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Abnormal p38α mitogen-activated protein kinase signaling in dilated cardiomyopathy caused by lamin A/C gene mutation

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We previously interrogated the transcriptome in heart tissue from *Lmna*$^{H222P/H222P}$ mice, a mouse model of cardiomyopathy caused by lamin A/C gene (*LMNA*) mutation, and found that the extracellular signal-regulated kinase 1/2 and Jun *N*-terminal kinase branches of the mitogen-activated protein (MAP) kinase signaling pathway were abnormally hyperactivated prior to the onset of significant cardiac impairment. We have now used an alternative gene expression analysis tool to reanalyze this transcriptome and identify hyperactivation of a third branch of the MAP kinase cascade, p38α signaling. Biochemical analysis of hearts from *Lmna*$^{H222P/H222P}$ mice showed enhanced p38α activation prior to and after the onset of heart disease as well as in hearts from human subjects with cardiomyopathy caused by *LMNA* mutations. Treatment of *Lmna*$^{H222P/H222P}$ mice with the p38α inhibitor ARRY-371797 prevented left ventricular dilatation and deterioration of fractional shortening compared with placebo-treated mice but did not block the expression of collagen genes involved in cardiac fibrosis. These results demonstrate that three different branches of the MAP kinase signaling pathway with overlapping consequences are involved in the pathogenesis of cardiomyopathy caused by *LMNA* mutations. They further suggest that pharmacological inhibition of p38α may be useful in the treatment of this disease.

INTRODUCTION

Mutations in the lamin A/C gene (*LMNA*) encoding nuclear A-type lamins cause dilated cardiomyopathy with or without associated skeletal muscular dystrophy (1,2). *LMNA* may be the most prevalent dilated cardiomyopathy gene, as mutations appear to be responsible for ~8% of inherited cases (3,4). *LMNA*-dilated cardiomyopathy is associated with arrhythmias, sudden death, myocardial remodeling and dilatation of left ventricle (LV) ultimately resulting in poor cardiac performance and heart failure. While sudden death from arrhythmias may be prevented by implantation of a pacemaker and defibrillator, progressive heart failure eventually becomes resistant to treatment, such as angiotensin converting enzyme inhibitors, angiotensin receptor blockers, beta-blockers, diuretics and aldosterone antagonists. No drugs are curative and heart transplantation is frequently necessary.

Despite the fact that the etiology and the symptoms of *LMNA*-dilated cardiomyopathy have been extensively investigated, little is known about the underlying pathogenic mechanisms. The *Lmna*$^{H222P/H222P}$ mouse, which develops cardiomyopathy that recapitulates the human disease, has been a useful small animal model (5). We previously interrogated the transcriptome in heart tissue from *Lmna*$^{H222P/H222P}$ mice and identified the extracellular signal-regulated kinase 1/2 (ERK1/2) and Jun *N*-terminal kinase (JNK) branches of the mitogen-activated protein (MAP) kinase signaling pathway as abnormally hyperactivated prior to the onset of significant cardiac impairment (6). We then hypothesized that small molecule inhibitors of ERK1/2 and JNK signaling

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would prevent LV dilatation and improve cardiac function in *Lmna*$^{H222P/H222P}$ mice and have seen these beneficial effects when administering such drugs (7–10).

Microarray analysis of RNAs generates large lists of differentially expressed genes; however, biological interpretation can be challenging. Bioinformatics tools that use functional information accumulated in public databases make it possible to dissect large gene lists to identify the most pertinent biological processes related to the microarray data. Our initial analysis of the transcriptome of hearts from *Lmna*$^{H222P/H222P}$ mice used ErmineJ, a tool based on gene set enrichment analysis (GSEA) that implements multiple algorithms including over-representation and resampling-based methods that focus on gene scores or correlation of gene expression profiles (11). Since then, additional analysis tools have emerged providing different approaches to analyze microarray data that could potentially identify pathways or functional groups of genes that were not detected using another bioinformatics tool. We therefore reanalyzed our microarray data sets from hearts of *Lmna*$^{H222P/H222P}$ mice using the Database for Annotation, Visualization and Integrated Discovery (DAVID), a program that uses different methodological and statistical approaches (12,13). Based on this reanalysis, we have identified a third branch of the MAP kinase cascade, p38α signaling, to be hyperactivated in *LMNA* cardiomyopathy. We further show that pharmacological inhibition of p38α prevents LV dilatation and prevents deterioration in LV fractional shortening (FS) in *Lmna*$^{H222P/H222P}$ mice.

RESULTS

Abnormal p38α signaling in hearts of *Lmna*$^{H222P/H222P}$ mice and humans with cardiomyopathy caused by *LMNA* mutations

We previously examined differential expression of mRNAs isolated from hearts of 10-week wild-type ($n = 8$) and *Lmna*$^{H222P/H222P}$ ($n = 6$) male mice using Affymetrix Mouse Genome 430 2.0 Arrays (6). These data are publicly available at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with accession numbers GSE6397 and GSE6398. We then used ErmineJ to analyze data from over 45 000 probe sets on the arrays to identify functional groups of genes, based on gene ontology (GO), that were differentially expressed in hearts of the *Lmna*$^{H222P/H222P}$ mice compared with wild-type mice. This analysis revealed alterations in the expression of genes in several signaling pathways, among them MAP kinases (6). However, ErmineJ has several limitations, including its incomplete coverage annotating the large database of existing signaling pathways. We therefore reanalyzed our data sets of genes differentially expressed in hearts of *Lmna*$^{H222P/H222P}$ mice using both ErmineJ and the DAVID program. These are based on different key computational methods: GSEA for ErmineJ and singular enrichment analysis (SEA) for DAVID (12,13).

