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Accessibility
Cardiovascular homeostasis dependence on MICU2, a regulatory subunit of the mitochondrial calcium uniporter

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Contributed by Christine E. Seidman, September 8, 2017 (sent for review June 26, 2017; reviewed by Kenneth E. Bernstein and Elizabeth Murphy)

Comparative analyses of transcriptional profiles from humans and mice with cardiovascular pathologies revealed consistently elevated expression of MICU2, a regulatory subunit of the mitochondrial calcium uniporter complex. To determine if MICU2 expression was cardioprotective, we produced and characterized \(Micu2^{-/-}\) mice. Mutant mice had left atrial enlargement and \(Micu2^{-/-}\) cardiomyocytes had delayed sarcomere relaxation and cytosolic calcium reuptake kinetics, indicating diastolic dysfunction. RNA sequencing (RNA-seq) of \(Micu2^{-/-}\) ventricular tissues revealed markedly reduced transcripts encoding the apelin receptor (\(A_{\text{Rp}}\)), which suppresses angiotensin II receptor signaling via allosteric transinhibition. We found that \(Micu2^{-/-}\) and wild-type mice had comparable basal blood pressures and elevated responses to angiotensin II infusion, but that \(Micu2^{-/-}\) mice exhibited systolic dysfunction and 30% lethality from abdominal aortic rupture. Aneurysms and rupture did not occur with norepinephrine-induced hypertension. Aortic tissue from \(Micu2^{-/-}\) mice had increased expression of extracellular matrix remodeling genes, while single-cell RNA-seq analyses showed increased expression of genes related to reactive oxygen species, inflammation, and proliferation in fibroblast and smooth muscle cells. We concluded that \(Micu2^{-/-}\) mice recapitulate features of diastolic heart disease and define previously unappreciated roles for \(Micu2\) in regulating angiotensin II-mediated hypertensive responses that are critical in protecting the abdominal aorta from injury.

mitochondrial calcium uniporter | diastolic dysfunction | aortic aneurysms | hypertension | calcium

The heart adapts to a variety of different stresses throughout life by adopting strategies to maintain excitation–contraction coupling and balance energy utilization and production. Genetic cardiomyopathies that cause ventricular hypertrophy (HCM) or dilatation (DCM) evoke chronic stress responses that have been identified in biochemical studies and transcriptional analyses of diseased cardiac tissues derived from human patients and model systems. Over a thousand genes with differential expression have been implicated in these responses, including molecules involved in cellular calcium flux (1–3).

Altered cardiac energetics is also a fundamental mechanistic component of HCM and DCM that is caused by mutations in sarcomere protein genes (4–8), and pharmacologic strategies are under study to correct energy deficits in patients with these disorders (9–11). Mutations in sarcomere proteins impact excitation–contraction coupling (12–15) and can alter cellular calcium flux, which in turn perturbs the balance of energy utilization and production in cardiomyocytes (16, 17). Changes in energy homeostasis can also reactivate fetal gene programs and promote profibrotic pathways and maladaptive remodeling that result in overt cardiomyopathy (18, 19).

Mutations that directly impact mitochondrial function also cause cardiomyopathies (20). These include mtDNA disorders such as MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) and MERRF (myoclonic epilepsy with ragged-red fibers) (21). Likewise, cardiomyopathy occurs from mutations in nuclear-encoded mitochondrial genes that function in oxidative phosphorylation, including \(TMEM70\) [mitochondrial complex V ATP synthase deficiency (22)], \(AGK\) [Sengers or mitochondrial depletion syndrome (23)], and \(AARS2\) [infantile hypertrophic mitochondrial cardiomyopathy (24)]. In addition to heart disease, the abnormal mitochondrial responses in these disorders cause diabetes, neurological disease, vision loss, deafness (20), and, less commonly, aortic dilation (25) and rupture (26).

We hypothesized that key mediators of altered calcium homeostasis and energetics, processes in which mitochondria play central roles, might be consistently dysregulated across many cardiomyopathies. To identify these, we performed bioinformatic comparisons of cardiac transcriptional datasets from humans and mouse ventricular tissues with HCM or DCM. Among these, we identified one consistently dysregulated gene, \(Micu2\), a calcium-sensing regulatory subunit of the mitochondrial calcium uniporter complex. Here we test the hypothesis that \(Micu2\) is a critical responder to cardiovascular stress. From studies of \(Micu2^{-/-}\) mice, we report unexpected phenotypes that link mitochondrial calcium sensing and cardiovascular stress responses.

Significance

Hypertension increases the risk for development of abdominal aortic aneurysms, a silent pathology that is prone to rupture and cause sudden cardiac death. Male gender, smoking, and hypertension appear to increase risk for development of abdominal aortic aneurysms by provoking oxidative stress responses in cardiovascular tissues. Here we uncovered unexpected linkages between the calcium-sensing regulatory subunit \(Micu2\) of the mitochondrial calcium uniporter and stress responses. We show that naive \(Micu2^{-/-}\) mice had abnormalities of cardiac relaxation but, with modest blood pressure elevation, developed abdominal aortic aneurysms with spontaneous rupture. These findings implicate mitochondrial calcium homeostasis as a critical pathway involved in protecting cardiovascular tissues from oxidative stress.

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Results

We performed a bioinformatic screen to identify stress-responsive molecules in cardiomyopathy by intersecting differentially expressed genes from nine left ventricle (LV) transcriptome datasets: seven from human heart tissue from patients with HCM (SI Appendix, Table S1), and two from mouse models of HCM (Myh6 p.R403Q) (1) and DCM (Ptn p.R9C) (27). Approximately 300 genes were differentially expressed in both mouse cardiomyopathy datasets. Intersecting this list with the seven human transcriptomes identified only 6 genes that were consistently expressed in the same direction among all nine transcriptome profiles that could plausibly function in the stress response. Three of these genes participate in pathways involving cell growth and metabolism, including IGFBP6 (insulin growth factor binding protein 6), PRKAB2 (the beta-2 non-catalytic subunit of AMP-activated protein kinase), and RHEB (a small GTPase superfamily member that functions in insulin/TOR/S6K signaling). Another gene, DAPK3 (expression of the death-associated protein kinase 3), participates in apoptosis pathways. Two mitochondrial proteins were also identified: UCP2 (mitochondrial uncoupling protein), that encodes an inner-membrane transporter, and MICU2 (mitochondrial calcium uptake 2), a regulatory subunit of the mitochondrial calcium uniporter complex that localizes to the mitochondrial intermembrane space, where it senses calcium levels to gate the activity of the mitochondrial calcium uniporter pore (28–31).

