Unrestrained p38 MAPK Activation in Dusp1/4 Double-Null Mice Induces Cardiomyopathy

Mannix Auger-Messier,* Federica Accornero,* Sanjeewa A. Goonasekera, Orlando F. Bueno, John N. Lorenz, Jop H. van Berlo, Robert N. Willette, Jeffery D. Molkentin

Rationale: Mitogen-activated protein kinase (MAPKs) are activated in the heart by disease-inducing and stress-inducing stimuli, where they participate in hypertrophy, remodeling, contractility, and heart failure. A family of dual specificity phosphatases (DUSPs) directly inactivates each of the MAPK terminal effectors, potentially serving a cardioprotective role.

Objective: To determine the role of DUSP1 and DUSP4 in regulating p38 MAPK function in the heart and the effect on disease.

Methods and Results: Here, we generated mice and mouse embryonic fibroblasts lacking both Dusp1 and Dusp4 genes. Although single nulls showed no molecular effects, combined disruption of Dusp1/4 promoted unrestrained p38 MAPK activity in both mouse embryonic fibroblasts and the heart, with no change in the phosphorylation of c-Jun N-terminal kinases or extracellular signal-regulated kinases at baseline or with stress stimulation. Single disruption of either Dusp1 or Dusp4 did not result in cardiac pathology, although Dusp1/4 double-null mice exhibited cardiomyopathy and increased mortality with aging. Pharmacological inhibition of p38 MAPK with SB731445 ameliorated cardiomyopathy in Dusp1/4 double-null mice, indicating that DUSP1/4 function primarily through p38 MAPK in affecting disease. At the cellular level, unrestrained p38 MAPK activity diminished cardiac contractility and Ca2+ handling, which was acutely reversed with a p38 inhibitory compound. Poor function in Dusp1/4 double-null mice also was partially rescued by phospholamban deletion.

Conclusions: Our data demonstrate that Dusp1 and Dusp4 are cardioprotective genes that play a critical role in the heart by dampening p38 MAPK signaling that would otherwise reduce contractility and induce cardiomyopathy. (Circ Res. 2013;112:48-56.)

Key Words: dilated cardiomyopathy ■ genetically altered mice ■ mitogen-activated protein kinases ■ signal transduction

The greater mitogen-activated protein kinase (MAPK) signaling cascade consists of a sequence of successively acting kinases that result in the dual phosphorylation and activation of terminal kinases p38, c-Jun N-terminal kinases (JNKs), and extracellular signal-regulated kinases (ERKs).1 The major upstream activators of ERK1/2 are two MAP kinase kinases, MEK1 and MEK2, whereas p38 kinases are directly activated by MKK6 and MKK3, and JNKs are directly activated by MKK4 and MKK7.1 Upstream of the MAP kinase kinases, multiple MAP kinase kinase kinases form a complex network that either directly sense stress stimulation or directly are regulated by effectors such as low-molecular-weight G-proteins (Ras, Rac, Rho, and others) and G-protein-coupled receptors.12 In the heart, MAPK signaling pathways regulate hypertrophic growth, dilated cardiomyopathy with ventricular remodeling, cellular apoptosis, fibrosis, contractility, and cellular proliferation.2,3 With respect to p38 MAPK, overexpression of an activated MKK3 mutant protein in the heart promoted dilated cardiomyopathy and early lethality in transgenic mice, whereas activated MKK6 induced restrictive cardiomyopathy with hypertrophy that also led to early lethality.4 However, deletion of the primary p38 gene in the heart, p38α, also predisposed to disease because mice subjected to transverse aortic constriction showed a worse phenotype.5 Cardiac-specific overexpression of dominant-negative p38α or dominant-negative MKK3 rendered the heart more susceptible to cardiac hypertrophy and ventricular remodeling with pressure overload stimulation.6 Thus, p38 appears to have both

*These authors contributed equally to this study.

The online-only Data Supplement is available with this article at http://circres.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.112.272963/-/DC1.