After analysis of only the 7805 differentially expressed genes that met a false discovery rate threshold of $q < 0.05$ (see Material and Methods), we identified 16 signaling pathways using ErmineJ, 13 signaling pathways using the KEGG database in DAVID and 12 using the BIOCARTA database in DAVID (Table 1). Overall, there was consistency in the results using ErmineJ and DAVID, as in a previous study that ran the same data sets with DAVID versus ErmineJ (14). For example, Wnt, TGFβ, MAP kinase, JAK-STAT and insulin signaling were identified using both DAVID and ErmineJ. However, only ErmineJ identified the I-kappaB kinase/NF-kappaB signaling pathway and only DAVID identified some signaling pathways, including ErbB, calcineurin/calcium, PDGF, AKT/mTOR and PPAR. In ErmineJ and both the KEGG and BIOCARTA pathway databases in DAVID, the GO term ‘MAP kinase signaling pathway’ considers the expression of genes in ERK1/2, JNK and p38α branches. However, the BIOCARTA database in DAVID includes a separate GO term that specifically considers only genes in the p38α branch, which was found to be significantly different in *Lmna*$^{H222P/H222P}$ mouse hearts using this analysis method.

Many individual genes in the ERK1/2, JNK and p38α branches of the MAP kinase signaling cascade were differentially expressed in hearts from *Lmna*$^{H222P/H222P}$ mice compared with wild-type mice (Fig. 1A). Differences in expression of 10 genes in the p38α branch were detected on the Affymetrix arrays (Fig. 1B). To validate the altered expression of four selected transcripts of genes encoding proteins in the p38α signaling branch detected on the arrays, we performed real-time quantitative RT–PCR using mRNA extracted from cardiac tissue of male *Lmna*$^{H222P/H222P}$ and *Lmna*$^{+/+}$ mice. We detected increased expression of *Atf2*, *Elk-1*, *Creb* and *Chop10* as early as 8 weeks of age in hearts from *Lmna*$^{H222P/H222P}$ mice and their expression becomes gradually more increased until 20 weeks of age (Fig. 1C).

We next examined activation of p38α signaling in cardiac tissue from male *Lmna*$^{H222P/H222P}$ mice at 8 weeks of age, when heart function is normal, and at 16 weeks of age, when the mice have LV dilatation and decreased ejection fraction. We performed immunoblotting to detect p38α phosphorylation (activation) as well as phosphorylation of MAP kinase kinase (MKK) 6, the kinase that activates p38α. There was increased phosphorylation of both p38α and MKK6 in extracts of whole hearts from *Lmna*$^{H222P/H222P}$ mice compared with wild-type mice at both 8 and 16 weeks of age (Fig. 2A). In cardiomyocytes isolated from *Lmna*$^{H222P/H222P}$ mouse hearts, there was a similar hyperactivation of p38α MAP kinase signaling compared with cardiomyocytes from wild-type mice (Fig. 2B). To determine whether the activation in p38α MAP kinase signaling in hearts of *Lmna*$^{H222P/H222P}$ mice occurs in the corresponding human disease, we examined the levels of phosphorylated and total p38α in heart tissue from human subjects with cardiomyopathy caused by *LMNA* mutations. A significant increase in phosphorylated p38α was detected in LV tissue from three patients with *LMNA* mutations compared with controls without *LMNA* mutations (Fig. 2C). These results show that p38α MAP kinase signaling is hyperactivated in *LMNA* cardiomyopathy.

Inhibition of p38α signaling prevents dilatation and deterioration of LV function in *Lmna*$^{H222P/H222P}$ mice

Given the enhanced p38α MAP kinase signaling in hearts of *Lmna*$^{H222P/H222P}$ mice, we asked whether we could improve the LV structure and performance in these mice. We

Table 1. GO analysis using ErmineJ and DAVID of genes differentially expressed in hearts from Lmna$^{H222P/H222P}$ mice at 10 weeks of age

	Rank	Signaling pathway (GO term)	*P*-value
GSEA -ErmineJ-			
	1	Wnt receptor signaling pathway	4.14E−10
	2	JNK kinase signaling pathway	7.21E−10
	3	Receptor serine/threonine kinase signaling pathway	1.04E−07
	4	MAP kinase signaling pathway	1.15E−05
	5	Transforming growth factor β receptor signaling pathway	1.57E−05
	6	Regulated secretory signaling pathway	2.67E−04
	7	G-protein signaling pathway	6.93E−04
	8	JAK-STAT signaling pathway	6.97E−04
	9	I-kappaB kinase/NF-kappaB signaling pathway	1.81E−03
	10	Frizzled signaling pathway	0.02308889
	11	Intracellular receptor-mediated signaling pathway	0.02352453
	12	Complement activation signaling pathway	0.02534146
	13	Insulin receptor signaling pathway	0.02737485
	14	Insulin-like growth factor receptor signaling pathway	0.03702819
	15	Cytokine- and chemokine-mediated signaling pathway	0.04445473
	16	Steroid hormone receptor signaling pathway	0.04874929
SEA -DAVID- KEGG			
	1	Wnt signaling pathway	5.20E−05
	2	TGF beta-signaling pathway	2.03E−04
	3	MAP kinase signaling pathway	2.18E−04
	4	Insulin signaling pathway	2.61E−04
	5	GnRH signaling pathway	6.42E−04
	6	Neurotrophin signaling pathway	0.00121274
	7	T cell receptor signaling pathway	0.001278196
	8	Calcium signaling pathway	0.022151913
	9	Jak-STAT signaling pathway	0.024891802
	10	ErbB signaling pathway	0.025551016
	11	Adipocytokine signaling pathway	0.02786599
	12	PPAR signaling pathway	0.03570115
	13	mTOR signaling pathway	0.043633556
BIOCARTA			
	1	Wnt signaling pathway	0.000151627
	2	TGF beta signaling pathway	0.003815475
	3	CXCR4 signaling pathway	0.007966109
	4	AKT/mTOR signaling pathway	0.008654229
	5	p38 MAPK signaling pathway	0.010403168
	6	IGF-1 signaling pathway	0.012208631
	7	PDGF signaling pathway	0.012444725
	8	MAP kinase signaling pathway	0.015196888
	9	T-cell receptor signaling pathway	0.015464509
	10	Integrin signaling pathway	0.016080005
	11	JNK signaling pathway	0.044987126
	12	Calcineurin signaling pathway	0.046356356

The most significantly changed GO terms in hearts from *Lmna*$^{H222P/H222P}$ mice were identified by canonical pathways analysis using either a GSEA enrichment method (ErmineJ) or a SEA enrichment method (DAVID). For each bioinformatics tool, signaling pathways identified were ranked according to their *P*-value. DAVID allows the identification of pathways from either the KEGG or BIOCARTA databases.