Recent studies link the mitochondrial calcium uniporter complex pore to both skeletal muscle and cardiac phenotypes. MCU is the uniporter’s pore-forming subunit that, in combination with essential MCU regulator (EMRE), forms a functional channel in vivo (32, 33). Under basal conditions, MCU–EMRE is kept in the closed position by the calcium-sensing MICU1–MICU2 heterodimer that resides in the mitochondrial intermembrane space. In response to a threshold spike in calcium, the MICU1–MICU2 complex disinhibits the pore to allow calcium entry into mitochondria. Mice lacking Mcu exhibit impaired skeletal muscle metabolism and peak performance (34, 35). Similarly, in the heart, MCU participates in matching cardiac metabolism to contractile stress (36, 37) and also impacts heart rate (38). Although a global Mcu knockout mouse and cardiac-specific Mcu deficiency did not cause overt heart deficits at baseline or with isoproterenol infusion or transverse aortic constriction, rapid physiologic fight-or-flight responses were impaired (34–38). Moreover, mice expressing a dominant-negative Mcu had altered cardiac oxygen utilization, cytoplasmic Ca\(^{2+}\) homeostasis, and pathologic responses to ischemia-reperfusion injury (39).

These data prompted us to prioritize MICU2 for further study. We hypothesized that if increased MICU2 expression was cardioprotective, deletion of MICU2 would promote cardiovascular dysfunction. To test this model, we generated a Micu2\(^{-/-}\) mouse using a gene-trap vector (Fig. 1A and SI Appendix, Fig. S1A). Micu2\(^{−/+}\) and Micu2\(^{-/-}\) mice bred and produced offspring in Mendelian ratios, had comparable sizes and activity levels to wild-type littermates, and routinely lived for >18 mo. Micu2\(^{−/-}\) mice had ~50% normal levels of Micu2 transcripts in hepatic, renal, and cardiac tissue. Transcript levels were further reduced in Micu2\(^{-/-}\) mice (SI Appendix, Fig. S1B). We did not detect Micu2 protein in LV, aorta, or liver tissues from Micu2\(^{-/-}\) mice by immunoblotting (Fig. 1B and C and SI Appendix, Fig. S2). Consistent with prior results of RNAi targeting Micu2 in mouse liver (28), both Micu1 and Mcu protein levels were significantly reduced in liver tissue from Micu2\(^{-/-}\) mice (SI Appendix, Fig. S2C). We also confirmed prior studies that demonstrate that Micu2\(^{-/-}\) mitochondria take up a high-concentration pulse of calcium more slowly than wild-type mitochondria (likely due to the reduction in Mcu levels) (28) but more rapidly take up lower-concentration pulses of calcium (SI Appendix, Fig. S3). These data are consistent with Micu2 setting the calcium threshold for the uniporter (30). Given these findings, we concluded that Micu2\(^{-/-}\) mice had reduced levels of Micu2 protein and activity.

Cardiac structure and function of Micu2\(^{-/-}\) mice were studied by histology, electron microscopy, and longitudinal echocardiography. Myocardial cellularity, cardiomyocyte size, and myocardial fibrosis in Micu2\(^{-/-}\) and wild-type littermate mice were indistinguishable and sarcomere structure was normal (Fig. 1D). By contrast, electron microscopy revealed that in Micu2\(^{-/-}\) mice (Fig. 1E), mitochondria were 20% smaller (P = 0.003) and 5% more eccentric (P = 0.002) than mitochondria from wild-type mice. Cistra structure appeared normal.

Longitudinal echocardiography revealed that Micu2\(^{-/-}\) mice had normal LV dimensions and fractional shortening (Table 1) but developed isolated left atrial (LA) enlargement at 16 to
Table 1. Echocardiographic assessment of cardiac structure and function in aged Micu2<sup>−/−</sup> mice

|                     | WT (SD) | Micu2<sup>−/−</sup> (SD) | P   |
|---------------------|---------|--------------------------|-----|
| Left atrial diameter, mm | 1.46 (0.06) | 1.76 (0.08) | 0.01 |
| Interventricular septum, mm | 0.93 (0.02) | 0.87 (0.02) | 0.11 |
| Posterior wall, mm    | 0.82 (0.04) | 0.85 (0.02) | 0.43 |
| LV diameter, mm       | 3.32 (0.18) | 3.48 (0.17) | 0.53 |
| Fractional shortening, % | 49 (4.0) | 48 (2.0) | 0.97 |

Statistics are mean (SD). P values were calculated from a two-sided t test with five or six animals, age 15 to 18 mo, in each group.

18 mo of age (20% increased diameter, P = 0.01). LA enlargement can reflect the cumulative effect of increased diastolic LV pressure due to diastolic dysfunction (40, 41).

To better understand this diastolic dysfunction in mutant mice, we profiled sarcomere contractility and cytosolic calcium transients in single cardiomyocytes from three pairs of wild-type mice and Micu2<sup>−/−</sup> littermates (ages 6 to 8 wk). Although the extent and rate of contraction were comparable in Micu2<sup>−/−</sup> and wild-type cardiomyocytes, relaxation rates were slower (P = 0.02) in Micu2<sup>−/−</sup> cardiomyocytes (SI Appendix, Table S2). The magnitude of calcium flux in Micu2<sup>−/−</sup> and wild-type cardiomyocytes was similar; however, the time constant for calcium reuptake was increased (P = 0.03) in Micu2<sup>−/−</sup> cardiomyocytes (Fig. 2 and SI Appendix, Table S3). From these profiles, we deduced that slower reuptake of cytosolic calcium by Micu2<sup>−/−</sup> cardiomyocytes could retard relaxation and contribute to diastolic dysfunction.

Next, we analyzed the LV transcriptome in 18-wk-old Micu2<sup>−/−</sup> and wild-type mice by RNA sequencing (RNA-seq). Among the differentially expressed genes, the sarcoplasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA2a) encoded by Atp2a (Dataset S1; P < 2 × 10<sup>−60</sup>) was significantly increased, likely as a compensatory response for increased cytosolic calcium in Micu2<sup>−/−</sup> cardiomyocytes. Database for Annotation, Visualization, and Integrated Discovery (DAVID) gene set enrichment analyses of 700 differentially expressed genes identified several gene ontologies with significant dysregulation (Fig. 3 and Datasets S1 and S2), including sarcomere genes (5.9-fold, P = 6 × 10<sup>−8</sup>), bZIP transcription factors (8.1-fold, P = 1.1 × 10<sup>−8</sup>), stress response genes (7.1-fold, P = 0.01), and ribosomal proteins (8.4-fold, P = 0.002).

