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Targeting MRTF/SRF in CAP2-dependent dilated cardiomyopathy delays disease onset

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Abstract

About one third of dilated cardiomyopathy (DCM) cases are caused by mutations in sarcomere or cytoskeletal proteins. Yet treating the cytoskeleton directly is not possible because drugs that bind to actin are not well tolerated. Mutations in the actin binding protein CAP2 can cause DCM and knockout mice, either whole body (CAP2 KO) or cardiomyocyte specific knockouts (CAP2 CKO), develop DCM with cardiac conduction disease. RNA-seq analysis of CAP2 KO hearts and isolated cardiomyocytes revealed over-activation of fetal genes including serum response factor (SRF) regulated genes such as Myl9 and Acta2 prior to the emergence of cardiac disease. To test if we could treat CAP2 KO mice, we synthesized and tested the SRF inhibitor CCG-1423-8u. CCG-1423-8u reduced expression of the SRF targets Myl9 and Acta2, as well as the biomarker of heart failure, Nppa. The median survival of CAP2 CKO mice was 98 days, while CCG-1423-8u treated CKO mice survived for 116 days and also maintain normal cardiac function longer. These results suggest that some forms of sudden cardiac death and cardiac conduction disease are under cytoskeletal stress and that inhibiting signaling through SRF may benefit DCM by reducing cytoskeletal stress.

Key words:
Cyclase associated protein; embryonic reprogramming; mechanical stress; coflin; dilated cardiomyopathy; 6p22 syndrome.
Introduction

Sudden cardiac death is responsible for 180,000 to 450,000 deaths a year in the United States, and is one of the leading causes of mortality (1). Non-ischemic cardiomyopathy, including dilated cardiomyopathy (DCM), is responsible for 5-20% of these deaths. About half of the cases of DCM are caused by mutations in the cytoskeletal proteins of the sarcomere (2, 3).

Heart disease can lead to remodeling of the heart, characterized by cardiomyocyte hypertrophy, fibrosis and expression of fetal genes in cardiomyocytes, or fetal repograming. The transcription factors that drive the fetal program include MEF2, NKX, NFAT, NKX2-5, SMAD, GATA, NRSF and SRF (4, 5). It is not known if the fetal reprogramming protects the heart from further damage or contributes to the pathology of heart disease.

One feature of fetal reprogramming is activation of the set of genes regulated by the serum response factor (SRF). Serum activates SRF through Rho signaling, but Rho does not activate SRF through typical kinase cascades but instead by promoting polymerization of actin into the filaments of the actin cytoskeleton (6). By responding to actin, SRF can be activated directly by changes in actin dynamics or activated indirectly by other causes of mechanical stress. Activation by modulating actin dynamics makes SRF unique among signaling molecules. A core component of SRF are the two forms of myocardin related transcription factor (MRTF), MRTFA and MRTFB, which are both regulated by an actin monomer-binding site. Through MRTF, SRF senses the balance of monomers and filaments and serves as an actin rheostat, permitting the heart to respond to levels of cytoskeletal stress (7).
CAP2 is an actin monomer binding protein that associates with the sarcomere and promotes actin depolymerization. CAP proteins were originally isolated in yeast as a subunit of adenylyl cyclase, hence the family name, cyclase associated proteins (8, 9). In yeast, CAP assists Ras in activating cyclase by binding to the farnesyl lipid tail on Ras (10), while mammalian CAP1 can bind to the Ras related protein Rap1, again through its lipid tail (11). All CAPs also bind to actin monomers and partner with coflin to depolymerize actin filaments and recharge actin with ATP (12, 13). The yeast CAPs also transduce signals from the cytoskeleton to Ras, but no role has been found for mammalian CAPs in transducing signals from the cytoskeleton (14). There are two CAP isoforms in mammals, CAP1 and CAP2 (15).

Substantial evidence points to the importance of CAP2 in cardiac function. CAP2 is located on chromosome 6 at 6p22, near a locus that confers a risk for sudden cardiac death and conduction disease (16-19). Deletion of 6p22 is associated with cardiac abnormalities (20). A mutation in CAP2 is likely the cause of childhood DCM in two related patients in a large pedigree that was analyzed by exome sequencing. Both affected family members were the only ones in the pedigree having the same homozygous CAP2 mutation, while 12 heterozygotes had no evidence of DCM. Thus, the CAP2 mutations segregate as an autosomal recessive DCM gene (21), though some mutations may be dominant or semi-dominant.

The DCM caused by CAP2 loss has been modeled in mice. CAP2 KO mice are characterized by dilated cardiomyopathy with conduction disease. Males are more severely affected than females. CAP2 KO mice are also short and develop eye abnormalities (22, 23). The various phenotypes in CAP2 KO mice are recessive. Cardiomyocytes are the cells that are
primarily affected in DCM since cardiomyocyte specific CAP2 knockouts (CAP2 CKO) are especially prone to sudden cardiac death (23).

Here we show that both CAP2 KO mice and CAP2 CKO mice have elevated levels of SRF gene activation in their hearts, and that treatment of mice with an SRF inhibitor improves their cardiac function and extends their lifespan. This study suggests that SRF activation may be an indication of cytoskeletal stress and that reducing levels of SRF activation can prevent fetal reprogramming and benefit some forms of DCM.

