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SUPPLEMENTARY MATERIALS

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HEART DISEASE

A small-molecule inhibitor of sarcomere contractility suppresses hypertrophic cardiomyopathy in mice

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Hypertrophic cardiomyopathy (HCM) is an inherited disease of heart muscle that can be caused by mutations in sarcomere proteins. Clinical diagnosis depends on an abnormal thickening of the heart, but the earliest signs of disease are hyperdynamic contraction and impaired relaxation. Whereas some in vitro studies of power generation by mutant and wild-type sarcomere proteins are consistent with mutant sarcomeres exhibiting enhanced contractile power, others are not. We identified a small molecule, MYK-461, that reduces contractility by decreasing the adenosine triphosphatase activity of the cardiac myosin heavy chain. Here we demonstrate that early, chronic administration of MYK-461 suppresses the development of ventricular hypertrophy, cardiomyocyte disarray, and myocardial fibrosis and attenuates hypertrophic and profibrotic gene expression in mice harboring heterozygous human mutations in the myosin heavy chain. These data indicate that hyperdynamic contraction is essential for HCM pathobiology and that inhibitors of sarcomere contraction may be a valuable therapeutic approach for HCM.

Hypertrophic cardiomyopathy (HCM) is the most common inherited disease of the heart muscle and occurs in ~1 in 500 adults (*1*). Patients are diagnosed with HCM when they present with left ventricular hypertrophy (LVH) that cannot be explained by another cardiac or systemic disease. Associated histopathologic findings include enlarged, disorganized cardiomyocytes and increased amounts of myocardial fibrosis. HCM also perturbs heart function, with characteristically hyperdynamic contraction (*2*, *3*) and impaired relaxation (*4*). Both the histopathologic and hemodynamic abnormalities of HCM contribute to patient symptoms (including exertional angina and shortness of breath) and increase the risk for atrial fibrillation, stroke, heart failure, and premature death (*5*). Contemporary pharmacologic therapy for HCM consists of off-label use of medications that nonspecifically reduce contractile strength by blocking β-adrenergic receptors or calcium channels (*6*). Although these medications can provide some symptom relief, none alter the progression of disease (*7*).

Dominant inherited and de novo mutations in genes that encode protein components of the sarcomere, the contractile unit of the heart, are identified in 35% of unselected patients who submit to gene-based testing (*8*) and 80% of patients with familial HCM (*9*). The two most frequently mutated HCM genes encode β-cardiac myosin heavy chain (*MYH7*), the predominant myosin isoform expressed in the adult human heart, and myosin-binding protein C (*MYBPC3*), a modulator of cardiac contraction. Pathogenic missense mutations in *MYH7* cluster within the globular motor domain, the head-rod junction region, and the rod domain, whereas most pathogenic *MYBPC3* variants encode truncated proteins (*8*, *10*). Studies of young carriers of either *MYH7* or *MYBPC3* mutations have shown that hyperdynamic contraction and impaired relaxation precede the appearance of LVH (*11*–*13*).

To study the role of sarcomere mutations in the development of HCM, we used previously generated mouse models of HCM, which we created by introducing human disease-causing mutations into the murine α-cardiac myosin heavy chain gene (*14*–*17*). This is the predominant myosin isoform expressed in the adult mouse ventricle and is 92% identical to human β-cardiac myosin heavy chain. Mice with heterozygous $Arg^{403}\rightarrow Gln^{403}$ (R403Q) (within the actin-binding site), $Arg^{453}\rightarrow Cys^{453}$ (R453C) (adjacent to the nucleotide-binding site), and $Arg^{719}\rightarrow Trp^{719}$ (R719W) (within the converter domain) missense residues (fig. S1) recapitulate most morphologic and functional features of human HCM.