We partitioned differentially expressed genes into those primarily expressed in cardiomyocytes (n = 64) and nonmyocytes (n = 89) based on greater than fivefold difference in normalized transcript levels (42). Differentially expressed genes found in both compartments were excluded. Notably, cell-specific pathway analysis identified enrichment in sarcomere genes (P = 2.5 × 10<sup>−4</sup>) and oxidative phosphorylation (P = 3.9 × 10<sup>−4</sup>) in cardiomyocytes and bZIP transcription factors in nonmyocytes (P = 2.9 × 10<sup>−5</sup>). Among 1,100 genes that encode mitochondrial proteins (43, 44), only 46 genes were differentially expressed [odds ratio 1.1, P = NS (not significant)]. Two of the most significant changes in transcripts from Micu2<sup>−/−</sup>-LV (Dataset S1) were a 1.6-fold decrease in the contractile protein gene Myh7 (P < 1e−50) and a 2.9-fold decrease in the apelin receptor (Aplnr; P = 1e−40), findings that were independently confirmed by qPCR (Fig. 3B). Notably, two dysregulated bZIP proteins, ATF3 (activating transcription factor 3) and C/EBP-B (CCAAT/enhancer-binding protein-beta), regulate apelin expression in response to stress (45).

Given these transcriptional data, we hypothesized that Micu2<sup>−/−</sup> mice would be particularly sensitive to angiotensin II, since the apelin receptor suppresses angiotensin II type 1 receptor signaling via allosteric trans-inhibition (46, 47). As angiotensin II stimulates cardiac hypertrophy (48), we also expected that this provocation could increase diastolic dysfunction in Micu2<sup>−/−</sup> mice. Homozygous Micu2<sup>−/−</sup> mice, heterozygous Micu2<sup>+/−</sup> mice, and wild-type littermates were infused with 1.2 mg·kg<sup>−1</sup>·d<sup>−1</sup> of angiotensin II to increase systolic blood pressure over baseline by 25 mmHg (P < 0.001; SI Appendix, Fig. S4). After 2 wk of treatment, all mice had equivalent increases in cardiac wall thickness, suggesting a comparable degree of hypertrophic remodeling (Table 2). However, Micu2<sup>−/−</sup> and Micu2<sup>+/−</sup> but not wild-type mice had significantly decreased fractional shortening in comparison with baseline (P = 0.04 and P = 0.002, respectively).

Unexpectedly, 3 of 14 Micu2<sup>−/−</sup> but no wild-type mice died during angiotensin II infusion (P = 0.03; Fig. 4A). As autopsies revealed that all three had ruptured abdominal aortic aneurysms, we extended these studies to assess whether loss of Micu2 impaired blood pressure and stress-induced vascular responses. Infusion of low (1.2 mg·kg<sup>−1</sup>·d<sup>−1</sup>) or high (2.4 mg·kg<sup>−1</sup>·d<sup>−1</sup>) doses of angiotensin II showed dose-dependent but equivalent increases in blood pressure in both Micu2<sup>−/−</sup> and wild-type mice (SI Appendix, Fig. S4).

Abdominal ultrasonography of untreated Micu2<sup>−/−</sup> mice (n > 10) showed slightly larger (5%) aortic artery diameters than wild-type mice (P = 0.003). Neither low nor high doses of angiotensin II infusion altered abdominal aortic diameters in wild-type mice, but Micu2<sup>−/−</sup> mice had dose-dependent increases in aortic diameters (P = 0.0006; Fig. 4B). Excluding mice with aortic rupture, a conservative estimate of the angiotensin II-induced aortic dilatation was at least 4.5-fold increased in Micu2<sup>−/−</sup> compared with wild-type mice.

Serial analyses of high-dose angiotensin II infusions revealed 10% increased abdominal aortic dimensions at day 2 in both Micu2<sup>−/−</sup> and wild-type mice. At day 4 and thereafter, aortic dimensions of wild-type mice remained unchanged while Micu2<sup>−/−</sup> mouse aortas continued to enlarge (P = 0.005; Fig. 4C and SI Appendix, Table S4).

To consider if this vascular pathology was blood pressure-mediated or angiotensin II-specific, we treated mice with nor epinephrine (5.6 mg·kg<sup>−1</sup>·d<sup>−1</sup>). Using nor epinephrine to increase blood pressures to equivalent levels induced by low-dose angiotensin II (SI Appendix, Fig. S4) did not induce aortic aneurysms in Micu2<sup>−/−</sup> or wild-type mice (Fig. 4B).

Fig. 2. Functional analysis of isolated Micu2<sup>−/−</sup> cardiomyocytes. Representative tracings of (A) single-myocyte sarcomere length (plotted as % of resting length) and (B) calcium flux (plotted as normalized Fura-2 intensity) in Micu2<sup>−/−</sup> mice and wild-type littermates. Micu2<sup>−/−</sup> cardiomyocytes had slower re-polarization kinetics, as evidenced by both decreased sarcomere relaxation velocity (A, Inset) and increased time constant (Tau, the exponential decay of the ventricular pressure during isovolumic relaxation) for calcium reuptake (B, Inset) compared with wild-type cardiomyocytes (Micu2<sup>−/−</sup>, n = 22 cells; wild type, n = 31 cells; t test). Error bars denote standard deviation.
Aneurysms arise from abnormalities of the intima, media, or adventitial layers of vascular beds that include fibroblast, smooth muscle, and endothelial cells. As histopathology of aortic aneurysms from Micu2−/− mice did not implicate a vascular layer or cell lineage (Fig. 4A, Inset), we attempted to deconvolute transcriptional changes by single-cell RNA-seq (49). From angiotensin II-treated Micu2−/− and wild-type aortas, we isolated 60 smooth muscle and fibroblast cells (SI Appendix, Fig. S6A) and 10 endothelial cells (likely due to smaller cell diameters that escaped microfluidic capture chips). The relative distribution of captured cell types was the same in Micu2−/− and wild-type aortas (P = NS).

Among aortic cells from wild-type mice, the distribution of Micu2 expression did not differ by cell type (P = NS). Micu2 was robustly expressed in 50% of endothelial, 23% of smooth muscle, and 18% of fibroblast cells, indicating that Micu2 deficiency could affect each cell lineage.