Results

Gene expression profiling in hearts from CAP2 knockout mice

To identify the gene expression differences caused by loss of CAP2, we isolated hearts and carried out RNA-seq analysis in both CAP2 KO and CKO mice. The RNA-seq identified 788 and 2274 differentially expressed genes, respectively, with >1.2-fold change in the CAP2 KO mice and CKO mice, with 50.3% or 41.2% of the genes up-regulated. Figure 1A shows the top 50 regulated genes in the CAP2 KO mice and CAP2 CKO mice. Volcano-plots of the changes in gene expression of CAP2 KO or CKO mice versus WT mice are shown in Supplementary Figure 1A. Comparing the two gene lists by Venn diagram gives 325 genes found in both two lists, with 172 genes up-regulated and 153 down-regulated (Figure 1C). The top up-regulated gene was Natriuretic peptide A (Nppa), a common marker of heart failure and hypertrophy. Other upregulated genes were heart failure and hypertrophy related genes such as α−actin-1 (Acta1), Ankyrin repeat domain 1 (Ankrd1), Myosin light chain kinase 2 (Mylk2), as well as several genes implicated in arrhythmia such as calsequestrin 1 (Casq1), potassium
voltage-gated channel subfamily A member 5 (Kcna5), and potassium channel, subfamily K, member 2 (Kcnk2). CAP2 was downregulated more than 20-fold in the mutants.

Next, we filtered the gene list with the pipeline shown in figure 1B and performed pathway analysis with IPA (Ingenuity Pathway Analysis) on the list of genes shown up-regulated in both KO and CKO mice. The top activated canonical pathways were actin related, such as RhoA signaling and actin cytoskeleton signaling, as well as several pathways related to heart disease such as cardiac hypertrophy signaling and calcium signaling. The top upstream regulators were SRF and TGFB. Among these, SRF was the top transcription regulator by IPA z-score calculation (Figure 1D). IPA analysis identified the SRF genes overexpressed in the hearts from CAP2 KO mice (Supplementary Figure 1B).

**SRF activation precedes heart failure in CAP2 KO mice**

To confirm expression levels of genes identified by RNA-seq and trace the progression of changes as mice age, we used qPCR to quantify the expression of genes representative of the most altered pathways. Myl9 is regulated by SRF, Bmp10 is regulated by TGFB, Nppa and Ankrd1 are markers of heart failure, and Casq1 is a marker of arrhythmia and calcium signaling. Figure 2A shows expression of these marker genes in hearts from CAP2 KO and CKO mice at different ages. Each data point shows the fold-change in mutant mice compared with age-matched WT mice. For genes over-expressed more than 10-fold in the mutants, we assigned a 10-fold change for better data visualization. These data confirm the expression changes that were identified by RNA-seq analysis. In comparing expression over time, we found that SRF genes, Myl9, Acta1 and Acta2 were activated up to 10-fold at 3 weeks of age and remained elevated until 90 weeks of age. Heart failure and arrhythmia indicators, Nppa, Ankrd1 and Casq1 were
elevated but at 8 weeks of age, delayed compared to SRF genes. The TNF gene, Bmp10 was activated sporadically.

Heart function was evaluated by echocardiography at 3 weeks and 13 weeks \((n = 6\) in each group). Loss of CAP2 caused mild left ventricular (LV) chamber dilation, with an increase in end-diastolic LV internal diameter (LVIDd) and end-systolic LV internal diameter (LVIDs) at 3 weeks and 13 weeks (Figure 2B), corrected for body-weight. At 13 weeks, the ratio of LV mass to body weight was increased in CAP2 KO (Figure 2C). We note that most parameters measured in CAP2 KO indicated a trend towards significance and we did not observe a difference in ejection fraction or fractional shortening (Supplementary figure 2A). In comparing the transcription data with the functional cardiac data, we note that though all genes are eventually activated, only SRF genes were activated at all ages tested, suggesting that SRF signaling activation precedes heart failure.

**SRF signaling is activated through MRTFB translocation in CAP2 KO mice**

To determine if SRF is activated by loss of CAP2 in other tissues where CAP2 is highly expressed, we examined expression Acta2 and Myl9 in brain and muscle but found that there were no changes in mRNA levels in these tissues (Figure 3A). Thus, even though CAP2 is expressed in brain and skeletal muscle as well as in the heart, SRF pathway activation is exclusive to the heart. SRF can drive two mutually exclusive gene regulation programs by associating with different transcriptional cofactors, Elk or MRTF (also known as Mlk1/2). To address which cofactor is most likely regulating the affected genes, we compared the expression of the Elk-dependent gene cfos with MRTF-dependent genes, Acta2 and Myl9. Levels of cfos
did not change (Figure 3B). These data suggested that only MRTF/SRF signaling was activated in the hearts of CAP2 KO mice.

To address if activation was cell autonomous, or specific to the CAP2 isoform, we investigated whether CAP loss could activate SRF in other cells (Supplementary Figure 3A and 3B). We performed microarray experiments in HeLa cells and found that CAP1 knockdown caused several SRF regulated genes including Acta2 to be up-regulated. We did not observe Acta2 activation when CAP2 alone was knocked down, but when CAP2 was knocked down in the CAP1 knockdowns, we observed additional activation of SRF markers. Thus, loss of CAP activation of SRF is cell-autonomous, though in some cells and tissues, CAP1 is rate-limiting.

SRF is activated by MRTF, which has two isoforms, MRTFA and MRTFB. To address these factors, we isolated the protein from the nuclei in the hearts from CAP2 KO mice, and performed western blots for MRTFA, MRTFB and SRF. Compared with age-matched CAP2 WT mice, MRTFB accumulated in the nuclei in the CAP2 KO mice, while there was no difference with MRTFA. SRF was also up-regulated in the CAP2 KO mice (Figure 3D). This suggests that CAP2 loss primarily activates MRTFB.