Characterizing the biochemical and biophysical effects of these mutations at the molecular level and connecting these properties to the clinical phenotype of HCM is an active field of study. In many biomechanical assays, mutant myosin molecules expressed in vitro or isolated from HCM mouse hearts or human myofibrillar preparations have shown enhanced enzymatic [adenosine triphosphatase (ATPase)] activity, increased tension development, and/or increased unloaded actin-filament sliding velocities (*10*, *18*). These molecular phenotypes could explain the hyperdynamic contraction observed in human HCM patients. Furthermore, as cardiac fibroblasts respond to heightened mechanical, paracrine, and electrophysiological signals from cardiomyocytes with increased profibrotic gene expression (*19*) and production of extracellular matrix proteins (*20*), the hyperdynamic biomechanical properties of mutant sarcomeres could contribute to the downstream development of HCM histopathology (*16*). However, some HCM mutant proteins in reconstituted systems do not demonstrate increased power production and appear to slightly decrease force production (*21*). Such discrepancies highlight the current limitations of the reconstituted systems: single- rather than double-headed myosins, as well as the absence of key protein components and posttranslational modifications. Thus, the question remains as to whether mutations found in HCM increase power output at the molecular level. To test this hypothesis by an alternate approach, we sought a small molecule that could reduce sarcomere power output.

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We reasoned that if excess sarcomere power is the primary defect in HCM, small-molecule inhibitors of sarcomere power might ameliorate the disease at its source and abolish hallmark features of HCM such as hypertrophy, cellular disarray, and myocardial fibrosis.

Sarcomere power output is the product of ensemble force generated by myosin heads and their velocity of movement along actin filaments (*22*). In principle, either component of power output could be a target for modulation, and we initially sought agents that reduce power by reducing ensemble force generation. Because increases in the cycle time of myosin ATPase reduce ensemble force generation, we conducted a chemical screen for molecules that reduced the maximal actin-activated ATPase rate of myosin in bovine myofibrils (Fig. 1A). Both α- and β-cardiac myosin normally spend ~1/10 of their ATPase chemomechanical cycle times strongly bound to actin (Fig. 1A, lower two structures). Lengthening the total cycle time reduces this proportion, resulting in fewer myosin molecules in a force-producing state and an overall reduction in force generation (*22*). Compounds identified via this screen underwent optimization of potency and pharmaceutical properties to yield MYK-461 (Fig. 1B).

Treatment of mouse cardiac myofibrils with MYK-461 reduced ATPase activity in a dose-dependent manner [median inhibitory concentration (IC_{50}) of 0.3 μM] (Fig. 1C). Maximal doses of MYK-461 (>10 μM) reduced the maximal ATPase rate by ~90%. The potent inhibition of