Two patterns of altered gene expression were observed: differences in the absolute expression levels (quantitated by a Wilcoxon test on median differences; SI Appendix, Fig. S6B) and differences in the number of cells expressing an appreciable level of genes (quantitated with a Fisher test; SI Appendix, Fig. S6C). Too few endothelial cells were captured to identify genes exceeding the significance threshold (Bonferroni adjustment for significance was set at P < 10−5 to reflect 5,000 expressed genes), but differential expression was identified in six smooth muscle cell genes and five fibroblast genes.

Among the six genes differentially expressed in Micu2−/− smooth muscle cells, we observed increased expression of genes associated with inflammation and reactive oxygen species (ROS) (SI Appendix, Figs. S6 B and C). Significantly more Micu2−/− (96%) than wild-type (29%) smooth muscle cells expressed extracellular glutathione peroxidase (Gpx3), the inflammatory cytokines Tnfα6 and Cell11, and the transcription factor Ifh1, which is associated with inflammation (50). Although the majority of Micu2−/− and wild-type smooth muscle cells expressed metallothionein (M1l) that contributes to cardiovascular protection in high-R0S states (51), the median expression was ninefold higher in Micu2−/− smooth muscle cells (P = 7.5 × 10−6).

The five differentially expressed genes in Micu2−/− fibroblasts are involved in proliferation, inflammation, and ROS (SI Appendix, Figs. S6 B and C). More Micu2−/− fibroblasts (80%) than wild-type fibroblasts (10%) expressed the proproliferative transcription factor Myc (P = 1.5 × 10−6) (52). Most Micu2−/− (88%) but few wild-type (10%) fibroblasts expressed Efnpl, which induces EGFR autophosphorylation and activates cell adhesion and migration (9.6 × 10−6) (53–55). Micu2−/− fibroblasts also showed significant differential gene expression of inflammatory and ROS genes, including decreased expression of Fno3 and Sod3, which reduce ROS free radicals (56, 57), and increased expression of the inflammatory mediator Il6 (58).

**Table 2. Cardiac structure and function in Micu2−/− mice after 2 wk of angiotensin II treatment**

| | WT (SD) | Micu2−/− (SD) | P  |
|---------------------------------|----------|---------------|----|
| Systolic blood pressure, mmHg   | 141 (9.8)| 142 (9.8)     | 0.94|
| Intraventricular septum, mm     | 0.89 (0.02) | 0.82 (0.06) | 0.28|
| Posterior wall, mm              | 0.82 (0.04) | 0.82 (0.02) | 0.93|
| LV diameter, mm                | 3.25 (0.11) | 3.58 (0.15) | 0.09|
| Fractional shortening, %        | 36 (1.2) | 28 (1.9) | 0.006|
| Systolic strain, %              | −19.3 (2.1) | −13.8 (2.5) | 0.007|

Statistics are mean (SD). P values were calculated from a two-sided t test with four to eight male mice, age 6 to 8 wk, in each group following 2 wk of angiotensin II infusion (1.2 mg·kg−1·d−1).
Discussion

Our data establish a critical role for Micu2, and presumably the mitochondrial calcium uniporter channel complex, in maintaining cardiovascular homeostasis in health and disease. Loss of Micu2 produced cardiac diastolic dysfunction with LA enlargement, abnormalities that are likely secondary to delayed calcium reuptake and decreased relaxation rates by cardiomyocytes. Micu2 depletion did not significantly alter gene expression of Mcu or Micu1, but protein levels of both were reduced. Hence, the cellular consequences of Micu2 loss are attributable to the combination of two effects: (i) a decrease in the threshold for mitochondrial calcium uptake due to loss of the gatekeeping activity of Micu2; and (ii) overall loss of mitochondrial calcium uptake by the uniporter due to destabilization of Muc1 and the entire complex. Moreover, changes in protein levels of Mcu and Micu1 after genetic ablation of Micu2 indicate that homeostatic interactions among components of the mitochondrial calcium uniporter complex may be important for stability. The resultant altered calcium dynamics was associated with pathologic transcriptional signatures in cardiomyocytes and nonmyocytes. Gene expression in Micu2−/− hearts was characterized by sarcomere dysregulation, stress response pathways, and altered expression of sarcomere genes and the bZIP family of transcription factors, molecules that contribute to cardiomyopathy pathogenesis and that regulate apelin signaling (2, 3, 59, 60).

In the setting of cardiovascular disease, Micu2 expression was particularly important. Hypertensive Micu2−/− mice had both diastolic and systolic dysfunction, as evidenced by decreased fractional shortening and altered systolic strain measurements. Despite these functional abnormalities, LV dimensions in Micu2−/− mice remained normal. As such, hypertensive Micu2−/− mice may provide a first step toward the development of an animal model for heart failure with preserved ejection fraction, a prevalent and poorly understood human disease (61).

The molecular mechanisms for diastolic dysfunction are incompletely understood. At the cellular level, cardiomyocyte relaxation requires tightly bound actin–myosin filaments to detach, a process mediated in part by calcium removal. Reduced or delayed calcium reuptake into the sarcoplasmic reticulum (SR) or mitochondria or removal from cardiomyocytes can delay relaxation (62). In addition, abnormal mitochondrial calcium homeostasis can influence NADPH levels and the redox state of cardiomyocytes to perturb relaxation. Consistent with these observations, malfunction of multiple calcium-handling proteins propel heart failure, including SERCA2a and its modulator phospholamban (PLB), the SR Calcium release channel ryanodine receptor RYR2, the sodium–calcium exchanger (NCX), and the mitochondrial calcium uniporter complex. The resultant altered cardiac calcium dynamics can preserve systolic function but lead to diastolic dysfunction (15, 63, 64). The mitochondrial calcium uniporter complex may be important for stability. The resultant altered calcium dynamics was associated with pathologic transcriptional signatures in cardiomyocytes and nonmyocytes. Gene expression in Micu2−/− hearts was characterized by sarcomere dysregulation, stress response pathways, and altered expression of sarcomere genes and the bZIP family of transcription factors, molecules that contribute to cardiomyopathy pathogenesis and that regulate apelin signaling (2, 3, 59, 60).

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Micu2 inhibition is relieved can trigger oxidative stress and vascular dysfunc-
tion. Recent studies of Micu2 in mice reduces aneurysm formation (74).
Mice and increased after angiotensin II in-
duction (transforming growth factor beta-2, TGFβ2). Matrix metalloproteinase 3 (Mmp3),
which degrades collagen, fibronectin, laminin, and elastin, had significant-
cantly increased expression (77). Mmp3 gene polymorphisms are
associated with aneurysm formation in human patients (78, 79),
and deletion of Mmp3 in mice reduces aneurysm formation (74). The
expression of Col3a1 and Col1a2, molecules that cause vascular
disease in Ehlers–Danlos syndrome (80–82), were increased in aneurysm II-treated Micu2+/− mice, a finding suggestive of active collagen degradation from increased Mmp3 expression.
Angiogenesis II increased the expression of other genes implicated in human aneurysms, most notably Fbn1 (fibrillin-1, mutated in Marfan syndrome) and TGFβ2 (transforming growth factor beta-2, mutated in Loeys–Dietz syndrome) (83–85).