**MRTF/SRF signaling is activated only in the cardiomyocytes of CAP2 KO mice**

MRTF/SRF signaling is up-regulated when fibroblasts are activated into myo-fibroblasts during heart remodeling, a process thought to be driven primarily by MRTFA. We addressed if fibroblasts are affected and found that the SRF genes, Acta2 and Myl9 were not up-regulated in MEF cells (Supplementary Figure 4A). We also evaluated these genes in isolated
cardiomyocytes from CAP2 KO mice (Figure 4A). Myl9, Acta1 and Acta2 were all upregulated, while c-fos was not changed (Figure 4B) in the isolated cardiomyocytes. We observed activation of SRF genes even in 3-week-old mice, while Nppa was not activated until 13 weeks of age (Figure 4C). Since the fibrosis pathway is activated in the RNA-seq data in both CAP2 KO and CKO mice, we also evaluated fibrosis by Sirius Red staining but, as we previously observed, did not observe significant fibrosis in the hearts from CAP2 KO mice (Supplementary Figure 4B) (23). This suggests cardiomyocytes are the primary cells for SRF activation in the heart.

**CCG-1423-8u inhibits SRF signaling through MRTF translocation in vitro and in vivo**

Whole body CAP2 KO males rarely survive to adulthood and the few males that survive develop DCM slowly, while females do not reliably develop DCM (23). However, cardiomyocyte-specific CAP2 knockout mice (CAP2 CKO) develop a robust DCM, so we used CAP2 CKO mice to test the functional importance of SRF in CAP2 DCM. qPCR analysis confirmed deletion of CAP2 in *Myh6Cre-cap2lox/cap2lox* compared with those of control *cap2lox/cap2lox* mice (Supplementary Figures 5A and 5B). CAP2 CKO mice die from heart failure during a narrow time window of 8-15 weeks. We found that mice sacrificed near this time window or subsequent necropsy of animals that died suddenly displayed robust heart dilation and pleural effusion. Histological analysis of CAP2 CKO hearts with haematoxylin and eosin (H&E) staining revealed marked cardiac enlargement, including both ventricular and atrial hypertrophy. MRTF/SRF gene expression was also elevated in the hearts from CAP2 CKO mice, as we observed in CAP2 KO mice (Supplementary Figure 5C).
To evaluate the contribution of over-activated MRTF/SRF signaling to the phenotype of the CAP2 CKO mice, we treated mice with the SRF inhibitor CCG-1423-8u, a more potent derivative of CCG-1423 (Figure 5A) (24, 25). CCG-1423 compounds inhibit SRF transcription and prevent MRTF translocation (Figure 5B, & Supplementary Figure 6A). Since CCG-1423-8u has not been used *in vivo*, we first performed a preliminary experiment to evaluate its effect on SRF markers. We treated 3 CAP2 WT mice and 3 CAP2 KO mice for 14 days intraperitoneally at a dose of 0.15mg/kg/d, sacrificed the mice the day after the last injection and then measured SRF target gene mRNA expression levels. Acta2 and Myl9 activation were attenuated after treatment, both in CAP2 WT and KO mice, while cfos was unaffected. Nppa was also down regulated in the treated CAP2 KO mice (Figure 5C). These data indicated that CCG-1423-8u inhibited SRF signaling, *in vivo*, without affecting Elk signaling.

**CCG-1423-8u restores cardiac structure and function**

After finding that CCG-1423-8u reduced the SRF marker genes *in vivo*, we next tested if the drug could affect the course of DCM in the CAP2 CKO mice. 95% of CAP2 CKO mice die from heart failure between 12 and 15 weeks of age. For comparison, we also treated *cap2<sup>loxp</sup>/cap2<sup>loxp</sup> mice as a control for the toxicity of CCG-1423-8u and additionally *cap2<sup>loxp</sup>/cap2<sup>loxp</sup> mice as a control for any cardiac toxicity of the *Myh6Cre-cap2<sup>loxp</sup>/cap2<sup>loxp</sup>* mice (26, 27). We assigned 10-week-old CAP2 CKO mice to two different treatment groups (DMSO, n=10; MRTF/SRF inhibitor CCG-1423-8u, n=10), and examined cardiac structure and function at 13 weeks of age, after 3 weeks of treatment.
The CAP2 CKO mice developed severe cardiomyopathy around 13 weeks of age, just before they die. As shown by haematoxylin and eosin (H&E) staining, hearts from CAP2 CKO mice had significantly larger hearts (figure 5D), with a 78.2% increase in the HW/BW ratio and a 77.5% increase in the ratio of heart weight to tibia length (HW/TL) compared to control mice (Figure 5E). In contrast, CCG-1423-8u treatment reduced the size of the CAP2 CKO hearts (Figure 5D), with a 35.4% decrease in the HW/BW ratio and 31.6% decrease in the HW/TL ratio (Figure 5E). Morphometric analysis of the cross-sectional area of cardiomyocytes from histological sections showed that knockout of CAP2 caused a 2.12-fold increase in the myocardial cell area. This hypertrophy was decreased by 21.3% by CCG-1423-8u treatment (Figure 5F and 5G). These findings demonstrated that treating with CCG-1423-8u decreased progression of DCM and pathologic myocyte remodeling in CAP2 CKO mice.

We also measured cardiac dimensions and contraction by echocardiography on anesthetized mice after 3 weeks of treatment with CCG-1423-8u. M-mode transthoracic echocardiography showed increased LV end-diastolic cavity dimension (LVEDD) and LV end-systolic cavity dimension (LVESD) in CAP2 CKO mice, compared with control mice. However, the CCG-1423-8u treated CAP2 CKO mice had significantly smaller LVEDD and LVESD (Figure 6A). The ejection fraction (EF) and fractional shortening was significantly reduced in the CAP2 CKO mice, compared with cap2lox/cap2lox and Myh6Cre- cap2lox/cap2lox, but restored in the CAP2 CKO mice treated with CCG-1423-8u (Figure 6B).