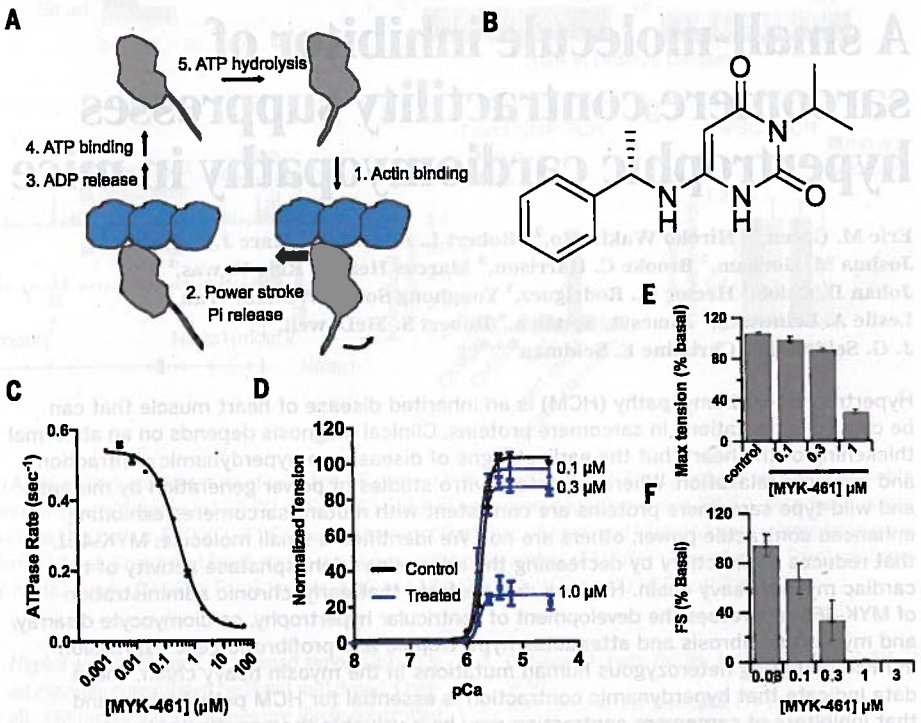

Fig. 1. MYK-461 inhibits myosin ATPase and contractility of cardiomyocytes. (**A**) Chemomechanical cycle of myosin, demonstrating the coupling between ATP hydrolysis and the myosin powerstroke. The swinging of the myosin lever arm (gray; bound light chains not shown) in step two is associated with movement of the actin filament (blue). (**B**) Chemical structure of MYK-461. (**C**) Decreasing rate of myosin ATPase in mouse cardiac myofibrils treated with increasing concentrations of MYK-461 (n = 6 biological replicates, mean ± SD). (**D**) Plots of tension versus pCA for skinned cardiac muscle fibers (from rats) exposed to increasing concentrations of MYK-461 (n = 3 to 4 fibers per concentration, mean ± SEM). (**E**) Decreasing maximal tension of skinned cardiac muscle fibers (from rats) exposed to increasing concentrations of MYK-461 (n = 3 to 4 fibers per concentration, mean ± SEM). (**F**) Decreasing fractional shortening (FS) of isolated adult rat ventricular cardiomyocytes treated with increasing concentrations of MYK-461 (n = 3 to 5 cells per concentration, mean ± SD).

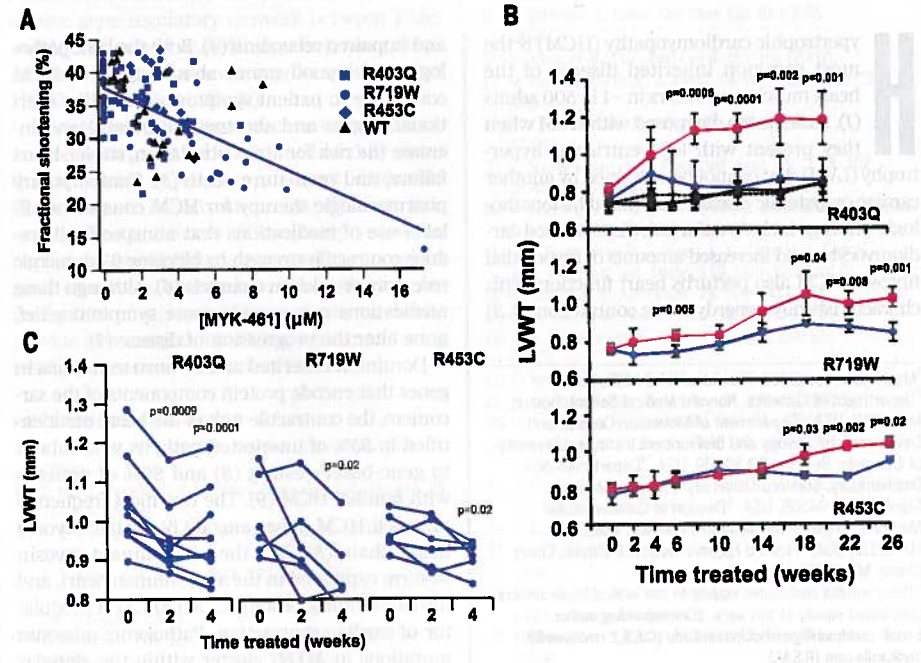

Fig. 2. MYK-461 reduces cardiac contractility in mouse models of HCM and prevents or ameliorates LV hypertrophy. (**A**) Reduction in fractional shortening with increasing MYK-461 plasma exposure in hearts from R403Q, R719W, R453C, and WT mice, fit by linear regression (n = 6 to 9 mice per background, Pearson's r = 0.57, $P < 10^{-5}$). (**B**) Changes in LVWT over time by echocardiography for WT mice (top panel: dashed black line, untreated; solid black line, treated; n = 6 mice each) and the indicated HCM mouse models (blue, treated with MYK-461; red, untreated; n = 3 to 6 mice per background). All data are expressed as mean ± SD, and statistical significance was tested by repeated-measures one-way analysis of variance. (**C**) Reduction in LVWT for the indicated HCM mouse models treated with MYK-461 after the onset of hypertrophy (15 to 30 weeks of age). Statistical significance was tested by paired t tests compared with baseline values.