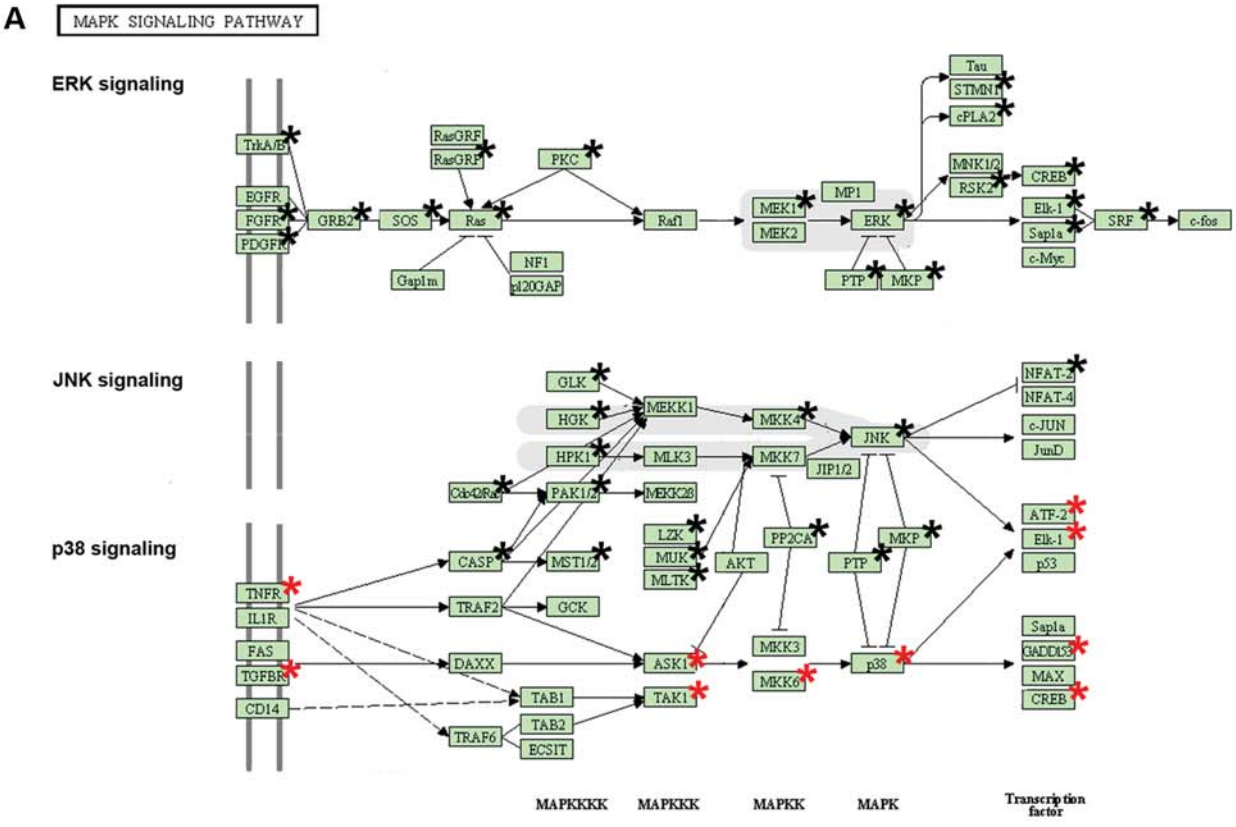
blocked p38α MAP kinase signaling in male *Lmna*$^{H222P/H222P}$ mice with twice daily oral doses of ARRY-371797 (30 mg/kg), a highly selective, potent inhibitor of p38α under investigation in a Phase II clinical trial for acute inflammatory pain (15). A treatment for 4 weeks was initiated at 16 weeks of age, when there were significant increases in LV end-diastolic diameter (LVEDD) and LV end-systolic diameter (LVESD) as well as a decrease in FS, a parameter directly proportional to the LV ejection fraction. Following 4 weeks of treatment, the mice were analyzed by echocardiography and then sacrificed for biochemical studies (Fig. 3A). ARRY-371797 reduced phosphorylated p38α in hearts compared with placebo, as shown by immunoblotting of proteins in tissue homogenates (Fig. 3B). ARRY-371797 did not reduce the phosphorylation of ERK1/2, suggesting that it is a selective inhibitor of p38α (Fig. 3B). We used M-mode echocardiography to image hearts *in vivo* and assess LV diameters (Fig. 3C). It showed that LVEDD and LVESD in *Lmna*$^{H222P/H222P}$ mice treated with ARRY-371797 were significantly smaller and FS was significantly increased compared with the placebo-treated mice (Fig. 3D; see also Table 2).

We also examined the effects of treatment with ARRY-371797 on the expression of genes encoding proteins involved in sarcomere organization and compensatory responses to LV dilatation such as natriuretic peptides and collagens. Hearts from the ARRY-371797-treated *Lmna*$^{H222P/H222P}$ mice had significantly reduced expression of *Mlc-1a* and *Acta2*, which encode proteins involved in sarcomere organization (Fig. 4A). They also had reduced expression of *Nppa* and *NppB*, respectively, encoding atrial natriuretic peptide A and brain natriuretic peptide B (Fig. 4B). ARRY-371797 treatment did not, however, reduce the expression of *Col1a1* and *Col1a2* mRNAs encoding collagens in hearts of *Lmna*$^{H222P/H222P}$ mice (Fig. 4C). These results show that inhibition of p38α signaling in hearts of *Lmna*$^{H222P/H222P}$ mice attenuates the expression of sarcomeric proteins and natriuretic peptides secreted in response to LV dilatation but may not prevent the myocardial fibrosis that occurs in these mice (5,8–10).

