Mitochondrial CaMKII causes adverse metabolic reprogramming and dilated cardiomyopathy

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Despite the clear association between myocardial injury, heart failure and depressed myocardial energetics, little is known about upstream signals responsible for remodeling myocardial metabolism after pathological stress. Here, we report increased mitochondrial calmodulin kinase II (CaMKII) activation and left ventricular dilation in mice one week after myocardial infarction (MI) surgery. By contrast, mice with genetic mitochondrial CaMKII inhibition are protected from left ventricular dilation and dysfunction after MI. Mice with myocardial and mitochondrial CaMKII overexpression (mtCaMKII) have severe dilated cardiomyopathy and decreased ATP that causes elevated cytoplasmic resting (diastolic) Ca2+ concentration and reduced mechanical performance. We map a metabolic pathway that rescues disease phenotypes in mtCaMKII mice, providing insights into physiological and pathological metabolic consequences of CaMKII signaling in mitochondria. Our findings suggest myocardial dilation, a disease phenotype lacking specific therapies, can be prevented by targeted replacement of mitochondrial creatine kinase or mitochondrial-targeted CaMKII inhibition.
Heart failure is a leading cause of death worldwide, and failing myocardium is marked by energetic defects. Loss of creatine kinase and decreased mitochondrial respiration are common findings in myocardium after pathological injury, including myocardial infarction (MI), a major cause of heart failure. Although failing hearts have reduced energy reserves, little is known about upstream control points contributing to decreased energy metabolism, nor the specific structural and functional consequences of these defects on myocardial responses to injury. MI triggers a complex set of changes in myocardium, including cardiomyocyte death and cardiac fibrosis, hypertrophy, and chamber dilation. Collectively, these changes are termed adverse structural remodeling. Much is known about cellular signals contributing to myocardial hypertrophy, fibrosis, and myocyte death, whereas relatively little is known about molecular signals causing cardiac dilatation.

Although hypertrophy has been proposed to be a precondition for myocardial chamber dilation, the connection between myocardial hypertrophy and cardiac chamber dilation is uncertain. The nature of the relationship between myocardial hypertrophy and cardiac chamber dilation is important clinically because current therapies are inadequate for treating or preventing pathological cardiac chamber dilation. Emergent evidence indicates that defects in mitochondrial function and energy depletion may contribute to heart failure and dilated cardiomyopathy. The multifunctional Ca²⁺ and calmodulin-dependent protein kinase II (CaMKII) is a pluripotent signal for promoting cardiomyopathy. Mouse models of myocardial CaMKII overexpression, without subcellular targeting, show activation of myocardial hypertrophy gene programs, increased cell death, fibrosis, chamber dilation, and premature death. Excessive CaMKII activity causes myocardial hypertrophy by catalyzing phosphorylation of HAD4, leading to cytoplasmic partitioning of HAD4, and derepression of hypertrophic transcriptional programs. CaMKII promotes myocardial death via multiple pathways, including induction of pro-apoptotic genes through NF-κB activation, defective DNA repair, promoting cytosolic Ca²⁺ overload, and triggering mitochondrial permeability transition pore opening. CaMKII also promotes dilated cardiomyopathy, but the mechanism is unknown. CaMKII is present in the mitochondrial fraction of heart and vascular lysates, and is found in the mioplast fraction, but responses to sustained, pathological mitochondrial activation in heart are unknown.

Here, we identify increased mitochondrial CaMKII activation in failing mouse hearts 1 week after myocardial infarction surgery, and find that mouse hearts with myocardial and mitochondrial CaMKII inhibition, due to transgenic expression of a potent, and selective CaMKII inhibitor polypeptide (mtCaMKIIN), are protected from left ventricular dilation and dysfunction 1 week after myocardial infarction. We develop a genetic mouse model with myocardial- and mitochondrial-targeted CaMKII overexpression, and find that excessive mitochondrial CaMKII causes dilated cardiomyopathy, without myocardial hypertrophy or death, by reducing expression of assembled complex I and the mitochondrial isoform of creatine kinase (CKmito). Genetic replacement of CKmito but not cytosolic myofibrillar creatine kinase (CK-M) is sufficient to rescue myocardial energetics, restore intracellular Ca²⁺ homeostasis, and prevent dilated cardiomyopathy. Mitochondrial CaMKII overexpression coherently affects TCA cycle dehydrogenases, causing increased phosphorylation, activity and heightened production of NADH, an essential electron donor for oxidative phosphorylation. These findings show that increased mitochondrial CaMKII can trigger dilated cardiomyopathy, independent of myocardial hypertrophy and cell death, by depressing energetics.

Our findings suggest that mitochondrial CaMKII selectively contributes to cardiac chamber dilation, potentially in acquired forms of myocardial injury with high importance to public health, and that dilated cardiomyopathy can be prevented by therapies capable of restoring mitochondrial energy metabolism.
between WT and mtCaMKII hearts were sustained for 3 weeks after surgery, suggesting mitochondrial CaMKII participated in structural remodeling on a subacute or chronic timescale (Fig. 1c). In order to verify that mitochondrial CaMKII activity was decreased in the mtCaMKII hearts, we quantified pMCU and found a trend for reduced phosphorylation of this CaMKII target 1 week following MI (Supplementary Fig. 1d). These data further support the role of mitochondrial CaMKII in structural remodeling following cardiac injury.