ATPase rates in both murine [>90% α-myosin (23)] and bovine myofibrils [>90% β-myosin (24)] indicates that MYK-461 is active against both the α and β isoforms of cardiac myosin. Because we observed similar activity (IC_{50} of 0.3 μM) (fig. S2A) in a basal system consisting of only purified bovine myosin S1, we concluded that MYK-461 acts directly on myosin. We next performed transient kinetic experiments to isolate individual steps of the myosin chemomechanical cycle by which MYK-461 inhibits myosin ATPase. Treatment with MYK-461 reduced the rate of phosphate release (Fig. 1A, step two, and fig. S2B), the rate-limiting step in the chemomechanical cycle, in a dose-dependent manner without slowing adenosine diphosphate (ADP) release (Fig. 1A, step three, and fig. S2C). These data suggest that MYK-461 reduces the myosin duty ratio (i.e., the ratio of strongly bound state time to total cycle time) and thus decreases the ensemble force, power, and contractility produced by the sarcomere.

We also directly studied the effect of MYK-461 on power generation in muscle, by measuring tension produced by skinned cardiac muscle fibers isolated from adult rats. Exposure to MYK-461 reduced maximal tension in a dose-dependent manner (~70% reduction at 1.0 μM) without altering the pCa_{50} (i.e., the pCa at half the maximal activity) or the tension at lower calcium concentrations (Fig. 1, D and E). Similarly, adult rat ventricular cardiomyocytes treated with MYK-461 showed a dose-dependent reduction in fractional shortening (IC_{50} 0.18 μM) (Fig. 1D) without changing the calcium transient (fig. S3).

To study in vivo effects and determine whether MYK-461 decreases fractional shortening, a measure of contractility (fig. S4A), we administered MYK-461 orally (2.5 mg/kg per day via drinking water) to young (ages 6 to 15 weeks) wild-type (WT) and HCM mice expressing α–cardiac myosin heavy chain missense mutations R403Q, R719W, or R453C. Chamber dimensions, fractional shortening, and plasma drug concentrations were measured at baseline and at 2- to 4-week intervals (fig. S4B). WT and mutant mice exhibited decreased fractional shortening that correlated linearly with MYK-461 plasma exposures (correlation coefficient $r = 0.57$; $P < 10^{-5}$) (Fig. 2A). Although MYK-461 inhibits the ATPase activity of skeletal myosin with a lower affinity (IC_{50} of 4.7 μM with rabbit skeletal myosin), treated rodents had no reduction in grip strength or voluntary exercise capacity (fig. S5). Thus, MYK-461 reduced cardiac contractility in a dose-dependent manner in normal and mutant mice without overt impairment of skeletal muscle function.

We next investigated whether MYK-461 administered to young prehypertrophic HCM mice [ages 8 to 15 weeks; left ventricular wall thickness (LVWT) ≤ 0.8 mm] affected the development of LVH, the cardinal manifestation of HCM. Both treated and untreated WT mice maintained stable LVWT throughout the experiment, whereas LVWT increased in placebo-treated HCM mice (Fig. 2B), indicating the emergence of overt disease. In contrast, HCM mice treated with MYK-461 had LVWT comparable to that of WT mice (Fig. 2B).

To determine whether MYK-461 could reverse pathologic remodeling, we administered this drug to older HCM mice (ages 30 to 35 weeks) with