Two calgranulins, S100a8 and S100a4, that were decreased in untreated Micu2+/− mice and increased after angiotensin II infusion are well-positioned to directly link altered calcium flux to aneurysm. Calgranulins are a family of small acidic calcium sig-
aling proteins that promote inflammation and vascular disease by activating the receptor for advanced glycation end products (RAGE) (86), and are linked to vascular dysfunction and ath-
erosclerosis (87, 88). S100a4 is strongly up-regulated in human thoracic aortic aneurysms (89) and intracranial aneurysms (90). Mechanistically, silencing S100a4 decreases vascular smooth pro-
floation and matrix metalloproteinase expression (89).

Notably, Micu2−/− mice did not develop aneurysms in the setting of norepinephrine-induced hypertension, a finding that implicates angiogenesis II-specific signaling. Angiogenesis II has been shown to activate noncanonical TGFβ signaling (ERK1/2)
via protein kinase C (83–85, 91–93). Recent studies of Fbn1−/− mice (modeling Marfan syndrome) treated with calcium channel blockers had accelerated thoracic aortic aneurysm expansion and rupture through a protein kinase C-mediated pathway, further implicating cross-talk between calcium signaling, TGFβ, and aneurysms (94). Clinical evidence also supports the link between calcium and aneurysm formation. A retrospective study of hu-
man patients identified the use of calcium channel blockers as an independent risk factor for abdominal aortic aneurysms (95). These data and our findings motivate further investigations into mitochondrial calcium homeostasis and aneurysm formation.

Angiogenesis II and loss of the Micu2 paralog and binding partner Micu1 can trigger oxidative stress and vascular dysfunc-
tion (96, 97). These data, combined with our transcriptional analyses, implicate oxidative stress in the pathogenesis of aneu-
rysm in angiogenesis II-treated Micu2+/− mice. While atherogenic mice are thought to develop abdominal aortic aneurysms via reactive oxygen species (98, 99), the Micu2−/− mice described here implicate abnormal mitochondrial calcium handling. It is notable that these two pathways, oxidative stress and mito-
chondrial calcium transport by the unipporter, have recently been mechanistically linked at a molecular level (71, 100). Further elucidation of the mitochondrial molecules and pathways that impact stress-induced calcium signaling are expected to further insights into disease pathogenesis and perhaps uncover novel therapeutic strategies to treat abdominal aortic aneurysms.

Materials and Methods

The Micu2 mice were derived from the gene-trap allele (Micu2; OST409343) that was generated by the Texas A&M Institute for Genomic Medicine. The

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**Fig. 5.** Transcriptional dissection of Micu2−/− aortic aneurysm. (A) RNA-seq and pathway enrichment analyses of untreated Micu2−/− mice (n = 3) compared with wild-type mice (n = 3) identified gene sets with increased (shaded bars) and decreased (clear bars) expression. GF, growth factor. Bonferroni-
corrected p values are shown. (B) RNA-seq pathway enrichment analysis of mouse aorta tissue after angiotensin II infusion (1.2 mg/kg for 2 wk) identified three notable changes in gene sets. (C) Fold change of selected genes with known roles in aneurysm formation after angiotensin II (1.2 mg/kg) versus basal state with significant differential expression by RNA-seq.
Vicr 37 viral plasmid was used to insert a splice acceptor, p-geo (p-galactosidase/ neomycin) cassette, synthetic polyA signal/transcriptional blocker, and PGK promoter/TK exo/nucleic donor cassette, all flanked by two viral long-terminal repeat segments in-between exons 4 and 5 of the Micu2 locus. Mice were de-

backcrossed onto the C57/B6 background for >10 generations. All animal experiments and procedures were reviewed and approved by the Institutional Animal Care and Use Committee at Harvard Medical School.

**Immunobots.** Antibodies were performed using antibodies from Sigma (Micu1, Micu, actin), Abcam (HSP60, ATP5a), and BD Biosciences (TIMM23). Antibodies against Micu2 and HNE were produced in collaboration with Bethyl.

**Mouse Liver Mitochondrial Isolation and Calcium Uptake Analysis.** Mitochon-
dria were isolated from mouse liver using differential centrifugation as previously described (28) and resuspended in 220 mM mannitol, 75 mM sucrose, 10 mM Hepes (pH 7.4), 1 mM EDTA, and 0.2% BSA and kept on ice. Calcium uptake assays were performed by adding 120 μg mitochondria to 150 μl buffer containing 125 mM KCl, 2 mM K2HPO4, 1 mM MgCl2, 20 mM Hepes at pH 7.2, 5 mM glutamate and malate, and 1 μM Oregon Green- Bapta6F. Fluorescence was monitored using a PerkinElmer EnVision plate reader in response to various pulses of CaCl2. The relative rate of calcium uptake is reported using a linear fit of fluorescence from 30 to 40 s (n = 3).

**Mouse Echocardiography and Abdominal Ultrasoundography.** Mice were anes-
thetized with an isoflurane vaporizer (VetEquip), and each limb was placed on an ECG leads on a Vevo Mouse Handling Table (VisualSonics), maintaining the body temperature at 37 °C during the study. Transthoracic echocardiography and transabdominal ultrasoundography were performed using the Vevo 2100 High-Resolution In Vivo Micro-Imaging System and MS550D transducer (VisualSonics), with heart rate at 500 to 550 beats per min. The images were acquired as 2D (left parasternal long and short axes), M-mode (left para-

ternal short axis), speckle tracking, and transabdominal 2D measurements. Measurements were averaged from images acquired during three consecu-
tive heart beats. All echocardiogram and sonogram measurements were performed with an experienced operator blinded to mouse genotype. Differences between groups of mice were determined using the unpaired Student’s t test.