DISCUSSION

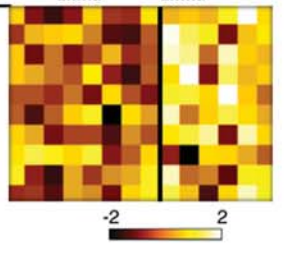
We previously used the gene expression analysis tool ErmineJ to analyze mRNA isolated from hearts of *Lmna*$^{H222P/H222P}$ mouse model of cardiomyopathy caused by *LMNA* mutation and identified the ERK1/2 and JNK branches of the MAP kinase signaling pathway as abnormally hyperactivated prior to the onset of cardiac disease (6). In the present study, we used an alternative analysis tool, DAVID, with two separate pathway databases, to obtain evidence for hyperactivation of the p38α branch of the MAP kinase signaling pathway using the same data sets. These results were confirmed by biochemical assays on isolated heart tissue showing hyperactivation of the p38α signaling branch. Our reanalysis using DAVID but not ErmineJ also detected differences in the AKT/mTOR signaling pathway in hearts from *Lmna*$^{H222P/H222P}$ mice, which prior to performing this analysis we found to be upregulated (16). DAVID additionally identified TGFβ signaling as being perturbed, which has been demonstrated previously using cell biological methods in hearts from older *Lmna*$^{H222P/H222P}$ mice and is consistent with the presence of cardiac fibrosis (5). Both analysis tools identified Wnt signaling as the highest ranked altered pathway by *P*-value; the role of Wnt in *LMNA* cardiomyopathy therefore may warrant further study. Overall, while for the most part ErmineJ and

A

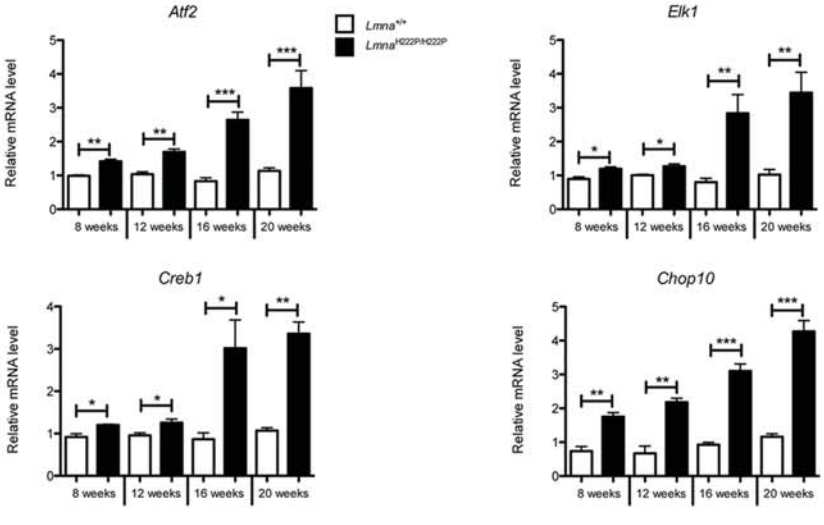


B

Probe set name	Gene symbol	Gene name	q-value
1420895_at	*TGFBR1*	transforming growth factor, beta receptor I	0.001166535
1452529_a_at	*CREB1*	cAMP responsive element binding protein 1 (Creb-1)	0.002516548
1459617_at	*MAPK14*	mitogen activated protein kinase 14 (p38 alpha)	0.005486382
1426850_a_at	*MAP2K6*	mitogen activated protein kinase kinase 6 (MEK6)	0.006352425
1439830_at	*MAP3K5*	mitogen activated protein kinase kinase kinase 5 (ASK1)	0.013138994
1419988_at	*MAP3K7*	mitogen activated protein kinase kinase kinase 7 (TAK1)	0.013430406
1452116_s_at	*ATF2*	activating transcription factor 2 (Atf-2)	0.014700189
1443897_at	*DDIT3*	DNA-damage inducible transcript 3 (CHOP10)	0.022766289
1421897_at	*ELK1*	E twenty-six (ETS)-like transcription factor 1 (Elk1)	0.028133111
1419508_at	*RIPK1*	receptor (TNFRSF)-interacting serine-threonine kinase 1	0.033654729



C



DAVID gave consistent results in analyzing alterations in cell signaling pathways, analysis with these two different bioinformatics tools identified non-overlapping alterations in additional pathways that were confirmed by biochemical methods.

Our reanalysis of this microarray data prompted us to examine the possible pathophysiological role of p38α hyperactivation in the development of dilated cardiomyopathy caused by *LMNA* mutation. Our experimental results provide several lines of evidence for such a role. First, activation of p38α is detectable as early as 8 weeks of age in $Lmna^{H222P/H222P}$ mice, before any measurable structural alterations or functional deterioration in the heart. Second, we observed hyperactivation p38α, albeit at a later stage, in heart tissue from human subjects with dilated cardiomyopathy caused by *LMNA* mutation. Third, pharmacological inhibition of p38α prevented LV dilatation and deterioration in LV function in $Lmna^{H222P/H222P}$ mice. The p38α hyperactivation in cardiomyopathy caused by *LMNA* mutation appears to occur concurrently with hyperactivation of ERK1/2 and JNK, two other MAP kinases