CaMKII has been shown to contribute to cardiac inflammation following ischemic injury. In order to determine if inhibition of mitochondrial CaMKII reduces inflammation after MI, we performed immunoblotting for Cox8a, CoxiVFlag merge, α-MHC promoter Flag, HGH1 polyA, and showed that WT mtCaMKII expression is significantly lower than that of WT sham. These findings further support the role of mitochondrial CaMKII in structural remodeling following cardiac injury.
MI, we measured chemokine expression and leukocyte infiltration 1 week after coronary ligation. We did find evidence of increased inflammation after MI surgery, but found no difference in CCL2 or CCL3 mRNA expression (Supplementary Fig. 1e), nor CD45+ cell infiltration (Supplementary Fig. 1f) in mtCaMKIIN hearts compared to WT littermates. These data suggest that inhibition of mitochondrial CaMKII does not affect the inflammatory response in this model of injury.

Increased mitochondrial CaMKII causes dilated cardiomyopathy. To directly test the effects of increased CaMKII activity in cardiac mitochondria, we developed a mouse model with chronic transgenic overexpression of CaMKII targeted to myocardial mitochondrial matrix (mtCaMKII). We fused the CaMKIIδ cDNA to a Cox8a mitochondrial localization sequence and a flag epitope tag, and expressed this construct under the control of the α-myosin heavy chain promoter (Fig. 1d). Cardiac myocytes isolated from mtCaMKII mice showed flag-CaMKII localized primarily with a mitochondrial marker (CoxIV) (Fig. 1e). The mtCaMKII mice developed dilated cardiomyopathy with reduced left ventricular ejection fraction, increased left ventricular volumes, and increased left ventricular wall thickness (Fig. 1f, g). We observed modest cardiac hypertrophy in the mtCaMKII mice compared to WT littermates, as measured by heart weight normalized to body weight (Fig. 1g, non-normalized data shown in Supplementary Fig. 2a), but cardiac myocyte cross-sectional area was not different in mtCaMKII hearts compared to WT littermate hearts (Supplementary Fig. 2b, c). Left ventricular dilation was apparent by 2 weeks of age, and preceded detectable hypertrophy in mtCaMKII hearts, compared to WT littermate controls (Fig. 1h). Mitochondrial ultrastructural integrity scoring25 (Fig. 1i, j), and abundance, reflected by the ratio of mitochondrial to nuclear DNA (Fig. 1k), were similar compared to WT littermate controls. These data show that mtCaMKII mice develop a severe dilated cardiomyopathy with minimal myocardial hypertrophy.

Mitochondrial CaMKII does not affect myocardial hypertrophy. In contrast to the dilated cardiomyopathy and near normal lifespan in mtCaMKII mice (Fig. 1l), myocardial CaMKII overexpression in the absence of subcellular targeting causes marked cardiac hypertrophy, left ventricular dilation, increased myocardial cell death, cardiac fibrosis, heart failure, and premature death12,13. The striking differences in the phenotypes of mtCaMKII, and mice with transgenic mitochondrial CaMKII overexpression lacking subcellular targeting appeared to confirm a specific role for mitochondrial CaMKII in dilated cardiomyopathy. However, while the Cox8a was effective in predominantly targeting CaMKII overexpression to myocardial mitochondria, we also detected small amounts of mtCaMKII in extramitochondrial compartments (Fig. 2a). To determine if extra-mitochondrial mtCaMKII contributed to cardiomyopathy in mtCaMKII mice, we crossed the mtCaMKII mice with mice expressing AC3-I, a CaMKII inhibitory peptide that is fused to enhanced green fluorescent protein (EGFP)22, and excluded from mitochondria (Fig. 2b). The double transgenic model, combining mitochondrial-targeted CaMKII overexpression and extra-mitochondrial CaMKII inhibition, showed persistent left ventricular dysfunction (see left ventricular ejection fraction) and dilation (see left ventricular end-systolic and diastolic volumes), but loss of left ventricular hypertrophy (see heart weight/body weight), compared to the mtCaMKII mice (Fig. 2c). The partial improvement of left ventricular ejection fraction in the mtCaMKII x AC3-I interbred mice suggested that mitochondrial and extramitochondrial CaMKII each have the potential to decrease myocardial function, but the unchanged end-diastolic volumes indicate that mitochondrial CaMKII exclusively promotes dilated cardiomyopathy, and not myocardial hypertrophy. We next crossed the mtCaMKII mice with mtCaMKII mice25. The coexpression of mtCaMKII reversed the dilated cardiomyopathy phenotype in the mtCaMKII mice (Fig. 2d), without reducing mtCaMKII expression (Fig. 2e, f), confirming that dilated cardiomyopathy is due to excessive mitochondrial CaMKII activity, and excluding the possibility that dilated cardiomyopathy is a non-specific consequence of mitochondrial-targeted transgenic protein overexpression. Considered together with previously published information13, these data suggest that the dilated phenotype of the mtCaMKII heart is due to increased CaMKII activity in mitochondria, while myocardial hypertrophy and premature death are predominantly related to extra-mitochondrial actions of CaMKII.