Correspondence to Jeffery D. Molkentin, Howard Hughes Medical Institute, Cincinnati Children’s Hospital Medical Center, 240 Albert Sabin Way, Cincinnati, OH 45229. E-mail: jeff.molkentin@cchmc.org

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

DOI: 10.1161/CIRCRESAHA.112.272963
pathologic and compensatory functions in the heart, although a nonoverexpression approach is needed to better examine its gain-of-function effects and to determine if p38 really could be a relevant target for treating heart disease.

The duration, extent, and subcellular compartment for p38, JNK, and ERK1/2 phosphorylation are critical determinants of the physiological response to any given mitogenic or stress stimulation. The terminal MAPKs (ERK1/2, JNK, and p38) are either activated or inactivated through the phosphorylation status of the threonine and adjacent tyrosine residue within the activation loop of these kinases. A specialized family of phosphatases has evolved that can dephosphorylate both serine/threonine and tyrosine residues within the activation loop, known as the dual-specificity protein phosphatases (DUSPs). There are 10 Dusp genes in the mouse genome that are specialized for the MAPKs and, hence, have been referred to as MAPK phosphatases (MKPs). A unique feature of most MKPs/DUSPs is their specificity for ERK1/2 vs JNK1/2 vs p38. DUSP1 (MKP-1) and DUSP4 (MKP-2) are each induced by stress stimulation in the terminal MAPKs and, hence, have been referred to as MAPK phosphatases (MKPs). A unique feature of most MKPs/DUSPs is their specificity for ERK1/2 vs JNK1/2 vs p38. DUSP1 (MKP-1) and DUSP4 (MKP-2) are each induced by stress stimulation in the heart is transitioning into failure, only DUSP4 remained active and capable of direct binding to the activation loop in MAPKs, resulting in dephosphorylation and their inactivation. Each of the 10 MKP/DUSP family members differs with respect to subcellular localization, tissue expression pattern, and exact specificity for ERK1/2 vs JNK1/2 vs p38. DUSP1 (MKP-1) and DUSP4 (MKP-2) are each induced by stress stimulation in the heart or cultured myocytes with agonist treatment, where they then reside mostly within the nucleus (although some cytoplasmic localization is observed) and have the highest degree of action against p38 MAPK, followed by JNK and then ERK1/2. Although the function of the Dusp6 gene has been investigated in the heart, where it serves as an exclusive regulator of ERK1/2 signaling, with effects on hypertrophic growth and myocyte proliferation, the function of the p38 inactivating DUSPs has not been evaluated, nor have their roles in heart disease been characterized.

Methods
Dusp1-null mice were described previously. The Dusp4 gene was targeted in embryonic stem cell using homologous recombination, after which gene-deleted mice were created using standard techniques. Mouse embryonic fibroblasts (MEFs) were generated from Dusp1/4 double-null embryos harvested at embryonic day 12.5 and cultured in 10% fetal bovine serum containing Dulbecco modified Eagle’s medium. Echocardiography was performed with a Hewlett Packard SONOS 5500 with a 15-mHz probe and images were collected in M-mode. Cardiac pressure overload was induced by transverse aortic constriction (TAC) in young adult mice as described previously. Myocytes were isolated from adult hearts and cultured for either Western blot analysis of MAPK phosphorylation or assessment of cellular shortening and Ca2+ handling as described previously. Results are shown as mean ± SEM, and significance between groups was evaluated by ANOVA or t test when appropriate. Detailed Materials are available in the Online Data Supplement.

Results

Generation of Dusp1/4 Double-Null Mice
DUSP1, DUSP4, and DUSP10 are the major regulators of p38 MAPK dephosphorylation to allow inactivation and recycling of this kinase. Both DUSP1 and DUSP4 are induced by hypertrophic agonists in cultured cardiomyocytes or during heart failure, in which they contribute to MAPK inactivation. Consistent with these previous observations, we observed an increase in DUSP1 and DUSP4 mRNA in the mouse heart after 7 days of hypertrophic pressure overload stimulation; however, by 8 weeks of stimulation when the heart is transitioning into failure, only DUSP4 remained high (Figure 1A). DUSP10, although expressed in the heart, was constitutively present and not subject to induction with hypertrophy (Figure 1A). To begin to address the physiologic
relevance of DUSP function in the heart in regulating p38 MAPK during disease, we inactivated the Dusp4 gene by targeting this locus in embryonic stem cells for the generation of gene-deleted mice (Figure 1B). Reverse-transcriptase polymerase chain reaction confirmed the deletion of the gene product in the heart (Figure 1C). Given redundancy in function of Dusp4 with Dusp1, we crossed these 2 gene-deleted mice together, and for control purposes we also analyzed DUSP1 mRNA from these hearts, which confirmed gene deletion as previously shown (Figure 1C).15