MAP kinases are activated by binding of mitogens, growth factors and cytokines to cell surface receptors and also by osmotic, mechanical and chemical stresses, such as reactive oxygen species, independent of such receptors (17,18). There is considerable crosstalk between different MAP kinase branches, particularly between JNK and p38α (Fig. 1A), and between MAP kinase and other signaling pathways. In dilated failing hearts, there is activation of multiple signaling pathways including ERK1/2, JNK and p38a MAP kinases as well as AKT (19). Given the crosstalk and overlap between these and other pathways, deciphering the specific pathogenic roles of any individual MAP kinase branch has been extremely complicated. Indeed, despite being the focus of extensive investigation, contradictive results have led to a perception that activated MAP kinases can have both protective and detrimental effects in the heart (20,21). With regards to p38α signaling, experimental studies have yet generated a clear picture regarding its role in cardiac pathogenesis. Ventricular-specific transgenic expression of MKK3 or MKK6 that activated p38α has been reported to cause systolic contractile depression, increased fibrosis and restrictive diastolic abnormalities in the absence of significant hypertrophy (22). Mice with cardiac-specific transgenic expression of dominant-negative mutants of p38α, MKK3 or MKK6 have been reported to have baseline cardiac hypertrophy (23). In contrast, mice with cardiac-selective depletion have been reported to have a normal life span and normal global cardiac structure and function (24). However, in response to pressure overload, these mice develop hypertrophy similar to controls but also develop LV dilatation and fibrosis (24). Treatment of hearts from hamsters with dilated cardiomyopathy with inhibitors of p38α has been reported to have beneficial effects, including decreased LV dimensions, increased ejection fraction and decreased fibrosis (25). The first two of these are consistent with our findings in $Lmna^{H222P/H222P}$ mice. However, we did not see a decrease in expression of genes encoding collagens that function in laying down fibrous tissue.

Overall, the hyperactivation of ERK1/2, JNK, AKT and p38α signaling that occurs prior to clinically detectable dilated cardiomyopathy in hearts of mice with *Lmna* mutation mimics what appears to occur later in dilated failing hearts (19). How this apparently detrimental pattern of cell signaling arises as a consequence of abnormalities in A-type lamins remains a mystery. MAP kinases can be activated by mechanical stress and A-type lamins are necessary to maintain proper mechanical stiffness as well as nuclear and cytoplasmic integrity in cells (26–28). Hence, defects in A-type lamins could predispose cells, especially contractile ones, to more readily activate stress–response signaling pathways in response to mechanical strain. Along these lines, cardiomyocyte nuclei in $Lmna^{H222P/H222P}$ mice are more elongated than in wild-type mice and treatment with MEK1/2 and JNK inhibitors reverses this alteration (7–10). Whether similar effects on nuclear structure occur with ARRY-371797 treatment remains to be determined. Lamin A also binds directly to ERK1/2, suggesting that defects in A-type lamins could affect its activation (29). Regardless of the mechanism responsible, the fact that ERK1/2, JNK, AKT and p38α are all hyperactivated in a mouse model of *LMNA* cardiomyopathy prior to the onset of clinical disease lead us to hypothesize that inhibiting their activities to restore a more physiological balance would be beneficial. In $Lmna^{H222P/H222P}$ mice, pharmacological inhibition of ERK1/2 prevents LV dilation, improves LV ejection fraction/FS, inhibits fibrosis and produces a modest survival benefit (7–9). Inhibition of JNK appears to have similar beneficial effects on LV structure, ejection fraction/FS and fibrosis (8). Inhibition of mTOR, a downstream target activated by AKT, correlates with enhancement of macroautophagy, prevention of LV dilation and improvement FS but does not appear to inhibit fibrosis (16). The present results show that p38α inhibition prevents LV dilatation and improves FS. Further preclinical studies in mice, including effects on lifespan of each of these drugs, could allow for the optimization of protocols that inhibit these signaling pathways to treat human subjects with cardiomyopathy caused by *LMNA* mutations.

Figure 1. Altered expression of genes in the p38α branch of the MAP kinase signaling cascade in hearts from $Lmna^{H222P/H222P}$ compared with wild-type mice at 10 weeks of age. (**A**) Representation of genes in the MAP kinase signaling pathway (MAPK signaling pathway) identified by the KEGG visual pathway resource, which includes the ERK1/2 (ERK signaling), JNK (JNK signaling) and p38α (p38 signaling) branches within this GO term. Genes with statistically significant differences ($q < 0.05$) in expression detected on Affymetrix Mouse Genome 430 2.0 Arrays in hearts of $Lmna^{H222P/H222P}$ mice compared with wild-type mice are indicated by asterisks; red asterisks indicate genes in the p38α branch. (**B**) Matrices showing array data of corresponding probe sets corresponding to 10 genes in the in the p38α branch with statistically significant differences ($q < 0.05$) in expression between wild-type ($Lmna^{+/+}$) and $Lmna^{H222P/H222P}$ mouse hearts. In the matrices, each probe set is visualized as a row of colored squares and each column is a biological replicate (sample form a different mouse heart). Darker color means lower expression and brighter color higher expression. The genes correspond to those indicated with red asterisks in (A) [*DDIT3*/CHOP10 is the same as GADD153 and *MAP2K6*/MEK6 the same as MKK6 in (A)]. (**C**) Validation of differential expression in hearts from $Lmna^{H222P/H222P}$ mice of four selected genes in the p38α MAP signaling pathway using real-time quantitative RT–PCR. RNA was obtained from hearts of mice at different ages as indicated on the *x*-axis. White bars show relative RNA expression levels in hearts of $Lmna^{+/+}$ mice and black bars in hearts of $Lmna^{H222P/H222P}$ mice. Values are means ± SEM for $n = 3$ samples per group. The real-time quantitative RT–PCR was performed in triplicate with the different RNA samples. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0005$.