**Single-Cardiomyocyte Functional Profiling.** Ventricular cardiomyocytes from three pairs of adult wild-type and Micu2−/− littersmates at 6 to 8 wk of age, on 3 separate days, were isolated via a Langendorff-perfused heart preparation using enzymatic digestion as previously described (101). After isolation, the cells were suspended in Tyrode’s buffer with gradually increasing Ca2+ concentrations (0.06, 0.6, and 1.2 mM, pH 7.4 at room temperature) and loaded with 1 μM Fura-2 AM (Molecular Probes) as pre-

viously described (102). Myocytes were washed three times for 10 to 15 min with 1.2 mM Ca2+. Tyrode’s solution containing 250 μM probenecid to retain the indicator in the cytosol. The experiments were then performed at room temperature in 1.2 mM Ca2+. Tyrode’s solution containing 140 mM NaCl, 4.5 mM KCl, 0.5 mM MgCl2, 5 mM glucose, and 10 mM Hepes, pH adjusted to 7.4 with NaOH.

Cardiomyocytes were electrically paced at 60 beats per min via platinum wires. Sarcomere shortening/relengthening and Fura-2 fluorescence ratios (which reflect the intracellular calcium transients) were simultaneously recorded and determined from discrete striation positions on the myocyte using a dual-excitation fluorescence imaging/contractility recording system (IonWizard SarcLen detection and PMT acquisition fluorescence system; IonOptix). Sarcomere length and Ca2+ transients were analyzed using Ion-

Optix transient analysis software. Myocytes included in the study were rod-

shaped with a clear striation pattern, quiescent in the absence of electrical stimulation, and with a resting sarcomere length of more than 1.6 μm. At least seven myocytes were profiled from each animal. Statistical analysis was performed with a two-sample, one-tailed Student’s t test.

**Electron Microscopy.** LV tissue from three pairs of wild-type and Micu2−/− littersmates at 18 wk of age were evaluated in a blinded fashion with seven independent examiners. Each mouse imaged at 1.900× magnification on a Tecnai G2 Spirit BioTWIN transmission electron microscope (FEI). The average mito-

chondrial size and eccentricity were automatically quantitated with Cell-

Profiler (Broad Institute).

**Pharmacologic Manipulation with Angiotensin II.** Hypertension was induced in mice by chronic infusion with angiotensin ii (Sigma-Aldrich) dissolved in saline at 1.4 mg kg−1 d−1 or 2.8 mg kg−1 d−1 or norepinephrine dissolved in saline at 5.6 mg kg−1 d−1, via an osmotic minipump for 2 wk (103).

**Blood Pressure Measurement.** Systolic blood pressure was measured in trained conscious mice, maintained at normal body temperature, using a BP-2000 Analysis System (Visitech Systems) as previously described (104). Mice were “trained” to the procedure twice daily for 3 consecutive days, and data were recorded over the following 2 d. Statistical analyses are from 10 readings for each mouse.

**Transcriptome-Wide Analyses.** Human samples from HCM patients were obtained from study participants undergoing either myocardy surgery heart, cardiac transplant surgery, or valve replacement surgery with informed consent, using IRB-approved protocols at Brigham and Women’s Hospital.

**RNA from human tissues and mouse LV was prepared and RNA-seq li-
braries were constructed as previously described (110, 111).** Uniform amplification of the cDNA library was achieved with amplification cycling before the re-

action reached saturation, as determined by quantitative PCR. Aorta RNA-

seq libraries were constructed with the Nextera library preparation method (106). To reduce biological variation in mouse specimens, RNA was pooled from three biological replicates for LV samples and angiotensin ii-treated aorta samples. For the basal aorta RNA-seq samples, libraries from three biological replicates for each genotype (Micu2−/− and wild type) were con-

structed and the libraries were sequenced individually.

Libraries were sequenced on an Illumina HiSeq 2000 sequencer with 50-bp paired-end reads. Following sequencing, alignment of reads to the mm10 genome was performed with Bowtie and TopHat (107). Gene expression profiles were constructed by tallying reads on gene loci, using a Bayesian P value to assess the significance of gene expression differences between pooled samples (108). Cuff-
diff 2 was used for assessing the significance of gene expression differences in aorta RNA-seq library replicates (109).

Genes were considered differentially expressed if there was a >40% increase or decrease in fold change with P < 10−4. The DCM mouse model and HCM mouse model transcriptome datasets were previously described (110, 111). Gene ontology pathway enrichment analysis was performed with DAVID (112).

**Single-Cell RNA-Seq.** Mouse aortas were dissected and digested into single cell suspensions using collagenase. Cells were captured, and RNA was extracted and amplified into cDNA libraries using the Fluidigm C1 system as previously described (110, 111). Libraries were sequenced on an Illumina HiSeq 2000 se-

quencer with 50-bp paired-end reads. Reads were aligned using TopHat. The expression of known markers for vascular lineages was used to classify each cell as a smooth muscle cell (Acta2, Tagln, Myh11), fibroblast (Vim), or en-

dothelial cell (Pecam1, Tek, Cd35). Cells lacking any of these markers were classified as “undetermined.” Wilcox and Fisher test P values were calculated in the R statistical computing environment, version 2.15 (www.r-project.org).

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1. Geisterfer-Lowrance AA, et al. (1996) A mouse model of familial hypertrophic car-
diomyopathy. Science 272:731–734.

2. Kita T, Chang S, Seidman JC, Seidman CE (2010) Genetics of hypertrophic cardiomyopathy. Curr Opin Cardio 25:205–209.

3. Teekarkulikul P, Padera RF, Seidman JC, Seidman CE (2012) Hypertrophic cardiomy-

opathy: Translating cellular cross talk into therapeutics. J Cell Biol 199:417–421.

4. Güçlü A, et al. (2013) E3LerGisits in hypertrophic cardiomyopathy: Translating be-

tween MRI, PET and cardiac myofilament function (ENGINE study). Neth Heart J 21: 567–571.

5. Ingwall JS (2004) Transgenesis and cardiac energetics: New insights into cardiac metabo-

lism. J Mol Cell Cardiol 37:613–623.

6. Maack C, O’Rourke B (2007) Excitation-contraction coupling and mitochondrial en-

ergetics. Basic Res Cardiol 102:369–392.

7. Suda S, Dhalla NS (1971) Excitation-contraction coupling in heart. VII. Calcium ac-

cumulation in subcellular particles in congestive heart failure. J Clin Invest 50:1019–1027.

8. Tardiff JC, et al. (2015) Targets for therapy in sarcomeric cardiomyopathies. Cardiovasc Res 105:457–470.

9. Abozguia K, et al. (2010) Metabolic modulator perhexiline corrects energy deficiency and improves exercise capacity in symptomatic hypertrophic cardiomyopathy. Circulation 122:1562–1569.