**Loss of Dusp1/4 Only Alters p38 MAPK Activity**

Part of the rationale for generating Dusp1/4 double-null mice was based on our inability to consistently measure a difference in p38, JNK, or ERK1/2 phosphorylation in single Dusp1 or Dusp4 gene-targeted mouse hearts or MEFs in culture (data not shown). Again, this is likely attributable to redundancy and compensation among the Dusp gene family members, of which Dusp1 and Dusp4 appear to function most similarly with respect to specificity for p38.7 Dusp1/4 double-null mice were viable and overtly normal, and fibroblasts from these mice proliferated normally (data not shown). Interestingly, Dusp1/4−/− MEFs cultured in serum-containing medium showed a remarkable and specific defect in p38 MAPK dephosphorylation at baseline, whereas none of the other MAPK family members were affected (Figure 2A).

Figure 2. Combined deletion of Dusp1 and Dusp4 results in unrestrained p38 mitogen-activated protein kinase (MAPK) phosphorylation. A, Western blot analysis of key MAPK pathway enzymes from serum-cultured mouse embryonic fibroblasts (MEFs) derived from wild-type (Wt) or Dusp1/4−/− embryos. Arrowheads indicate changed band intensities detected from Dusp1/4−/− cells. B, Western blot analysis of MAPK phosphorylation from Wt or Dusp1/4−/− MEFs stimulated with anisomycin (1 µg/mL) for the indicated period of time under serum-starved conditions. *Expanded p38 phosphorylation. C, Western blot analysis of the indicated proteins and phospho-proteins from hearts of adult mice subjected to sham or transverse aortic constriction (TAC) procedure (15 minutes of stimulation) in Wt or Dusp1/4−/− mice previously fed vehicle or SB731445-formulated chow. Asterisks and arrowheads in the gel picture or bottom of picture show bands that are differentially regulated by loss of Dusp1/4, whereas the 2 arrowheads on the right show the migration of MK2. D, Western blot analysis of phosphorylated and total p38 MAPK from the hearts of 2-week-old Wt or Dusp1/4−/− mice injected with anisomycin systemically (10 mg/kg) for the indicated period of time. *Expanded p38 phosphorylation. E, Western blot analysis of phosphorylated and total p38 MAPK from the hearts of 2-month-old Wt or Dusp1/4−/− mice subjected to sham or TAC procedure for the indicated periods of time in minutes or hours. **Expanded p38 phosphorylation.
Typically, MAPKs are activated within seconds to minutes of stress or mitogen stimulation, and thereafter the DUSPs are transcriptionally induced within 15 to 45 minutes to mediate inactivation of the MAPKs. To analyze the kinetics of p38 MAPK inactivation over time, we used serum-free cultures of Wt vs Dusp1/4−/− MEFs treated with the stress agent anisomycin (Figure 2B). This agent induced a transient activation of p38 MAPK in Wt MEFs at 10 and 30 minutes, but levels were back to baseline by 180 minutes. By comparison, Dusp1/4−/− MEFs showed no inactivation at 180 minutes, with even slightly greater total activation at all time points analyzed (Figure 2B). No effect was observed in JNK or ERK1/2 phosphorylation status, again suggesting that loss of Dusp1/4 genes only impacted p38 MAPK in MEFs (Figure 2B). We next investigated MAPK responsiveness in the heart at baseline or with acute TAC stimulation for 15 minutes in the presence or absence of the p38 inhibitor SB731445 administered to the mice starting 2 days before surgery (Figure 2C). The data show greater p38 and MK2 activation at baseline in the hearts of Dusp1/4−/− mice, with even greater activation after TAC stimulation compared with Wt controls (Figure 2C, arrowheads). Hearts from Dusp1/4−/− mice showed no changes in ERK1/2 or JNK1/2 phosphorylation at baseline or with TAC stimulation (Figure 2C). We also analyzed hearts from mice subjected to sham or TAC procedures for p38 and MK activation in either the cytoplasm or the nucleus (Online Figure I). The data show greater p38 and MK2 phosphorylation in hearts from Dusp1/4−/− mice, both at baseline and with TAC stimulation, as well as greater MK2 nuclear content in the absence of Dusp1/4, again suggesting greater activation (Online Figure I). We also injected 2-week-old Wt and Dusp1/4−/− mice with anisomycin to induce a systemic stress response and, at select times thereafter, mice were euthanized and the hearts were removed for analysis of p38 MAPK phosphorylation (Figure 2D). Similar to the results observed in MEFs, anisomycin induced a transient increase in p38 MAPK phosphorylation in the hearts of Wt mice at 5 to 60 minutes, which was inactivated by 180 minutes. In contrast, hearts from Dusp1/4−/− mice showed no inactivation up to 180 minutes and, in fact, activation attained a higher level as time progressed (Figure 2D). We also repeated this same type of analysis in Wt vs Dusp1/4−/− mice subjected to pressure overload by TAC for varying lengths of time to gauge the temporal aspects of regulation within the heart more thoroughly (Figure 2E). Hearts from Wt mice showed variable and intermittent activation of p38 at different time periods of pressure overload, as shown previously. In contrast, hearts from Dusp1/4−/− mice subjected to TAC showed uniform and maximal p38 phosphorylation at every time point analyzed, with no periods of inactivation (Figure 2E). Taken together, these results suggest that loss of Dusp1/4 from the heart or cultured MEFs removes a break on p38 MAPK signaling, rendering this pathway unrestricted and with greater net activity.