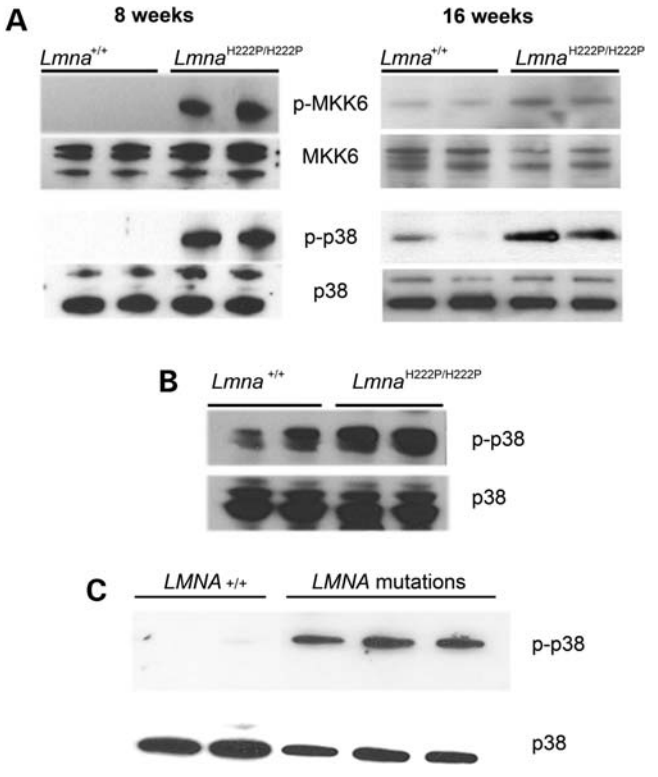


Figure 2. p38α signaling is hyperactivated in hearts of mice and humans with cardiomyopathy and lamin A/C gene mutaitons. (**A**) Immunoblots showing phosphorylated p38α (p-p38α), total p38α, phosphorylated MKK6 (p-MKK6) and total MKK6 in protein extracts of hearts of wild-type ($Lmna^{+/+}$) and $Lmna^{H222P/H222P}$ mice at 8 weeks (left panel) and 16 weeks (right panel) of age. Each lane contains protein extracts from a different mouse. (**B**) Immunoblot showing p-p38α and total p38α in protein extracts of cardiomyocytes isolated from hearts of wild-type ($Lmna^{+/+}$) and $Lmna^{H222P/H222P}$ mice at 16 weeks. Each lane contains protein extracts from a different mouse. (**C**) Immunoblots showing p-p38α and total p38α in protein extracts of heart tissue from two human controls ($LMNA^{+/+}$) and three individuals with dilated cardiomyopathy caused by *LMNA* mutations.

MATERIALS AND METHODS

Microarray data analysis

Data files were obtained through the National Center for Biotechnology Information's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), accessible through accession numbers GSE6397 and GSE6398. Genes were identified as being differentially expressed if they met a false discovery rate threshold of $q < 0.05$ in a two-tail Student's *t*-test, using GeneSpring GX software (Agilent Technologies). Gene expression changes related to functional groups were analyzed using the class score method in the bioinformatics tools DAVID (http://david.abcc.ncifcrf.gov/) and ErmineJ (http://www.chibi.ubc.ca/erminej/) to provide a statistical confidence to groupings. These bioinformatics tools take as input the *q*-values of differentially expressed genes and identify statistically significant functional groupings (GO terms) using modified Fisher exact test in DAVID and Wilcoxon rank-sum test in ErmineJ. Significant GO terms were identified using a false discovery rate of $P < 0.05$.

Mice

$Lmna^{H222P/H222P}$ mice were bred and genotyped as previously described (5). Mice were fed chow and housed in a disease-free barrier facility with 12 h/12 h light/dark cycles. The Institutional Animal Care and Use Committee at Columbia University Medical Center approved the use of animals and the study protocols.

Isolation of mouse cardiomyocytes

$Lmna^{+/+}$ and $Lmna^{H222P/H222P}$ mice (16 weeks of age) were anesthetized with pentofurane. Ventricular cardiomyocytes were isolated as described in the Alliance for Cellular Signaling procedure protocol PP00000125 (http://www.signaling-gateway.org/data/ProtocolLinks.html). Briefly, hearts were removed and the aorta cannulated. After Ca^{2+}-free buffer was perfused for 2 min, 0.25 mg/ml collagenase I/II (Roche) solution was perfused through the coronary arteries for 6 min with 12.5 μM Ca^{2+}. Left ventricular tissue was teased apart and pipetted to release individual cells. After enzymatic dispersion, Ca^{2+} concentration in the buffer containing bovine serum albumin was elevated in three steps up to 500 μM.

Human tissue samples

Sections of explanted hearts from human subjects with *LMNA* mutations were obtained from Myobank-AFM of l'lnstitut de Myologie. The subjects were a 23-year-old man with dilated cardiomyopathy associated with muscular dystrophy (*LMNA* delK61 mutation), a 47-year-old woman with isolated dilated cardiomyopathy (*LMNA* R60G mutation) and from a 62-year-old woman with dilated cardiomyopathy associated with muscular dystrophy (*LMNA* c.IVS9 + 1g sup a mutation). Control human heart samples from a 57-year-old man who died from an intracranial bleed and a 15-year-old woman who died of drug overdose were obtained from the National Disease Research Interchange. All tissue samples were obtained with appropriate approvals and consent (not specifically for this study) from l'lnstitut de Myologie and the National Disease Research Interchange and provided without patient identifiers.

Quantitative real-time RT–PCR analysis

Total RNA was extracted using the Rneasy isolation kit (Qiagen). cDNA was synthesized using superscript first strand synthesis system according to the manufacturer's instructions (Invitrogen) on total RNA. For each replicate in each experiment, RNA from tissue samples of different animals was used. Primers were designed corresponding to mouse RNA sequences using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (9). Real-time quantitative RT–PCR reactions contained HotStart-IT SYBR green qPCR Master Mix (Affymetrix), 200 nM of each primer and 0.2 μl of template in a 25 μl reaction volume. Amplification was carried out using the ABI 7300 Real-Time PCR System (Applied Biosystems) as described previously (9). Relative levels of mRNA expression were calculated using the $\Delta\Delta C_T$