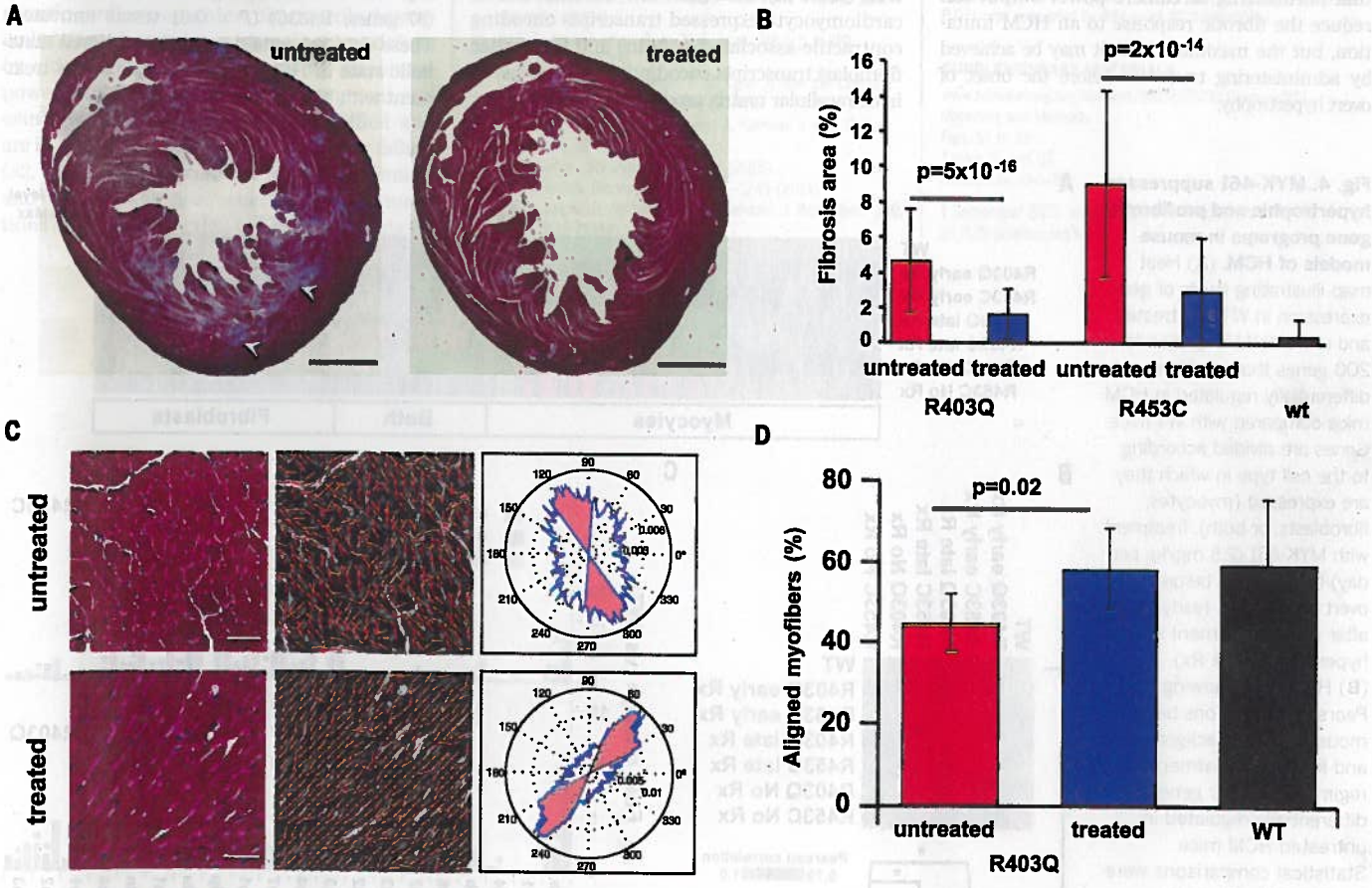

Fig. 3. MYK-461 reduces the development of myocardial disarray and fibrosis in mouse models of HCM. (**A**) Sections from untreated (left) and MYK-461–treated (right) 30-week-old R403Q mice stained with Masson's trichrome (arrowheads point to fibrotic areas). Scale bars, 1 mm. (**B**) Reduction in fibrosis area with MYK-461 treatment assessed in more than eight sections per mouse for R403Q and R453C HCM mice (n = 5 to 6 animals per group). (**C**) Representative regions of interest for analysis of cell orientation from R403Q mouse heart sections. Regions are shown (left) stained with Masson's trichrome (scale bars, 50 μM), (middle) with local gradient vectors illustrating cell orientations for analysis, and (right) as rose plots of the distribution of myofibril orientation angles (blue line; myofibrils oriented within 20° of the mean are shaded in red). (**D**) Percentage of aligned myofibers in WT and R403Q mice with and without MYK-461 treatment (n = 6 animals per condition). All data expressed are as mean ± SD; statistical comparisons were performed by t test.

overt LVH (LVWT ≥ 0.9 mm). Serial echocardiograms showed a significant reduction in the fractional shortening by week 2 (fig. S4C) and in LVWT by week 4 (Fig. 2C), after which these parameters remained stable with continued treatment. Thus, reduction of the pathologic power generated by the mutant sarcomere blunted the development of LVH and promoted partial regression of hypertrophy in older HCM mice.