10. Beadle RM, et al. (2015) Improvement in cardiac energetics by perhexiline in heart failure due to dilated cardiomyopathy. JACC Heart Fail 3:202–211.
11. Dass S, et al. (2015) Exacerbation of cardiac energetic impairment during exercise in hypertrophic cardiomyopathy: A potential mechanism for diastolic dysfunction. Eur Heart J 36:1547–1554.
12. Gao WD, Pérez NG, Seidman CE, Seidman JG, Marban E (1999) Altered cardiac excitation-contraction coupling in mutant mice with familial hypertrophic cardiomyopathy. J Clin Invest 103:661–666.
13. Hasenfus G, et al. (1992) Alteration of contractile function and excitation-contraction coupling in dilated cardiomyopathy. Circ Res 70:1225–1232.
14. Palmer BM, et al. (2004) Differential cross-bridge kinetics of FHC myosin mutations R430Q and R453C in heterozygous mouse myocardium. Am J Physiol Heart Circ Physiol 287:H415–H425.
15. Palmer BM, et al. (2013) Elevated rates of force development and MgATP binding in F764L and S352P myosin mutations causing dilated cardiomyopathy. J Mol Cell Cardiol 57:23–37.
16. Taegtmeyer H, Hi, LM Jones, BE (1998) Energy substrate metabolism, myocardial ischemia, and targets for pharmacotherapy. Am J Cardiol 82(Suppl 1):54K–60K.
17. Territo PR, Motha VK, French SA, Balaban RS (2000) Ca(2+) activation of heart mitochondrial calcium oxidative phosphorylation: Role of the Fo/F1-ATPase. Am J Physiol 278:C423–C435.
18. Degré C, Davies PJA, Taegtmeyer H (1999) Transcriptional adaptation of the heart to mechanical unloading. Am J Cardiol 83(Suppl 1):58H–63H.
19. Teekakirikul P, et al. (2010) Cardiac fibrosis in mice with hypertrophic cardiomyopathy is mediated by non-myocyte proliferation and requires Tgf-β. J Clin Invest 120:3520–3529.
20. Koopman WJH, Willems PHGM, Smetsink JAM (2012) Monogenic mitochondrial disorders. N Engl J Med 366:1132–1141.
21. Liéder DS, et al. (2011) Exome sequence of suspected mitochondrial disorders. Neurology 80:1762–1770.
22. Calvo S, et al. (2006) Systematic identification of human mitochondrial disease genes through integrative genomics. Nat Genet 38:576–582.
23. Calvo SE, et al. (2012) Molecular diagnosis of infantile mitochondrial disease with targeted next-generation sequencing. Sci Transl Med 1035:48.
24. Götz A, et al. (2011) Exome sequencing identifies mitochondrial alanyl-tRNA synthetase mutations in infantile mitochondrial cardiomyopathy. Am J Hum Genet 88:635–642.
25. Brunetti-Pierri N, et al. (2011) Dilatation of the aortic root in mitochondrial heart disease patients. Mol Genet Metab 103:167–170.
26. Tay SH, et al. (2006) Aortic rupture in mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes. Arch Neurol 63:281–283.
27. Schmitt JP, et al. (2005) Cardiomyopathy and heart failure caused by a mutation in phospholamban. Science 299:1410–1413.
28. Plovnich M, et al. (2013) MICU2, a paralog of MICU1, resides within the mitochondrial uniporter complex to regulate calcium handling. PLoS One 8:e55785.
29. Kamer JK, Motha VK (2015) The molecular era of the mitochondrial calcium uniporter. Nat Rev Mol Cell Biol 16:545–553.
30. Kamer JK, Motha VK (2014) MICU1 and MICU2 play nonredundant roles in the regulation of mitochondrial calcium uniporter. EMBO Rep 15:299–307.
31. Kamer JK, Grabarek Z, Motha VK (2017) High-affinity cooperative Ca(2+) binding by MICU1-MICU2 serves as an on-off switch for the uniporter. EMBO Rep 18:1397–1411.
32.neck Y, et al. (2013) EMRE is an essential component of the mitochondrial calcium uniporter complex. Science 342:1379–1382.
33. Kovács-Bogdán L, et al. (2008) Restoration of the mitochondrial calcium uniporter in yeast. Proc Natl Acad Sci USA 111:8985–8990.
34. Holmström KM, et al. (2015) Assessment of cardiac function in mice lacking the mitochondrial calcium uniporter. J Mol Cell Cardiol 85:178–182.
35. Fan X, et al. (2012) Mitochondrial calcium release revealed by mice lacking the mitochondrial calcium uniporter. Nat Cell Biol 15:1464–1472.
36. Kwong JQ, et al. (2015) The mitochondrial calcium uniporter selectively matches metabolic output to acute contractile stress in the heart. Cell Rep 12:15–22.
37. Luongo TS, et al. (2015) The mitochondrial calcium uniporter is essential for metabolic energy supply with cardiac workload during stress and modulates permeability transition. Cell Rep 12:23–34.
38. Wu Y, et al. (2015) The mitochondrial uniporter controls flight or flight heart rate increases. Nat Commun 6:6081.
39. Rasmussen TP, et al. (2015) Inhibition of MCU forces extramitochondrial adaptations to acute contractile stress in the heart. Circulation 132:1320–1325.
40. Rasmussen TP, et al. (2012) Plasma profiling by a protein array approach identifies IGBP1–2 as a novel biomarker of abdominal aortic aneurysm. Atherosclerosis 221:544–550.
41. Shinomiya K, Mitchell RN, Libby P (2006) Inflammation and cellular immune responses in abdominal aortic aneurysm. Arterioscler Thromb Vasc Biol 26:987–994.
42. Sukhanov S, et al. (2007) IFG-1 reduces inflammatory responses, suppresses oxidative stress, and decreases atherosclerosis progression in ApoE-deficient mice. Arterioscler Thromb Vasc Biol 27:2684–2690.
43. Palmomo D, et al. (1999) Mitochondrial metalloproteinases. Their role in degradative chronic diseases of abdominal aorta. J Cardiovasc Surg (Torino) 40:257–260.
44. Morris DR, Biro E, Cronin O, Kuivaniemi H, Collinge J (2014) The association of genetic variants of matrix metalloproteinases with abdominal aortic aneurysm: A systematic review and meta-analysis. Heart 100:295–302.
45. Saracini C, et al. (2012) Polyphosphomins of genes involved in extracellular matrix remodeling and abdominal aortic aneurysm. J Vasc Surg 55:171–179.e2.
46. Sasaki AS, et al. (2018) Ehlers-Danlos syndrome. A variant characterized by the de novo loss of pro-alpha 2(A) chain collagen. Arch Dermatol 124:76–79.
47. Superti-Furga A, Gugler E, Gitzelmann R, Steinmann B (1988) Ehlers-Danlos syndrome type IV: A multi-exon deletion in one of the two COL3A1 alleles affecting
structure, stability, and processing of type III procollagen. J Biol Chem 263: 6226–6232.