Table 1 shows the echocardiographic parameters for the CCG-1423-8u treated CAP2 CKO mice. The CAP2 CKO mice had significantly increased LVIDd and LVIDs compared with
control mice. The EF of CAP2 CKO mice at 13 weeks was 16.3±3.8%, which was decreased 39.5% compared to control mice. CAP2 CKO mice treated with CCG-1423-8u had a reduction in the LVIDd and LVIDs, and an EF of 31.4±11.9%, an increase of 15.1% (P=0.0002) compared with un-treated CAP2 CKO mice. Overall, these results showed that CCG-1423-8u had positive effects on cardiac function during the progression of DCM in CAP2 CKO mice. The control mice treated with the same dosing schedule of CCG-1423-8u did not show any differences in echocardiographic parameters compared with untreated control mice indicating that CCG-1423-8u does not cause any obvious cardiac toxicity or changes in baseline cardiac parameters.

**Effects of CCG-1423-8u on survival of CAP2 CKO mice with heart failure**

We next analyzed the effects of CCG-1423-8u treatment on CAP2 CKO survival. CAP2 CKO mice die between 8 and 15 weeks of age. We followed the same dosing schedule as described above; beginning at 6~7 weeks (45±3 days) of age CAP2 CKO mice were injected intraperitoneally for 21 days with 0.15mg/kg/d of CCG-1423-8u. DMSO-treated CAP2 CKO mice had a maximum survival of 117 days and a median survival of 98 days. CCG-1423-8u-treated CAP2 CKO mice had a significant increase in survival, with a median survival of 116 days (log rank P=0.004), with some mice living up to 127 days of age (Figure 7A). Treatment had no effect on the survival of control mice. We repeated this experiment with a different treatment protocol, treating daily, beginning at seven weeks of age, and again mice lived for a maximum of 126 days with a median survival of 114 days (log rank P=0.0005) (data not shown). Hence, suppressing SRF overactivation prolongs the lifespan of CAP2 CKO mice, consistent with its beneficial effects on cardiac fetal reprogramming, cardiac structure and heart function.
Discussion:

The SRF transcription factor stimulates expression of numerous sarcomere proteins. Knocking out SRF in the mouse heart causes a rapid onset of DCM indicating that it is essential for both heart development and maintenance of cardiac homeostasis (5, 28, 29). But hearts must balance SRF levels because overexpression of SRF also causes DCM (30, 31). SRF is overexpressed in many cardiac diseases, both ischemic and non-ischemic, but it is not established if the overexpression is protective or pathological. Since we find beneficial effects of inhibiting SRF in CAP2 KO, we suggest that overexpression is pathological and that cardiomyocytes can overcompensate when they activate SRF in response to cytoskeletal stress.

Cardiomyocyte transcriptional dedifferentiation, or fetal reprogramming, is common in both non-ischemic and ischemic cardiomyopathy and is characterized by re-expression of fetal genes and isoforms. It is not known if fetal transcription factor activation is a protective, detrimental or noncontributory response to the pathology of heart failure. There is some evidence that fetal reprogramming is a pathological contributor because beta blocker treated patients recovering from heart failure partially reverse the fetal gene reactivation, but the recovery of fetal gene expression was only correlative and is not seen in the partial recovery of patients on a left ventricular assist device support (5, 32, 33). Indeed, HDAC inhibitors, which are classic dedifferentiation agents, show some effectiveness in experimental models of cardiomyopathy but clinical use of multiple HDAC inhibitors to treat cancer is associated with adverse cardiac side effects (34-37). HDAC inhibitors are broad acting epigenetic transcription regulators that affect the expression of many genes, suggesting that effective use of transcription modulators may require more specific drugs. SRF, which directly responds to cytoskeletal
stress, is activated as part of the cardiac myoblast fetal expression program. Though transcription factors are generally poor drug targets, we find here that SRF can be successfully targeted with a small molecule inhibitor to delay DCM.

Fibrosis is commonly observed in DCM and ischemic heart damage. To initiate fibrosis, factors that activate fibroblasts, especially TGFB, are released from damaged tissues. Activated fibroblasts, or myofibroblasts express high levels of smooth muscle actin and secrete collagen (38). However, myofibroblasts are not the likely to be the target of CCG-1483-8u in our CAP2 knockout animals, since we do not reliably observe high levels of collagen expression in qPCR assays or observe collagen deposits by Sirius Red staining (supplementary figure 3B). Moreover, we find that there are extremely high levels of Acta2 and Myl9 in isolated cardiomyocytes from CAP2 KO animals. There is some debate as to the ideal level of fibrosis in damaged hearts. While some level of fibrosis can assist tissue repair, excessive fibrosis compromises cardiac contraction. In any event CCG-1483-8u inhibits SRF in fibroblasts and can reduce levels of lung fibrosis, suggesting that it may be beneficial in reducing excessive cardiac fibrosis as well as acting on cardiomyocytes.

The optimal dose and dosing schedule of CCG-1423-8u has not been determined. Only a few structurally related SRF inhibitors have been tested in vivo before. Though they are metabolized relatively rapidly, they have been successfully used in multiple models of fibrosis. This is the first study to test an SRF inhibitor in animals with compromised cardiac function (25, 39). We did not observe any obvious discomfort in the control mice that were treated with CCG-1423-8u, suggesting that they will tolerate higher doses and more potent SRF inhibitors. We
tested two dosing schedules, continuous dosing, and interrupted treatment and found that both extended survival about the same. This suggests that there may be an early time window for treatment and that treating beyond the window does not improve efficacy.