Dusp1/4−/− Mice Show Cardiomyopathy

Whereas Dusp1/4−/− mice were overtly normal at birth and young adulthood, we did observe a significant decrease in survival with aging, such that by 8 months, 30% of the double-null mice had died compared with no lethality in Wt or single Dusp1-null or Dusp4-null mice (Figure 3A). We suspected that some of these mice may have died of heart failure, because careful analysis by echocardiography showed progressive dilation of the ventricular chambers in the double-knockout (DKO) mice, reductions in fractional shortening, and secondary increases in heart weight normalized to body weight (Figure 3B–3D), although no increase in TUNEL was observed suggesting the failure was not associated with...
apoptosis (Online Figure II). As a whole, *Dusp1* and *Dusp4* single-null mice showed no cardiac abnormalities up to 8 months of age, except for a small, albeit significant, reduction in fractional shortening in *Dusp4−/−* mice at 8 months of age (Figure 3B–D). The large increase in heart weight observed in *Dusp1/4−/−* mice at 8 months of age was characterized mostly by myocyte thinning, as measured from isolated adult myocytes from these hearts (Figure 3E), as well as frank dilation of the left ventricle at the whole-organ level (Figure 3F). *Dusp1/4* DKO mice also were highly susceptible to lethality after TAC stimulation, showing their propensity toward rapid failure (Figure 3G), and the few DKO mice that survived this procedure to 14 days showed much greater cardiac hypertrophy than Wt controls (Online Figure III). These phenotypic characteristics likely were attributable to unrestrained p38 MAPK activity, at least partially consistent with the cardiac phenotype described previously for transgenic mice overexpressing activated MKK3/MKK6. Isolation and culturing (serum-free) of adult myocytes from hearts of wild-type vs *Dusp1/4−/−* mice showed again that only p38 MAPK was hyperphosphorylated in double-null cells, with activation of the p38-specific downstream kinase MK2 (Figure 3H). No changes were observed in JNK or ERK1/2 phosphorylation, again suggesting that DUSP1/DUSP4 are the primary regulators of p38 MAPK dephosphorylation in the heart, and their absence permits unrestrained activity that is associated with cardiomyopathy.