Seminal histopathologic features of HCM include cardiomyocyte disarray and fibrosis, which may contribute to ventricular arrhythmias and heart failure (*25*). We hypothesized that counteracting the biomechanical effects of sarcomere mutations with MYK-461 would normalize these histopathologic phenotypes. Untreated HCM mice (R403Q or R453C) developed variable amounts of patchy fibrosis encompassing ~4.5 and ~9.6% of the left ventricle (LV), respectively (Fig. 3, A and B). Prehypertrophic mice treated for 20 to 26 weeks (Fig. 3, A and B, and fig S6) displayed ~80% less fibrosis. However, when MYK-461 was administered after the development of substantial hypertrophy, it did not significantly reduce the occurrence of fibrosis. These data suggest that normalizing sarcomere power output can reduce the fibrotic response to an HCM mutation, but the maximum benefit may be achieved by administering treatment before the onset of overt hypertrophy.

To characterize the effect of MYK-461 on myofibrillar disarray, we used an algorithm (*26*, *27*) that determines the regional orientation of myofibrils across histological sections (Fig. 3C and fig. S7). We defined the mean myofiber orientations for a given region (Fig. 3C) and the percentage of aligned cells (cells oriented within 20° of the mean; see supplementary materials and methods). At 30 weeks of age, untreated HCM R403Q mice had an average of 45 ± 7% aligned cardiomyocytes per region (Fig. 3D, red). Prehypertrophic R403Q mice treated with MYK-461 had 30% more aligned cardiomyocytes (59 ± 10%; $P = 0.02$) (Fig. 3D, blue), comparable to the mean alignment of cardiomyocytes observed in WT heart sections (Fig. 3D, black). In contrast, mice treated with MYK-461 after the development of overt hypertrophy showed no significant increase in cardiomyocyte alignment.

To characterize the cardiac transcriptional responses to reduced contractility from MYK-461 in HCM hearts, we focused on 200 genes with significantly altered expression in hypertrophic R403Q and R453C mice compared with WT controls (table S1). This gene set was enriched for cardiomyocyte-expressed transcripts encoding contractile-associated proteins and for cardiac fibroblast transcripts encoding proteins involved in extracellular matrix production (table S2). RNA expression of this 200-gene set in R403Q and R453C mice exposed to MYK-461 either before (ages 8 to 15 weeks; early Rx) or after (ages 30 to 35 weeks; late Rx) the development of overt LVH was significantly closer to WT LV levels than to untreated HCM LV levels (Fig. 4, A and B) ($P < 0.0001$). Although we did not detect regression of myocardial fibrosis in these mice, both cardiomyocyte contractility and profibrotic gene expression were normalized (Fig. 4C), indicating that reduction in sarcomere power in the prehypertrophic and overt HCM hearts promoted maintenance or restoration of normal transcriptional pathways.

Because LV hypertrophy has been linked with inefficient energy utilization (*28*), we also compared gene expression for 1158 genes (*29*) that encode proteins localized to mitochondria (fig. S8A) in WT and HCM mice (R403Q or R453C). In untreated HCM mice, we observed dysregulated expression in 19.7% (228 genes; R403Q) and 28.7% (332 genes; R453C) of genes encoding mitochondrial proteins, as compared with WT levels ($P = 0.01$) (fig. S8B). Early treatment with MYK-461 reduced the percentage of dysregulated gene expression to 4.2% (49 genes; R403Q) and 7.5% (87 genes; R453C) ($P < 0.01$ versus untreated). These data are consistent with an altered metabolic state in HCM that is normalized by treatment with MYK-461.