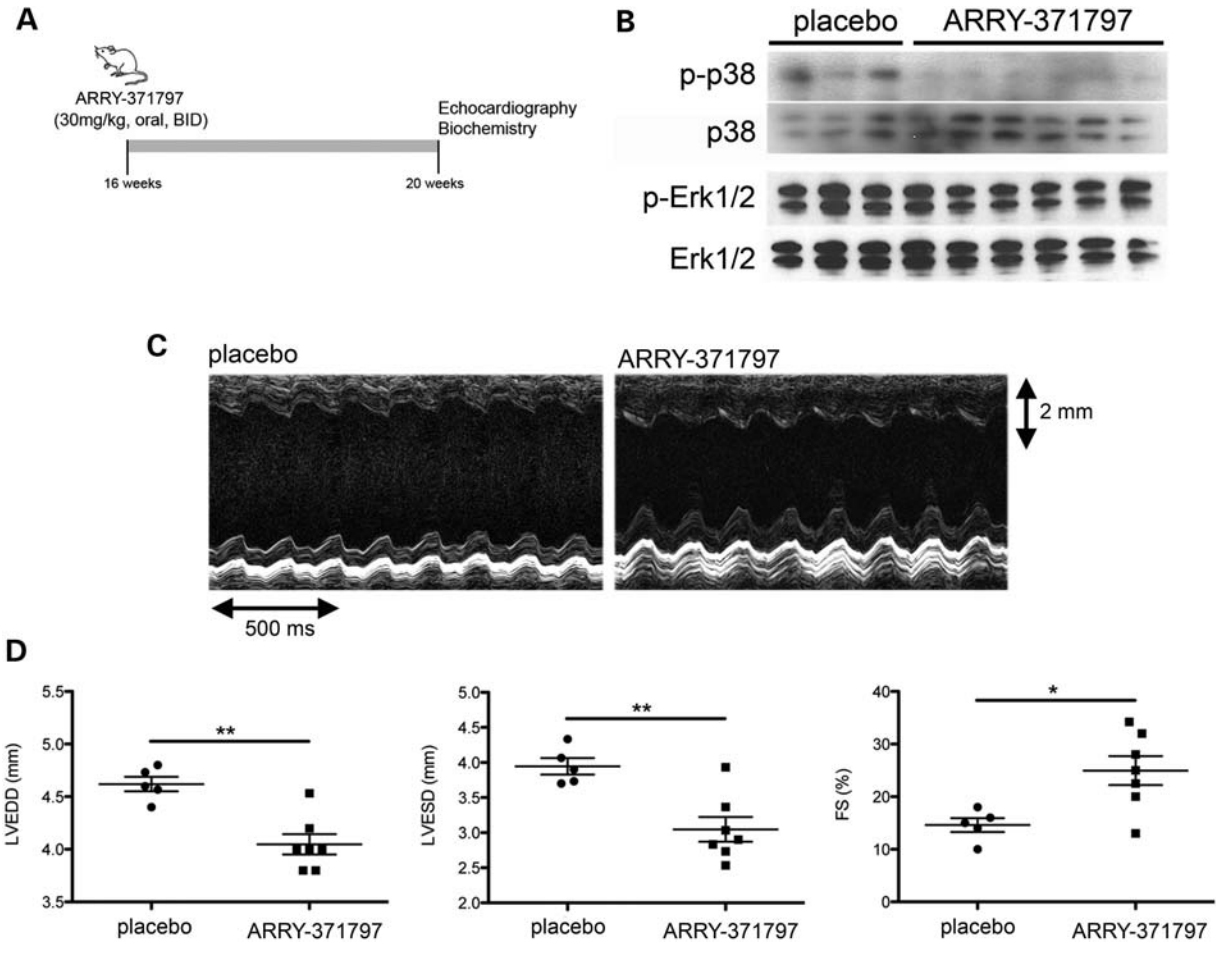


Figure 3. ARRY-371797 prevents LV dilatation and deterioration of FS in *Lmna*$^{H222P/H222P}$ mice. (**A**) Schematic representation of the treatment protocol of *Lmna*$^{H222P/H222P}$ mice with ARRY-371797. (**B**) Representative immunoblots using antibodies against phosphorylated p38α (p-p38α), total p38α, phosphorylated ERK1/2 (pERK1/2) and total ERK1/2 (ERK1/2) to probe proteins extracted from hearts from *Lmna*$^{H222P/H222P}$ mice treated with placebo or ARRY-371797. Each lane contains protein extracts from a different mouse. (**C**) Representative M-mode transthoracic echocardiographic tracings from 20-week-old male *Lmna*$^{H222P/H222P}$ mice treated with placebo or ARRY-371797. (**D**) Graphs showing mean LVEDD, mean LVESD and FS in 20-week-old male *Lmna*$^{H222P/H222P}$ mice treated with placebo ($n = 5$) or ARRY-371797 ($n = 7$). Values for each individual mouse receiving placebo (circles) or ARRY-371797 (squares) as well SEMs of means (bars) are shown. $^{*}P < 0.05$, $^{**}P < 0.005$.

Table 2. Effect of ARRY-371797 on LV diameters and FS in Lmna$^{H222P/H222P}$ mice

Treatment	Heart rate (bpm)	LVEDD (mm)	LVESD (mm)	FS (%)
Placebo ($n = 5$)	509.0 ± 4.5	4.6 ± 0.1	3.9 ± 0.1	14.6 ± 1.3
ARRY-371797 ($n = 7$)	496.0 ± 2.7	4.0 ± 0.1*	3.0 ± 0.2*	25.0 ± 2.7**

$^{**}P < 0.05$, $^{*}P < 0.005$ between placebo-treated and ARRY-371797-treated mice.

method (30). Individual expression values were normalized by comparison to *Gapdh* mRNA.

Protein extraction and immunoblotting

Human or mouse heart tissue was homogenized in sample extraction buffer (Cell Signaling) as previously described (10). Extracted proteins were separated by SDS–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and blotted with primary antibodies against p38α (No 9212, Cell Signaling), phosphorylated p38α (No 9216, Cell Signaling), MKK6 (No 9264, Cell Signaling) and phosphorylated MKK6 (No 9236, Cell Signaling). Secondary antibodies were horseradish peroxidate-conjugated (GE Healthcare). Recognized proteins were visualized by enhanced chemiluminescence (GE Healthcare).

Mouse treatment protocols

ARRY-371797 (Array BioPharma) was dissolved in Water for Injection (WFI) (Gibco) at a concentration of 0.5 mg/ml. The placebo control consisted of the same volume of WFI. ARRY-371797 was delivered at a dose of 30 mg/kg, twice a day. ARRY-371797 and WFI were administered orally by gavage starting when mice were 16 weeks of age and continuing until 20 weeks of age.