**Inhibition of p38 Protects Dusp1/4−/− Mice From Cardiomyopathy**

DUSP1 and DUSP4 also can dephosphorylate JNK, albeit less efficiently than p38 MAPK. Moreover, there are other DUSPs with greater specificity for JNK over p38 that likely would compensate when *Dusp1/Dusp4* are deleted. Our analysis of *Dusp1/4−/−* MEFS and hearts failed to show any effect on JNK phosphorylation status, suggesting that the phenotype observed in the DKO mice was attributable solely to unrestrained p38 activity. However, to better establish specificity for p38 MAPK, we treated *Dusp1/4−/−* mice with a selective and orally active p38 inhibitor, SB731445, which was formulated in mouse chow at a dosage of 50 mg/kg per day. Mice achieved a blood and heart tissue level of ≈350 ng/mL (or per gram for heart). In Wt control mice administered SB731445, we observed an increase in MKK6 phosphorylation in both heart and skeletal muscle, as well as an increase in total MKK6 protein, suggesting that p38 was inhibited because it normally reduces MKK6 expression through feedback, as discussed previously (Figure 4A). SB731445 binds to the ATP pocket in p38 and reduces its ability to phosphorylate substrates, but it does not prevent p38 itself from being phosphorylated and, hence, in skeletal muscle p38 was hyperphosphorylated because of greater MKK6 activity (Figure 4A). MK2 was hypophosphorylated (faster migration on a gel) in skeletal muscle and, to a lesser extent, in cardiac muscle, again suggesting that the inhibitor was blocking p38 activity (Figure 4A). Wt and *Dusp1/4−/−* mice were administered SB731445 chow beginning at 8 weeks of age for 2 months of treatment (6 mice per group). Mice were subjected to echocardiography every 2 weeks and euthanized at 4 months of age for measurement of heart weights (echo started 4 weeks before treatment began). p38 inhibition with SB731445 prevented the increase in heart weight, prevented ventricular dilation, and prevented loss of fractional shortening in *Dusp1/4−/−* mice compared with vehicle-treated DKO mice over the next 2 months of treatment (Figure 4B–E). Moreover, the known lethality in DKO mice with acute TAC stimulation was partially protected by SB731445 (Figure 4F). Inhibitor treatment of Wt mice had no effect in any of the parameters evaluated here. These results suggest that unrestrained p38 activity is the primary reason for dilated cardiomyopathy and hypertrophy in *Dusp1/4−/−* mice,
The sensitivity of myofilaments to Ca\(^{2+}\), thus acting as a negative inotrope.\(^{21}\) Consistent with these results, 2-month-old Dusp1/4\(^{-/-}\) mice showed reduced systolic contractile performance with a Millar pressure-transducing catheter (Figure 5A). Dusp1/4\(^{-/-}\) mice also showed decreased relaxation, collectively suggesting that increased p38 activity affected systolic and diastolic performances (Figure 5B). However, a parallel group of mice also was acutely treated with SB731445 chow for 2 weeks and assessed for contractile changes, which showed no rescue in systolic performance but showed a significant rescue in relaxation and diastolic performance (Figure 5A–B). These results suggest that p38 activity is acutely involved in cardiomyocyte contractility.

Isolation of adult myocytes from 2-month-old Wt control and Dusp1/4\(^{-/-}\) hearts also revealed a reduction in cellular contractile performance from DKO mice (Figures 5C and 5G). Associated with this reduction in myocardial contractility was a reduction in peak Ca\(^{2+}\) release, a reduction in sarcoplasmic reticulum Ca\(^{2+}\) content, and prolonged relaxation times (Figure 5C–F). Acute administration of the p38 inhibitor SB239063 for 10 minutes reversed all these effects in the DKO adult cardiomyocytes, suggesting that acute phosphorylation events by p38 directly dampened contractility, although it is not clear if such effects are through phosphorylation of myofilament proteins, Ca\(^{2+}\) handling proteins, or both. These results provide additional mechanistic insight into how enhanced p38 activity negatively impacts the heart and how DUSP1/4 are protective by maintaining contractile performance.