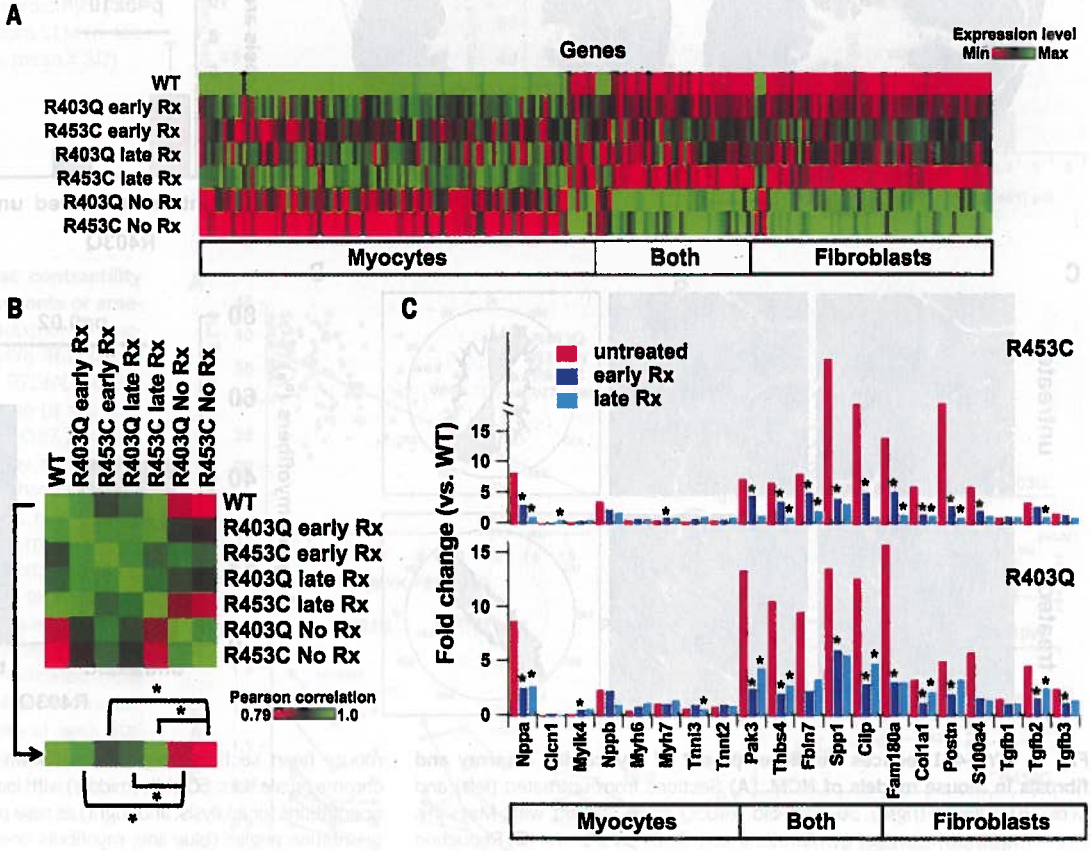

Fig. 4. MYK-461 suppresses hypertrophic and profibrotic gene programs in mouse models of HCM. (**A**) Heat map illustrating levels of gene expression in WT and treated and untreated HCM mice for 200 genes that are highly differentially regulated in HCM mice compared with WT mice. Genes are divided according to the cell type in which they are expressed (myocytes, fibroblasts, or both). Treatment with MYK-461 (2.5 mg/kg per day) began either before overt hypertrophy (early Rx) or after the development of hypertrophy (late Rx). (**B**) Heat map showing Pearson correlations between mouse genetic backgrounds and MYK-461 treatment regimens for 200 genes differentially regulated in untreated HCM mice. Statistical comparisons were performed between the Pearson correlations for WT animals or untreated mutants and each treatment condition for that mutant. Significance was assessed by Fisher *r*-to-*z* transformation (*$P < 0.0001$). (**C**) Fold change in gene expression levels (as compared with WT animals) for selected genes expressed in myocytes, fibroblasts, or both across MYK-461 treatment regimens in R453C mice (top) and R403Q mice (bottom; *$P < 0.05$).

In summary, our data demonstrate that a small-molecule inhibitor of sarcomere power administered early in the course of disease can attenuate the development and progression of the morphologic, histopathologic, and molecular changes that characterize HCM. Our findings support a mechanistic model for HCM in which sarcomere mutations lead to increased molecular power output, hyperdynamic contraction, and ultimately, pathologic remodeling of the heart. We suggest that analyses from reconstituted systems (*18*, *21*) have not consistently validated this model because key components (e.g., MYBPC or titin) are missing and/or important parameters for power production have not been examined (*30*). Nevertheless, our in vivo results indicate that reduction of sarcomere power output by direct manipulation of their biomechanical properties—with a small molecule that targets cardiac myosin—may present a therapeutic approach to HCM.

As other heritable cardiomyopathies are associated with mutations that disrupt physiologic power production by the sarcomere (*31*), the salutary effects of inhibiting myosin power in HCM suggest a more general paradigm for treating genetic cardiomyopathies: pharmacologic normalization of underlying biomechanical defects by either increasing or decreasing sarcomere power (*22*). Small molecules that augment myosin power have already been identified and are in clinical trials for patients with heart failure (*32*, *33*). Further elucidation of the biochemical and biophysical consequences of human mutations on cardiac function can form the basis for future efforts to treat these diseases at their mechanistic source.

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SUPPLEMENTARY MATERIALS

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