Comparison of the CAP2 KO and CAP2 CKO mice with human CAP2-DCM is in the early stages because only two patients have been described with homozygous CAP2 mutations (21). Their DCM was diagnosed at ages 5 and 12 respectively and the 12-year-old died shortly after diagnosis and was not completely phenotyped. The mutation that both patients had was at a splice site and generated a protein that lacked two exons but left most functional domains intact. Expression of the mutant protein was low, so it is not clear if the deleted regions reduced protein function or only affected stability. Phenotypically, the patients had DCM and conduction disease without the eye or height abnormalities observed in CAP2 KO suggesting that they resembled CAP2 CKO more closely than CAP2 KO. Though we note that the CAP2 KO mice are a biased population because few mice survive infancy, so the survivors that we study may have less severe DCM than those that do not live long enough to characterize.

Though this study utilized CAP2 knockout mice to study DCM, many mutations seen in DCM are in genes for sarcomere proteins including actin. Actin is difficult to target safely because actin-binding drugs, such as latrunculin A, cytochalasin D, phalloidin and jasplakinolide are not well tolerated, often causing cardiac toxicity (40-42). However, drugs that target signals to the actin cytoskeleton offer a less toxic alternative than those targeting actin itself. The most common signaling pathways to the cytoskeleton are through Rho family small GTPases. Druggable targets from the Rho pathway include Rho kinases, JNK and p38 kinases. Inhibitors
of these kinases, as well as ERK, have shown some effectiveness in DCM models caused by lamin A/C mutations, the most common mutation in DCM (3, 37, 43-48). Lamin A/C mutant mice develop DCM and conduction disease, with reduced levels of SRF activation, suggesting that a common element of lamin A/C and CAP2 KO DCM may be the mis-regulation of Rho signaling (49). Given the common pathway leading back to Rho and actin, treatment of DCM by SRF inhibition may benefit patients with DCM caused by mutations in genes other than CAP2 or even benefit patients with heart failure, where cytoskeletal stress and fetal reprograming are also observed (5, 50).
Materials and Methods

Generation of mice used in this study  CAP2 knockout (KO or CAP2 KO) mice and CAP2 cardiomyocyte specific knockout (CKO or CAP2 CKO) mice were generated and genotyped as described (23). Mice were euthanized by CO₂ and hearts were isolated from CAP2 KO mice and CKO mice at the indicated ages. For all western blots, RNA-seq and real-time PCR experiments, CAP2 KO mice were compared directly with age and gender-matched wild-type (WT) littermates taken from the litters of 6 different crosses between cap2+/− and cap2+/−. For CCG-1423-8u treatment experiments, CAP2 CKO mice were taken from the litters of 10 different crosses between cap2lox/cap2lox, or Myh6Cre-cap2+/cap2+ and Myh6Cre-cap2+/cap2+ Myh6-Cre-cap2+/cap2lox. Control cap2lox/cap2lox and Myh6Cre-cap2+/cap2+ were taken from each of the litters from which the CAP2 CKO mice were used. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania and studies were carried out in accordance with the approved guidelines.

CCG-1423-8u studies  CCG-1423-8u was prepared as described (25). An analysis of the compound used in these studies is shown in Supplementary Figure 6. CCG-1423 was purchased from SelleckChem. For animal experiments, CCG-1423-8u was dissolved in DMSO at a concentration of 15mg/mL, and then dissolved in PBS at a final concentration of 0.15mg/mL before injection. Mice were injected intraperitoneally with 0.1ml/10g for 14 days or 21 days at a dose of 0.15mg/kg/d. Control mice were injected with 0.1ml/10g PBS containing 0.1% DMSO.

RNA isolation from whole hearts  Total RNA was extracted using the RNeasy fibrous tissue kit (Catalog 74704, QIAGEN) according to the manufacturer’s instructions. The RNA concentration of each sample was measured by using Nanodrop 2000/2000c spectrophotometers (Thermo Fisher).
**RNA-seq and analysis** RNA sequencing and analysis were performed by *PrimBio Research Institute LLC* (Exton, PA, US), Next Generation Sequencing Core (University of Pennsylvania), and *ITMAT Bioinformatics* (University of Pennsylvania).

**Library Preparation and Sequencing** For the CAP2 KO RNA-seq samples, cDNA libraries were constructed using Ion Total RNA-Seq Kit v2 from Life Technologies (Cat# 4479789) and manufacturers recommended protocol. The prepared libraries were run on the Agilent 2100 Bioanalyzer to determine the yield and size distribution of each library. Approximately 100pM of pooled barcoded libraries were used for templating using Life Technologies Ion Chef 200 kit (Cat# 4488377) and manufacturers recommended protocol. The loaded chip was then placed into an Ion Proton sequencer and the run was started using an Ion Torrent RNA-seq run plan that was configured based on type of library, species, number of run flows required, type of plug-in required, adapter-trimming as well as other parameters specific to the transcriptome run.

For the CKO RNA-seq samples, total RNA quantity and quality were assayed with an Agilent 2100 Bioanalyzer instrument using the RNA 6000 Nano Kit (Agilent Technologies). Libraries were prepared using TruSeq Stranded mRNA HT Sample Prep Kit (Illumina) as per standard protocol in the kit’s sample prep guide. Libraries were assayed for size with D1000 ScreenTape kit of Agilent 2200 TapeStation System (Agilent Technologies) and quantified using KAPA Library Quantification Kit for Illumina platforms (KAPABiosystems). 100bp single-read sequencing of multiplexed samples was performed on an Illumina HiSeq 4000 sequencer. Illumina’s bcl2fastq version 2.20.0.422 software was used to convert bcl to fastq files.