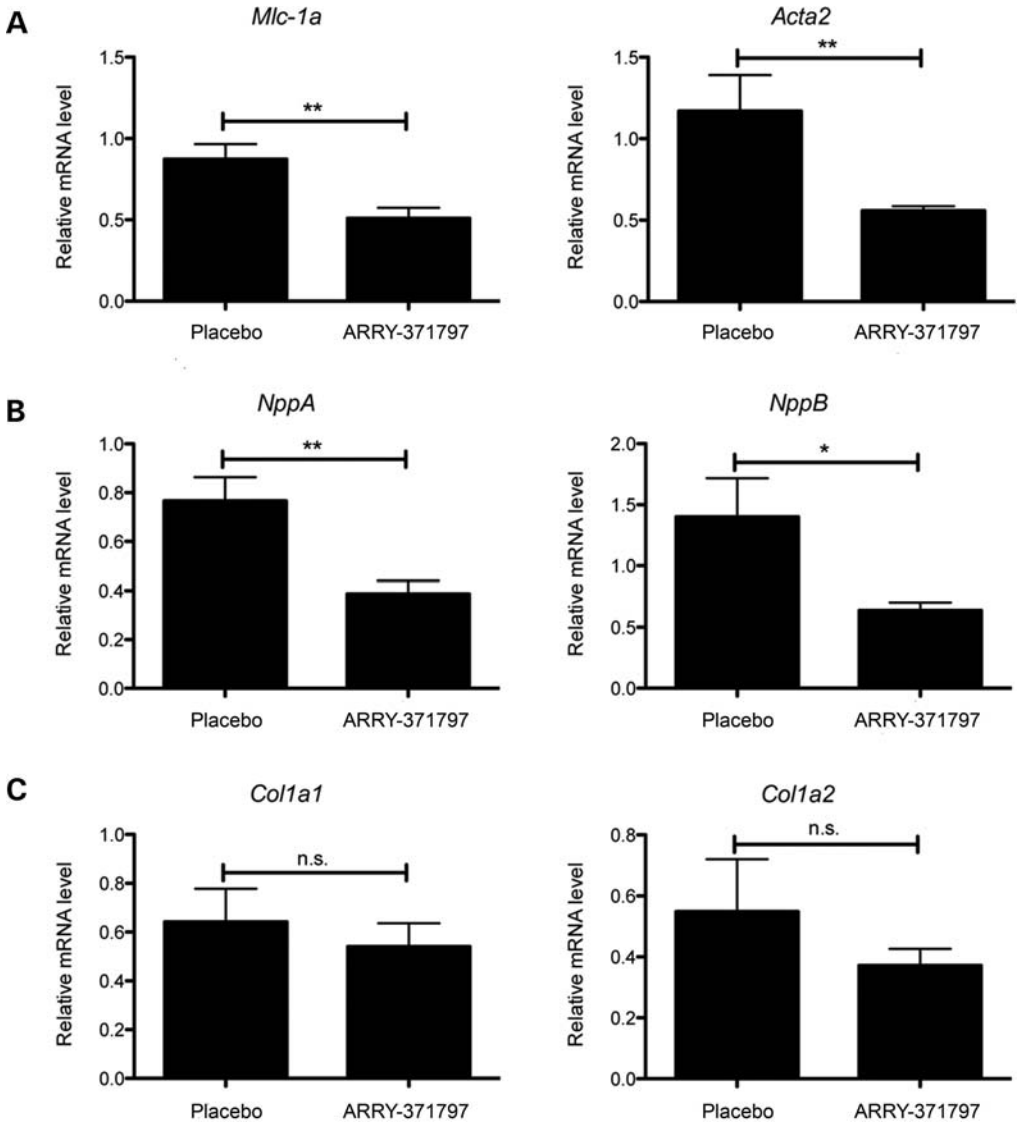


Figure 4. ARRY-371797 decreases expression of genes encoding proteins involved in sarcomere organization and natriuretic peptides but not collagens in *Lmna*$^{H222P/H222P}$ mice. (**A**) Relative expression of mRNAs from genes encoding myosin light chain (*Mlc-1a*) and cardiac-specific actin (*Acta2*) in *Lmna*$^{H222P/H222P}$ mice treated with placebo or ARRY-371797. (**B**) Relative expression of mRNAs from genes encoding atrial natriuretic peptide A (*NppA*) and brain natriuretic peptide B (*NppB*) in *Lmna*$^{H222P/H222P}$ mice treated with placebo or ARRY-371797. (**C**) Relative expression of mRNAs from genes encoding type 1 collagen isoforms (*Col1a1* and *Col1a2*) in *Lmna*$^{H222P/H222P}$ mice treated with placebo or ARRY-371797. Real-time quantitative RT–PCR, performed in triplicate on three separate samples, was used to measure mRNAs in hearts from *Lmna*$^{H222P/H2222P}$ mice treated with placebo ($n = 5$) or ARRY-371797 ($n = 7$). Values shown are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.005$, n.s., not significant.

Thansthoracic echocardiography

Lmna$^{H222P/H222P}$ mice were anesthetized with 1.5% isoflurane in O_2 and placed on a heating pad (37°C). Echocardiography was performed using a Visualsonics Vevo 770 ultrasound with a 30 MHz transducer applied to the chest wall. Cardiac ventricular dimensions and FS were measured in 2D mode and M-mode three times for the number of animals indicated.

Statistics

Values for real-time quantitative RT–PCR were compared using an unpaired Student *t*-test. Comparisons of echocardiographic parameters between ARRY-371797-treated and placebo-treated *Lmna*$^{H222P/H222P}$ mice were performed using a Welch *t*-test; to validate these results, a non-parametric test (Mann–Whitney) was performed and concordance checked. Statistical analyses were performed using GraphPad Prism software.

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Conflict of Interest statement. H.J.W. and A.M. are inventors on a pending patent application (PCT/US09/42614) on methods for treating and/or preventing cardiomyopathies by ERK and JNK inhibition filed by the Trustees of Columbia University in the City of New York.

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