To gain greater insight into the mechanism of how p38 affects contractility and secondary propensity to cardiomyopathy, we crossed Dusp1/4\(^{-/-}\) mice with mice lacking Pln (phospholamban). The rationale here is that if deletion of Pln complemented (rescued) the contractile deficits in Dusp1/4\(^{-/-}\) mice, then it would suggest a mechanism primarily involving sarcoplasmic reticulum Ca\(^{2+}\) handling but not myofilament Ca\(^{2+}\) sensitivity. The mice were analyzed at 4 months of age and show that DKO/Pln null mice had a restoration in fractional shortening and prevention of ventricular dilation compared with Dusp1/4 DKO–only mice (Figure 6A–C). These results suggest that loss of Pln, which enhances sarcoplasmic reticulum Ca\(^{2+}\) cycling, can partially restore function and prevent aspects of cardiomyopathy in DKO hearts, further suggesting that p38 regulates Ca\(^{2+}\) cycling as a mechanism for mediating decreased contractility when activated.

**Discussion**

Of the 10 *Dusp* genes, 5 have been reported to prefer p38 and JNK for dephosphorylation over ERK1/2, including *Dusp1, Dusp4, Dusp8, Dusp10*, and *Dusp16*.\(^{7}\) However, some literature is in disagreement and differences in substrate specificity have been reported. For example, DUSP4 (MKP-2) was suggested to primarily regulate ERK1/2, then JNK, but not p38 in cultured cells, although in vivo regulation of ERK1/2 was not observed.\(^{22}\) Before the generation of gene-deleted mice (and null MEFs), it was often difficult to ascertain the true specificity of a given DUSP protein for a MAPK subfamily member in vitro because of promiscuity associated with reconstitution assays that fail to mimic true physiologic concentrations and subcompartmentation, as it would occur in

**p38 Negatively Regulates Cardiac Contractility in Dusp1/4\(^{-/-}\) Mice**

To probe more deeply into the mechanism of cardiomyopathy associated with unrestrained p38 activity in Dusp1/4\(^{-/-}\) mice, we assessed cardiac contractility at the whole organ and cellular levels. p38 activity was previously suggested to reduce...
a cell. Analysis in cultured cells also was confusing because it often relied on overexpression or partial gene knockdown approaches, which again did not mimic known physiologic concentrations of the DUSPs. Hence, the potentially only reliable means of assessing DUSP “physiologic” specificity is using gene-deleted cells or tissues to then probe for upregulation in selected MAPK members. Moreover, regulation even varies by tissue or cell type, depending on the levels of each DUSP expressed, the levels of other interacting proteins that support a complex between DUSP-MAPKs, and the levels of the individual MAPKs themselves and even their nuclear versus cytoplasmic localization. Thus, every tissue or cell type could have a unique profile of MAPK subfamily member regulation by an individual DUSP, depending on what other DUSPs are expressed and the relative content of each MAPK subfamily member. We have generated or obtained gene-deleted mice for 7 of the 10 Dusp genes, and only Dusp1, Dusp4, and Dusp10 appear to dominantly regulate p38 MAPK dephosphorylation in the heart and MEFs (results shown here and data not shown). DUSP8 appears to be mostly specific for JNK in the heart (data not shown), and DUSP6 is entirely specific for ERK1/2 in the heart and MEFs.15

Single disruption of Dusp1, Dusp4, or Dusp10 did not appreciably impact baseline or inducible p38 MAPK phosphorylation status attributable to compensation, although Dusp1/4 double-null MEFs and hearts from these DKO mice showed a dramatic and specific effect on only p38 MAPK phosphorylation, suggesting that these 2 DUSPs are the primary regulators of p38 in vivo, or suggesting that no remaining DUSPs are present in the heart with sufficient activity or specificity for p38. However, our loss-of-function results do not prove that DUSP1/4 cannot additionally affect JNK, because other DUSPs are still present that could potentially compensate for the loss of DUSP1/4, especially if they had preference for JNK over p38 (ie, DUSP8). The critical aspect underlying our results is that loss of DUSP1/4 appears to account for the majority of DUSP activity toward p38 MAPK in fibroblasts and the heart, because the remaining 8 Dusp genes are not sufficient to fully inactivate p38 MAPK.