**Alignment and Data Analysis** For the KO experiment, the raw sequence files were aligned to the mouse genome (mm10) reference sequence by the alignment plugin tool in Ion Torrent Suite software using the default parameters. Quality control, quantification and normalization was performed by the StrandNGS program using its DEseq algorithm. Differential expression was analyzed using Limma-Voom. For the CKO experiment, the raw sequence files were
aligned to the mouse genome (mm10) using STAR version 2.6.0c. One sample, a male CKO, had abnormally low number of reads and was removed from further analysis. The remaining samples had their reads quantified and normalized using PORT 0.8.5d (https://github.com/itmat/Normalization). Differential expression between WT and CKO was analyzed by Limma-Voom with a gender cofactor. For both experiments, pathway analysis was performed with Ingenuity IPA software using the top genes by differential expression with FDR < 0.2 and fold-change at least 1.2. The RNA-seq data for all the samples in both experiments were deposited on GEO under accession number GSE125603.

**Cardiomyocyte Isolation** Cardiomyocytes were isolated by the Langendorff method, using retrograde perfusion through the aorta with enzyme-containing solutions, as described (51). Briefly, mice were anesthetized with an *intraperitoneal* injection of Ketamine/Xylazine, 100 mg/kg/12.5mg/kg with 100 IU of Heparin respectively. After prepping with antiseptic, the chest was opened and a cardiectomy was performed. The heart was mounted on a perfusion apparatus and perfused with a calcium-free Krebs-Henseleit buffer followed by a collagenase-based enzyme solution to digest the extracellular matrix through aorta. After digestion, myocytes were dispersed into a single-cell suspension in Krebs-Henseleit buffer.

**Real-time quantification PCR** Immediately after RNA was extracted, 100µg of RNA was used to synthesize cDNA, using the High-Capacity RNA-to-cDNA Kit (catalog 4387406, Thermo Fisher Scientific). Predesigned SYBR Green primers were obtained from Integrated DNA Technologies (IDT) for the following genes: Myl9 (Mm.PT.58.41726289), Nppa (Mm.PT.58.12973594.g), Acta1 (Mm.PT.58.7312945), Acta2 (Mm.PT.58.163206644), cFOS (Mm.PT.58.29977214), Ankrd1 (Mm.PT.58.11977470), Gapdh (Mm.PT.39a.1), Casq1 (Mm.PT.58.42990820), Bmp10 (Mm.PT.58.5708693). Cap2 primers (Mm00482645) were
obtained from Thermo Fisher Scientific. SYBR Green PCR Master Mix was purchased from Thermo Fisher Scientific (catalog 4309155). The real-time PCR reaction contained 5µl mastermix, 100nM of each primer, and 0.5 µl of template in a 10-µl reaction volume. Amplification was carried out on a ViiA7 Real-Time PCR system from Applied Biosystems. Relative levels of mRNA expression were calculated according to the ΔΔCT method. Individual expression values were normalized by comparison with Gapdh mRNA.

**Subcellular protein fraction extractions from hearts and western blot analysis** Subcellular protein fractions were extracted from whole hearts using a Subcellular Protein Fractionation Kit for Tissues (catalog: 87790, Thermo Fisher Scientific). Protein concentration was quantified with a Bradford Protein Assay Kit (catalog 23246, Thermo Fisher Scientific). 30 µg of protein was resolved by SDS-PAGE (4-12% gel, Invitrogen). The proteins were transferred to PVDF (Invitrogen) membranes and probed with an anti-MRTFA antibody (sc-21558, Santa Cruz Biotechnology, 1:1000, Santa Cruz, CA, USA), an anti-MRTFB antibody (TE2565871, Invitrogen, 1:500, Rockford, IL, USA) or an anti-SRF antibody (sc-335, Santa Cruz Biotechnology, 1:1000, Santa Cruz, CA, USA). Antibodies against Lamin-B (sc-374015, Santa Cruz Biotechnology, 1:1000, Santa Cruz, CA, USA), or GAPDH (sc-25778, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as loading controls. MRTF is ~150 kDa, SRF is ~67 kDa. Immunoreactivity was visualized by use of ECL according to manufacturer’s protocol (catalog: 32106, Thermo Fisher Scientific). Band intensity was measured and quantified using ImageJ.

**Histopathology Analysis** Sections (4µm) of paraffin-embedded hearts stained with hematoxylin and eosin (H&E) were examined using light microscopy. Myocyte sectional-cross area was measured using ImageJ. For each heart the mean cross-sectional area was determined from 120
myocytes in 3 different visual, measured by using ImageJ. Heart size was calculated using the weight after sacrifice of the heart, corrected for the length of tibia and body weight weighed before sacrificing.

**Echocardiography** Transthoracic echocardiography was performed as described before (23). Mice were anesthetized by inhalation of 4% isoflurane in a glass chamber and then maintained during procedures in 1.5% isoflurane via nose cones. Echocardiography was performed using a Vevo 770 (VisualSonics) with a linear 30-MHz probe (RMV 707B). Left ventricle (LV) parameters were obtained from M-mode recordings in short-axis view. Interventricular septal wall thickness in diastole (IVSd), posterior wall thickness in diastole (PWd), LV end-diastolic (LVEDD) and LV end-systolic (LVESD) diameters were calculated from the mean of at least three separate cardiac cycles. LV Ejection fraction (EF) was calculated by dividing stroke volume by the end diastolic volume (EDV). LV mass was calculated with following formula:

\[
\text{LV mass (g)} = 1.05 \times \frac{5}{6} (\text{epicardial} \times \text{long axes (epicardial)} - (\text{endocardial} - \text{long axes endocardial}))
\]

and corrected for body weight.

**Statistics** Differences between groups were compared using 2-tailed Student’s t test, 2-way ANOVA with Bonferroni’s correction, or 2-tailed paired t test. Differences in survival of groups were compared using Log-rank (Mantel-Cox) test using GraphPad Prism software (version 7.0, CA, USA). Data are expressed as means ± SEM. P values are provided in the figures or in the legends. P < 0.05 was considered statistically significant.

