAPKs in Dusp8 WT and KO MEFs following PE (10 µmol/L) stimulation for indicated time shown in Figure 3A. Data are representative of at least three independent experiments. *p<0.05 vs WT ERK for each time point. B, Western blot analysis of MAPK phosphorylation and total MAPK levels in hearts and brains of 2 month-old Dusp8 WT and KO mice. C-E, Quantitative analysis of phosphorylated MAPKs relative to total MAPKs in Dusp8 WT and KO hearts following PE or TAC stimulation shown in Figure 3C and 3D. N=4 for each group. *p<0.05 vs WT PE; #p<0.05 vs WT TAC. F, Quantitative analysis of phosphorylated ERK/total ERK shown in Figure 3E. At least 3 samples were quantified from each group at each time point. *p<0.05 vs WT.
Online Figure IV. Cardiac overexpression of DUSP8 leads to reduced MAPK activity and impaired cardiac function. 

A. Quantitative analysis of phosphorylated MAPKs relative to total MAPKs in tTA and DTG mice shown in Figure 6D. At least 3 independent heart samples were processed in each group. *p<0.05 vs tTA.

B. Echocardiographic parameters of hearts from tTA and DTG mice, 6 weeks after Dox removal to induce DUSP8 expression. Abbreviations: LVIDs, left ventricular end-systolic chamber diameter; IVSd and IVSs, intraventricular end-diastolic and end-systolic septal thickness; LVWd and LVWs, left ventricular end-diastolic and end-systolic posterior wall thickness. Five mice were analyzed in each group. * p<0.05 vs tTA.

C. Quantitative analysis of phosphorylated MAPKs relative to total MAPKs in hearts from tTA and DTG mice following TAC stimulation for the indicated times as shown in Figure 6K. At least 3 independent samples were processed for each. *p<0.05 vs tTA for each respective MAPK following TAC.

D. Echocardiography assessment of fractional shortening percentage (FS%) from hearts of tTA and DTG mice after 2 weeks of TAC or a sham procedure. Number of mice assessed is shown in the graph. Mice were 9 weeks-old, with 6 weeks no Dox. *p<0.05 vs tTA TAC.
### Online Table I. Echocardiographic parameters in *Dusp8* WT and KO mice at baseline

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>KO</th>
<th>P value</th>
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<tbody>
<tr>
<td><strong>2 months</strong></td>
<td></td>
<td></td>
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<tr>
<td>LVIDd(mm)</td>
<td>3.62±0.09</td>
<td>3.43±0.06*</td>
<td>0.009</td>
</tr>
<tr>
<td>LVIDs(mm)</td>
<td>2.49±0.10</td>
<td>2.43±0.09</td>
<td>0.65</td>
</tr>
<tr>
<td>LVWd(mm)</td>
<td>0.92±0.03</td>
<td>0.89±0.02</td>
<td>0.41</td>
</tr>
<tr>
<td>LVWs(mm)</td>
<td>1.09±0.03</td>
<td>1.13±0.02</td>
<td>0.28</td>
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<tr>
<td>IVSs(mm)</td>
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<td>1.23±0.03*</td>
<td>0.001</td>
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<tr>
<td>HR(bpm)</td>
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<td>541.86±7.09</td>
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<td><strong>6 months</strong></td>
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<tr>
<td>LVIDd(mm)</td>
<td>3.76±0.04</td>
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<tr>
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<td>0.93±0.01</td>
<td>0.08</td>
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<tr>
<td>LVWs(mm)</td>
<td>1.15±0.03</td>
<td>1.15±0.02</td>
<td>0.36</td>
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<tr>
<td>IVSs(mm)</td>
<td>1.15±0.03</td>
<td>1.28±0.02*</td>
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<tr>
<td>HR(bpm)</td>
<td>473.62±11.13</td>
<td>441.56±22.01</td>
<td>0.46</td>
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</table>

Data are presented in millimeters (mm) as mean±SE. LVIDd and LVIDs: left ventricular end-diastolic and end-systolic chamber diameters; LVWd and LVWs: left ventricular end-diastolic and end-systolic posterior wall thickness; IVSs: end-systolic intraventricular septal thickness. HR: heart rate, which is given in beats per minute (bpm). *p<0.05 vs WT.
### Online Table II. Echocardiographic parameters in Dusp8 WT and KO mice after 2 weeks of surgery.

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>AngII/PE</th>
<th>Sham</th>
<th>TAC</th>
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<td></td>
<td>WT</td>
<td>KO</td>
<td>N=6</td>
<td>N=6</td>
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<tr>
<td>LVIDd(mm)</td>
<td>3.72±0.08</td>
<td>3.99±0.09</td>
<td>3.74±0.07</td>
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<td>LVIDs(mm)</td>
<td>2.91±0.07</td>
<td>2.88±0.13</td>
<td>2.82±0.05</td>
<td>2.73±0.08</td>
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<tr>
<td>LVWd(mm)</td>
<td>0.92±0.02</td>
<td>0.89±0.03</td>
<td>0.91±0.02</td>
<td>1.00±0.03</td>
</tr>
<tr>
<td>LVWs(mm)</td>
<td>1.08±0.03</td>
<td>1.07±0.04</td>
<td>1.16±0.01</td>
<td>1.18±0.03</td>
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<tr>
<td>IVSd(mm)</td>
<td>0.70±0.02</td>
<td>1.07±0.04</td>
<td>0.72±0.01</td>
<td>0.82±0.03</td>
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<tr>
<td>IVSs(mm)</td>
<td>1.09±0.04</td>
<td>1.19±0.04</td>
<td>1.03±0.03</td>
<td>1.21±0.05</td>
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<tr>
<td>HR(bpm)</td>
<td>476±13</td>
<td>503±24</td>
<td>468±33</td>
<td>472±11</td>
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<tr>
<td>KO</td>
<td>N=6</td>
<td>N=8</td>
<td>N=10</td>
<td>N=8</td>
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<td>3.47±0.07#</td>
<td>3.65±0.11#</td>
<td>3.40±0.10*</td>
<td>3.48±0.09*</td>
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<td>2.43±0.08#</td>
<td>2.62±0.06*</td>
<td>2.38±0.07*</td>
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<tr>
<td>LVWd(mm)</td>
<td>0.89±0.02</td>
<td>0.88±0.04</td>
<td>0.93±0.07</td>
<td>1.01±0.02</td>
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<tr>
<td>LVWs(mm)</td>
<td>1.13±0.02</td>
<td>1.16±0.06</td>
<td>1.12±0.06</td>
<td>1.22±0.05</td>
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<td>IVSd(mm)</td>
<td>0.80±0.02#</td>
<td>1.16±0.06#</td>
<td>0.85±0.05*</td>
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<tr>
<td>IVSs(mm)</td>
<td>1.23±0.03#</td>
<td>1.14±0.03</td>
<td>1.12±0.03*</td>
<td>1.32±0.03*</td>
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<tr>
<td>HR(bpm)</td>
<td>436±21</td>
<td>476±20</td>
<td>471±20</td>
<td>446±16</td>
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</table>

Data are presented as millimeters (mm) as the mean±SE. LVIDd and LVIDs: left ventricular end-diastolic and end-systolic chamber diameters; IVSd and IVSs: end-diastolic and end-systolic intraventricular septal thickness. LVWd and LVWs, left ventricular end-diastolic and end-systolic posterior wall thickness. HR: heart rate, which is given in beats per minute (bpm). #p<0.05 vs WT Sham-AngII/PE; *p<0.05 vs WT Sham-TAC.