Another issue to consider is how MAPK activity is assessed in interpreting the effects of DUSP deletion. Although the field relies heavily on direct assessment of ERK1/2, JNK, and p38 phosphorylation using phosphospecific antibodies, it is still arguably better to examine direct downstream targets of each MAPK. However, we have determined that this is not a trivial order, because MK2 appears to be the only truly specific target of any of the MAPK family members (at least for p38 activity in heart). We surveyed a number of reported ERK-specific phosphorylation sites in different target proteins, such as Elk-1, but none was affected in hearts of our Dusp1/4 DKO mice, nor was it affected in Erk1/2 double heart-specific deleted mice that we generated previously.23 c-Jun also is thought to be a specific target of JNK1/2, but p38 also can phosphorylate this target. However, c-Jun phosphorylation levels also were not changed in the hearts of our Dusp1/4 DKO mice, which is still consistent with DUSP1/4 only dominantly regulating p38 MAPK.

DUSP1 and DUSP4 both are thought to be primarily localized to the nucleus, thus it is difficult to envision how a specific alteration in nuclear p38 phosphorylation would lead to acute changes in myocyte contractility. However, DUSP1/4 both show some degree of localization and function within the cytosol. For example, some DUSP1 was observed within the cytosol of arterial smooth muscle cells, and some DUSP4 was similarly observed in the cytosol of cultured endothelial cells.24,25 Another issue related to subcellular compartments was suggested by the observation that p38 within the nucleus can activate MK2 and mediate its nuclear extrusion into the cytosol.26 Wang et al.27 showed that the failure and hypertrophic cardiomyopathic phenotype observed in activated MKK3 transgenic mice was reduced in mice lacking the gene encoding MK2. However, activated p38 promoted greater MK2 nuclear occupancy, not its extrusion, suggesting that in the adult heart of Dusp1/4−− mice MK2 might have deleterious functions in the nucleus that are p38-dependent.

Dusp1/4−− mice have development of cardiomyopathy with aging, such that by 8 months the ventricular chambers have dilated and function is significantly diminished, the start of which can be observed as early as 2.5 months of age. In addition, 2-month-old Dusp1/4−− mice show exaggerated hypertrophic enlargement after 2 weeks of pressure overload stimulation compared with a normal hypertrophic response in the single-null mice and Wt mice. These results clearly indicate that Dusp1/4 are cardioprotective genes, and because the only identifiable molecular effect consistent with the known function of the Dusp gene family is an increase in p38 MAPK activity, we assume that unrestrained p38 activity underlies the cardiomyopathic phenotype in these DKO mice. Proof for this assumption came from the use of SB731445 over 2 months.
in $Dusp1/4^{-/-}$ mice, which prevented cardiac disease. These results suggest that prolonged p38 MAPK activity in the heart induces pathology, consistent with the phenotype of transgenic mice expressing an activated mutant of MKK6 specifically in cardiomyocytes of the heart, which showed restrictive hypertrophy and lethality, whereas activated MKK3 transgenic mice showed dilated cardiomyopathy with lethality. However, unlike MKK3/6 transgenic mice that presumably show near-maximal p38 activity at all times, combined loss $Dusp1/4$ does not induce p38 activity; it merely removes a necessary “brake,” thus allowing prolongation in activation as long as a “physiologic” stimulus is present. Hence, our results suggest that increased p38 activity in the heart is profoundly pathologic and could be a novel target for inhibition in treating patients with heart failure or select disease states that lead to hypertrophy and fibrosis, such as muscular dystrophy. A hamster model of muscular dystrophy and associated heart disease showed inhibition of cardiac myocytes, which was reversed with a p38 inhibitory compound. Whereas Liao et al. never directly identified a mechanism of action whereby p38 negatively impacted contractility, they failed to observe a change in Ca$^{2+}$ handling, suggesting that the effect was at the level of the myofilament. Detergent-skinned papillary muscle strips from transgenic mice expressing an activated MKK6 mutant protein in the heart showed a reduction in maximum tension and ATPase activity, suggesting that p38 was dampening contractility at the level of myofilament proteins. We similarly observed a reduction in myocyte contractility with enhanced p38 activity attributable to $Dusp1/4$ deletion, which was reversed with SB239063 in minutes, suggesting that regulation of contractility was attributable to immediate phosphorylation of ≥1 proteins. Crossing into the $Phn$-null background, which partially prevented disease and enhanced cardiac contractility in $Dusp1/4$ DKO mice, suggests that part of the regulation by p38 is through affecting ≥1 Ca$^{2+}$ handling proteins, presumably at the level of sarcoplasmic reticulum.