**Multiple Hypothesis Testing Consideration** A total of 62 two-sample tests were performed, all with a significance threshold of 0.05. A total of 31 of the 62 p-values are significant at the 0.05 level. We therefore would conservatively expect 0.05×62=3.1 false positives from 62 tests if all null hypotheses were true. Thus we (conservatively) expect approximately 28 of our rejected null
hypotheses to be correctly rejected. With an expectation of 28 true positives we can recalculate the expected number of falsely rejected null hypotheses to conservatively be $0.05 \times 34 = 1.7$. Therefore, we expect approximately two false positives from the 31 rejected null hypotheses. This number is conservative since many of the observed p-values are in fact much smaller than 0.05. The overall conclusions of this paper are robust to such a small number of false positives among all rejected null hypotheses, particularly among the hypotheses with marginal p-values.

**Study approval** All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania and studies were carried out in accordance with the approved guidelines.

**Author contributions** YX, KB, BKA and KW design and execution of experiments; SB, design of synthesis and testing of SRF inhibitors; TB, execution of bioinformatics part in this project; KBM, JF, project supervision and manuscript preparation.

**Acknowledgments** The authors thank Professor Gregory R Grant (University of Pennsylvania) for insightful discussion on the bioinformatics and statics analysis in this manuscript. This project was supported by R01HL134923-01 to JF.
References


Table 1. Echocardiographic parameters in 13-week-old CAP2 CKO mice and controls with or without CCG-1423-8u treatment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DMSO</th>
<th>CCG-1423-8u</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=9)</td>
<td>CKO (n=9)</td>
<td>Control (n=7)</td>
<td>CKO (n=12)</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>24.8 ± 5.4</td>
<td>24.4 ± 2.5</td>
<td>24.0 ± 3.3</td>
<td>23.1 ± 4.0</td>
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<tr>
<td>Heart Rate (bmp)</td>
<td>447 ± 36</td>
<td>499 ± 92</td>
<td>407 ± 38</td>
<td>454 ± 62</td>
</tr>
<tr>
<td>EF (%)</td>
<td>55.8 ± 9.1</td>
<td>16.3 ± 3.8*</td>
<td>52.6 ± 5.5</td>
<td>31.4 ± 11.9*#</td>
</tr>
<tr>
<td>CO (mL/min)</td>
<td>18.1 ± 4.1</td>
<td>10.9 ± 4.1*</td>
<td>16.1 ± 2.9</td>
<td>13.2 ± 2.6</td>
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<tr>
<td>SV (µL)</td>
<td>40.2 ± 7.3</td>
<td>21.4 ± 5.4*</td>
<td>39.5 ± 6.1</td>
<td>30.9 ± 6.2*#</td>
</tr>
<tr>
<td>IVSd (mm)</td>
<td>0.7 ± 0.1</td>
<td>0.6 ± 0.1*</td>
<td>0.7 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>IVSs (mm)</td>
<td>1.1 ± 0.1</td>
<td>0.7 ± 0.1*</td>
<td>0.9 ± 0.1</td>
<td>0.8 ± 0.1#</td>
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<tr>
<td>LVIDd (mm)</td>
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<td>5.0 ± 0.3*</td>
<td>4.0 ± 0.3</td>
<td>4.7 ± 0.7*</td>
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<td>LVIDs (mm)</td>
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<td>2.9 ± 0.3</td>
<td>4.0 ± 0.8*</td>
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<tr>
<td>LVPWd (mm)</td>
<td>0.7 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>LVPWs (mm)</td>
<td>0.9 ± 0.1</td>
<td>0.7 ± 0.1*</td>
<td>0.9 ± 0.1</td>
<td>0.7 ± 0.1</td>
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<tr>
<td>LVFS (%)</td>
<td>27.4 ± 4.6</td>
<td>9.4 ± 3.7*</td>
<td>26.5 ± 0.4</td>
<td>17.3 ± 6.2*#</td>
</tr>
<tr>
<td>LVmass/BW (mg/g)</td>
<td>3.3 ± 0.8</td>
<td>3.6 ± 0.4*</td>
<td>3.2 ± 0.5</td>
<td>3.6 ± 0.5*</td>
</tr>
</tbody>
</table>

*P<0.05 compared with control mice receiving the same treatment
#P<0.05 compared with DMSO-treated CKO mice