82. Vissing H, et al. (1991) Multixen deletion in the procollagen III gene is associated with mild Ehlers-Danlos syndrome type IV. J Biol Chem 266:5244–5248.

83. Boileau C, et al.; National Heart, Lung, and Blood Institute (NHHLBI) GO Exome Sequencing Project (2012) TGFB2 mutations cause familial thoracic aortic aneurysms and dissections associated with mild systemic features of Marfan syndrome. Nat Genet 44:916–921.

84. Lerner-Eliis JP, et al. (2014) The spectrum of FBN1, TGFβRI, TGFβRII and ACTA2 variants in 594 individuals with suspected Marfan syndrome, Loey-Dietz syndrome or thoracic aortic aneurysms and dissections (TAAD). Mol Genet Metab 112:171–176.

85. Milewicz DM, et al. (1996) Fibrillin-1 (FBN1) mutations in patients with thoracic aortic aneurysms. Circulation 94:2708–2711.

86. Hofmann MA, et al. (1999) RAGE mediates a novel proinflammatory axis: A central cell surface receptor for S100/calgranulin polypeptides. Cell 97:889–901.

87. Averill MM, Kerkhoff C, Bornfeldt KE (2012) S100A8 and S100A9 in cardiovascular biology and disease. Arterioscler Thromb Vasc Biol 32:222–229.

88. Hofmann Bowman M, et al. (2010) S100A12 mediates aortic wall remodeling and aortic aneurysm. Circ Res 106:145–154.

89. Cao J, et al. (2013) Spatiotemporal expression of matrix metalloproteinases (MMPs) is regulated by the Ca2+ signal transducer S100A4 in the pathogenesis of thoracic aortic aneurysm. PLoS One 8:e70057.

90. Coen M, et al. (2013) Smooth muscle cells of human intracranial aneurysms assume a phenotype features similar to those of the atherosclerotic plaque. Cardiovasc Pathol 22:339–344.

91. Olson ER, Shamhart PE, Naugle JE, Meszaros JG (2008) Angiotensin II-induced extracellular signal-regulated kinase 1/2 activation is mediated by protein kinase Cdelta and intracellular calcium in adult rat cardiac fibroblasts. Hypertension 51: 704–711.

92. Regalado ES, et al.; NHHLBI GO Exome Sequencing Project (2011) Exome sequencing identifies SMAD3 mutations as a cause of familial thoracic aortic aneurysm and dissection with intracranial and other arterial aneurysms. Circ Res 109:680–686.

93. Shah BH, Catt KJ (2002) Calcium-independent activation of extracellularly regulated kinases 1 and 2 by angiotensin II in hepatic C9 cells: Roles of protein kinase Cdelta, Src/proline-rich tyrosine kinase 2, and epidermal growth receptor trans-activation. Mol Pharmacol 61:343–351.

94. Doyle JJ, et al. (2015) A deleterious gene-by-environment interaction imposed by calcium channel blockers in Marfan syndrome. Elife 4:e08648.

95. Wilminck AB, et al. (2002) Are antihypertensive drugs associated with abdominal aortic aneurysms? J Vasc Surg 36:751–757.

96. Doughan AK, Harrison MG, Dikalov S (2008) Molecular mechanisms of angiotensin II-mediated mitochondrial dysfunction: Linking mitochondrial oxidative damage and vascular endothelial dysfunction. Circ Res 102:488–496.

97. Hoffman NE, et al. (2013) MICU1 motifs define mitochondrial calcium uniporter binding and activity. Cell 153:515–528.

98. Daugherty A, Cassis LA (2004) Mouse models of abdominal aortic aneurysms. Arterioscler Thromb Vasc Biol 24:429–434.

99. Kuhlenkordt PJ, et al. (2001) Accelerated atherosclerosis, aortic aneurysm formation, and ischemic heart disease in apolipoprotein E endothelial nitric oxide synthase double-knockout mice. Circulation 104:448–454.

100. Choi S, et al. (2017) Mitochondrial calcium uniporter transfer calcium between the endoplasmic reticulum and mitochondria in oxidative stress-induced cell death. J Biol Chem 292:14473–14485.

101. Lim CC, Apstein CS, Golucci WS, Liao R (2000) Impaired cell shortening and re-lengthening with increased pacing frequency are intrinsic to the senescent mouse cardiomyocyte. J Mol Cell Cardiol 32:2075–2082.

102. Nagata K, et al. (1998) Early changes in excitation-contraction coupling: Transition from compensated hypertrophy to failure in Dahl salt-sensitive rat myocytes. Cardiovasc Res 37:467–477.

103. Zhan Y, et al. (2005) Ets-1 is a critical regulator of Ang II-mediated vascular inflammation and remodeling. J Clin Invest 115:2508–2516.

104. Chen PC, et al. (2010) Activation of multiple signaling pathways causes developmental defects in mice with a Noonan syndrome-associated Sos1 mutation. J Clin Invest 120:4353–4365.

105. Christodoulou DC, Gorham JM, Herman DS, Seidman JG (2011) Construction of normalized RNA-seq libraries for next-generation sequencing using the crab duplex specific nuclease. Curr Protoc Mol Biol 94:12.1–12.11.

106. Trombetta JJ, et al. (2014) Preparation of single-cell RNA-seq libraries for next generation sequencing. Curr Protoc Mol Biol 107:4.21.1–4.21.17.

107. Trapnell C, et al. (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat Protoc 7:562–578.

108. Christodoulou DC, et al. (2011) Quantification of gene transcripts with deep sequencing analysis of gene expression (DSAGE) using 1 to 2 μg total RNA. Curr Protoc Mol Biol 93:258.9.1–258.9.16.

109. Trapnell C, et al. (2013) Differential analysis of gene regulation at transcript resolution with RNA-seq. Nat Biotechnol 31:46–53.

110. Burke MA, et al. (2014) Proliferation of cardiac fibroblasts defines early stages of genetic dilated cardiomyopathy and precedes myocardial metabolic derangement. Circ Res 115:A290.

111. Christodoulou DC, et al. (2014) 5′RNA-seq identifies Flh1 as a genetic modifier in cardiomyopathy. J Clin Invest 124:1364–1370.

112. Huang W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4:44–57.

113. Wu AR, et al. (2014) Quantitative assessment of single-cell RNA-sequencing methods. Nat Methods 11:41–46.