Hence, $Dusp1/4^{-/-}$ mice most likely have a chronic reduction in cardiac contractility because of attenuated Ca$^{2+}$ cycling, which then likely requires neuroendocrine enhancement to maintain cardiac output, leading to a secondary hastening of disease. Sustained neuroendocrine drive is known to cause hypertrophy or augment heart failure. More importantly, because p38 MAPK is activated in a semisustained manner in failing human hearts, and because p38 inhibitors appear to slightly but significantly augment cardiac contractile performance in diseased myocytes, drugs that block p38 could be of further benefit in heart failure patients. Finally, in addition to alterations in contractility and associated Ca$^{2+}$ handling, it is likely that unrestrained activation of p38 is pathologic and leads to dilated or hypertrophic cardiomyopathy for additional reasons, such as enhanced fibrosis and remodeling.

**Acknowledgments**

We thank Dr Evangelia G. Kranias for supplying the $Phn$-targeted mice (University of Cincinnati, Cincinnati, OH).

**Sources of Funding**

This work was supported by grants from the National Institutes of Health (to Dr Molkentin and Dr Lorenz). Dr Molkentin also was supported by the Howard Hughes Medical Institute. Dr van Belo and Dr Accornero were supported by local affiliate grants from the American Heart Association. Dr Auger-Messier was supported by a Heart and Stroke Foundation of Canada postdoctoral fellowship. Dr van Berlo also was supported by a Rubicon fellowship from the Netherlands Organization for Scientific Research, and Dr Accornero also was supported by a 1-year fellowship from the Italian Intesa SanPaolo SpA.

**Disclosures**

None.

**References**

Unrestrained activation of p38 activity by removing the brake of 
• DUSP1 and DUSP4 are the primary regulators of p38 activity in the heart.

Unrestrained activation of p38 activity by removing the brake of 
• DUSP1 and DUSP4 are cardioprotective factors.

DUSP1 and DUSP4 protect the heart by specifically inactivating p38, allowing this kinase to maintain a dynamic range of activity.

DUSP1 and DUSP4 are the primary regulators of p38 activity in the heart.

Novelty and Significance

What Is Known?
• Constitutive activation of p38 in the heart using cardiac-specific transgenesis induces cardiac dysfunction.
• Dual-specificity phosphatase (DUSP) proteins dephosphorylate mitogen-activated protein kinase (MAPK), leading to their inactivation.
• p38 activity is associated with decreased cardiac contractility.

What New Information Does This Article Contribute?
• DUSP1 and DUSP4 are cardioprotective factors.

Deletion of Pin prevents loss of contractile performance and ventricular dilation in Dusp1/4-null mice, suggesting that p38 affects contractility by altering calcium handling at the level of the sarcoplasmic reticulum.

This study was designed to examine the function of Dusp genes as negative regulators of the MAPK superfamily to better understand which kinases might be pathologic and the best targets for pharmacotherapy. We show that deletion of Dusp1 and Dusp4 together in gene-targeted mice leads to cardiomyopathy with aging and enhances decomposition and disease with pressure overload stimulation by leading to prolongation and maintenance of p38 MAPK activity. Our results suggest that p38 MAPK inhibitors may be useful in the treatment of heart failure patients.
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Circ Res. 2013;112:48-56; originally published online September 19, 2012; doi: 10.1161/CIRCRESAHA.112.272963

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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. Pln null mice were also previously described. 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. Cardiac hypertrophy was induced by transverse aortic constriction (TAC) to produce pressure overload as previously described. 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, 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. 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 2x10⁶ 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 Ca²⁺ 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. After perfusion, the ventricles were disassociated into individual myocytes, filtered and Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ signal was acquired, and data were analyzed using Felix and Clampfit software. Contractility measurements were acquired using video edge detection as previously described.

**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. 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; 9132), 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'-ctccaagggatgataggcg-3' (DUSP1-for), 5'-ctccagcatcttggagttc-3' (DUSP1-rev), 5'-gtggaatctttctcttctc-3' (DUSP4-for), and 5'-gatgtgctggctgtgcttctc-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


Online Figure I. Western blot analysis of the indicated proteins and phosphoproteins 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.