EF= Ejection fraction; CO = Cardio output; SV = Stroke volume; IVSd = Interventricular septal wall thickness in diastole; IVSs = Interventricular septal wall thickness during systole; LVIDd = Left ventricular diameter during diastole; LVIDs = Left ventricular diameter during systole; LVPWd= Left ventricular posterior wall thickness during diastole; LVPWs = Left ventricular posterior wall thickness during systole; LVFS = Left ventricular fractional shortening; LVmass/BW = the ratio of left ventricle mass with body weight
Figure 1. Genes and pathways altered in whole body CAP2 knock out (KO) mice and conditional CAP2 knock out (CKO) mice. For KO mice and its controls, RNA was isolated from female wild-type mice (n=3) and mutant CAP2 KO hearts (n=3), ages ~86-weeks-old. For CKO mice, RNA was isolated from male (n=2) and female (n=3) rescued mice and male (n=1) and female (n=3) CAP2 CKO mice hearts, ages ~13-weeks-old. The samples were subjected to RNA sequence analysis and genes were ranked by differential expression p-values as determined by the Limma-Voom software. Pathway analysis by Ingenuity Pathway Analysis (IPA) software was performed on the list of all genes that were differentially expressed genes with FDR < 0.2 and fold change at least 1.2 in both KO and CKO experiments. (A) Top 50 altered genes in hearts from CAP2 KO mice (n=3) compared with wild type (n=3) (left) and CAP2 CKO mice.
(n=4) compared with age-matched rescued mice (n=5) (right); **(B)** The RNA-seq analysis pipeline used in this study; (C) The overlap genes with altered expression levels in both CAP2 KO and CKO mice; **(D)** Regulators analysis shows serum responsive factor (SRF), TGFB and TNF signaling were significantly activated; red means predicted activation by IPA software.
Figure 2. SRF pathway activation is an early step in CAP2 mutant hearts, and precedes dilated cardiomyopathy. RNA was isolated from mouse hearts at the indicated ages and individual gene expression levels were determined by qPCR. Normalization was done by using GAPDH expression as a standard (A) RT-qPCR analysis of representative genes of each pathway and associated phenotypes were selected and tested in the hearts of 10 pairs of WT and CAP2 KO and 5 pairs of WT and CAP2 CKO age-matched mice. Heat map shows the fold-change in gene expression levels with a cut-off of 10-fold, CAP2 KO or CKO mice compared with age-matched control mice; the age and gender of each pair of mice are labeled in the figure. Three technical triplicates were used in each sample. (B and C) Left ventricle (LV) function
parameters were measured from M-mode recordings in the short-axis view. (B) LV diameter during diastole (LVIDd) and systole (LVIDs) (n=5~6) and (C) LV mass, corrected for body weight, was increased in CAP2 KO mice at 3 weeks and 13 weeks of age, compared with WT mice, (n=5~6). Bars represent mean values ± SEM. P value between groups was based on Student’s t test.
Figure 3. The SRF pathway is specifically activated in hearts from CAP2 KO mice and associated with MRTFB translocation. (A) SRF target genes are activated in hearts but not brains or skeletal muscle from CAP2 KO mice. Expression levels of SRF targets Myl9 and Acta2 were determined in CAP2 KO mice (n=3), data is presented as mean±SEM, P value compared between groups was based on Student’s t test. Three technical triplicates were used in each sample. (B) (left) Relative expression level of Myl9 and c-fos in hearts from CAP2 KO mice (n=5), and age matched WT mice (n=5). (right) SRF regulation through two pathways. Note the difference in scales from panel A. Data is presented as mean±SEM. P value compared with WT mice was based on the Student’s t test. Three technical triplicates were used in each sample. (C)
Representative western blots and quantification showing MRTFB nuclear accumulation in CAP2 KO mice hearts (n=2). Data is presented as the mean of the values; P value was based on Student’s t test. Western blot of SRF and MRTFA and MRTFB in nuclear and cytoplasmic extracts from mice hearts (n=2). Right panel shows quantification of the western blot using Image J software. Three technical replicates were used in each sample.
Figure 4. SRF genes are activated in cardiomyocytes of CAP2 KO mice. Cardiomyocytes were isolated by the Langendorff method from CAP2 KO mice and age-matched WT mice at 3 weeks (n=3) and 13 weeks of age (n=4), respectively. (A) Schematic of this experiment (B) Relative expression of SRF pathway representative genes, Myl9, Acta1, Acta2 and cfos in isolated cardiomyocytes. Data is presented as mean±SEM; P value compared with WT mice was based on Student’s t test. Three technical triplicates were used in each sample. (C) Nppa and CAP2 expression in isolated cardiomyocytes. Data is presented as mean±SEM. P value compared with WT mice was based on Student’s t test. Three technical triplicates were used in each sample.
Figure 5. SRF inhibition reduces severity of cardiac pathology in CAP2 KO mice. (A) Structure of the SRF inhibitor CCG-1423-8u. (B) The inhibitory activity of CCG-1423-8u was measured using a luciferase reporter for SRF. Three technical triplicates were used in each
sample. (C) Expression of SRF target gene expression detected by qPCR in the hearts of whole body CAP2 KO (n=6) mice or WT mice (n=6) treated for fourteen days with CCG-1423-8u (n=3) or DMSO (n=3), at six weeks of age. Data is presented as mean±SEM. P value compared with WT mice was based on Student’s t test. Three technical triplicates were used in each sample. (D) H&E staining of the CAP2 CKO or control mouse hearts treated with CCG-1423-8u or DMSO; scale bar is 500µm. (E) Heart weight to body weight and heart weight to tibia length of the treated control or CAP2 CKO mice (n= 6~11). Data is presented as mean±SEM. P value compared with WT mice was based on the Student’s t test. (F) Detail of stained hearts; scale bar is 50µm and (G) myocyte sectional-cross area was measured in different groups (n=3) using ImageJ. For each heart, the mean cross-sectional area was determined from 120 myocytes in 3 different visual fields. Data is presented as mean±SEM. P value compared with WT mice was determined using the 2-tailed paired t test.
Figure 6. **SRF inhibition delays DCM in CAP2 CKO mice.** CAP2 CKO mice and controls were treated with CCG-1423-8u for 3 weeks starting 6~7 weeks of age, and heart function was measured by echocardiography. (A) Echocardiogram of CAP2 CKO mice and control treated with CCG-1423-8u or DMSO, respectively. (B) Ejection fraction was significantly reduced in treated CAP2 CKO mice (n=12) compared with age-matched controls (n=9), while CCG-1423-8u treatment delayed the reduction in contraction. Data is presented as mean±SEM. P value compared with WT mice was determined using the Student’s t test.
Figure 7. SRF inhibition extends the lifespan of CAP2 CKO mice. (A) Survival curves of CAP2 CKO mice treated with CCG-1423-8u (n=10) or DMSO (n=10). P value was determined by Log-rank (Mantel-Cox) test. (B) Schematic model of MRTF activated SRF signaling in CAP2 KO hearts.