Normal mitochondrial Ca2+ in mtCaMKII hearts. CaMKII plays a prominent role in myocardial biology, in part, by enhancing activity of intracellular Ca2+ homeostatic proteins11. Mitochondrial-targeted CaMKII inhibition, in mtCaMKIIIN mice, protects against acute responses to myocardial injury by reducing cardiomyocyte death, Ca2+ overload, and loss of ΔΨm (ref. 25). Mitochondria isolated from the hearts of mtCaMKII mice showed significantly increased pMCU (Fig. 2g, h), suggesting excess phosphorylation at these sites in mtCaMKII compared to WT MCU. Based on these findings, we initially anticipated that
Fig. 2 mtCaMKII does not affect hypertrophy, MCU, mitochondrial Ca\(^{2+}\), ROS, nor \(\Delta \Psi_{\text{mito}}\). a Western blot for flag-CaMKII, VDAC1, and GAPDH in cytoplasm and mitochondria fractions from WT (n = 4) and mtCaMKII hearts (n = 4). b Quantification AC3-I-GFP fusion protein expression in cytoplasm and mitochondria from WT and AC3-I mouse hearts (n = 3). c Summary data for echocardiographic measurements from WT (n = 16), AC3-I (n = 13), mtCaMKII (n = 18), and mtCaMKII x AC3-I (n = 12) mice. d Summarized echocardiographic measurement data from WT (n = 12), mtCaMKIIIN (n = 8), mtCaMKII (n = 10), and mtCaMKII x mtCaMKIIIN (n = 10) mice. e Western blot and f summary data for flag-CaMKII normalized to GAPDH in mtCaMKII (n = 4) and mtCaMKII x mtCaMKIIIN (n = 4) hearts. g Western blot and h summary data for phosphorylated MCU normalized to total MCU in mitochondria from WT (n = 4) and mtCaMKII (n = 4) hearts. i Mitochondrial Ca\(^{2+}\) uptake assay with cell membrane permeabilized adult ventricular myocytes from WT (n = 3) and mtCaMKII (n = 3) mice; arrows indicate addition of Ca\(^{2+}\). j Rate of Ca\(^{2+}\) uptake in permeabilized myocytes calculated from the first 3 peaks of Ca\(^{2+}\) addition and total Ca\(^{2+}\) uptake before mPTP opening in WT (n = 3) and mtCaMKII (n = 3) hearts. k Total mitochondrial Ca\(^{2+}\) content normalized to total protein in isolated mitochondria from WT (n = 10) and mtCaMKII (n = 10) hearts. l Mitochondrial membrane potential (\(\Delta \Psi_{\text{mito}}\)) measured with TMRM under state 2 (substrate alone) and state 3 (substrate plus ADP) respiration in isolated mitochondria from WT (n = 4) and mtCaMKII (n = 4) hearts. Data are represented as mean ± SEM, significance was determined using a two-tailed Student’s t test or one-way ANOVA with Tukey’s multiple comparisons test. ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05, NS = not significant. Source data, including exact p values, are provided as a Source data file.
dilated cardiomyopathy in mtCaMKII mice was related to mitochondrial Ca\(^{2+}\) overload, loss of \(\Delta \Psi_{\text{mito}}\) and increased myocardial death.

To test if mitochondrial Ca\(^{2+}\) entry was enhanced in mtCaMKII mitochondria, we measured MCU-mediated mitochondrial Ca\(^{2+}\) uptake, and mitochondrial Ca\(^{2+}\) content. We found that mitochondria from mtCaMKII and WT littermate control hearts had similar Ca\(^{2+}\) uptake rates (Fig. 2i, j), and no differences in mitochondrial Ca\(^{2+}\) content (Fig. 2k). In order to determine whether the mitochondrial transition pore contributed to the apparent rate of mitochondrial Ca\(^{2+}\) uptake, we also included cyclosporine A (CsA), an inhibitor of the mPTP, in the Ca\(^{2+}\) uptake assays (Fig. 2i, j). We found an increase in the apparent rate of Ca\(^{2+}\) uptake in WT mitochondria, and a modest reduction in apparent rate of Ca\(^{2+}\) uptake in mtCaMKII compared to WT mitochondria in the presence of CsA, suggesting a reduction in mPTP activity in the mtCaMKII mitochondria. Taken together, these studies supported a view contrary to our starting hypothesis: that chronic mitochondrial CaMKII overexpression did not cause dilated cardiomyopathy by disrupting mitochondrial Ca\(^{2+}\) homeostasis. Furthermore, mtCaMKII mice did not exhibit increased mitochondrial ROS (Fig. 2l), cardiomyocyte TUNEL staining (Fig. 2m), or fibrosis (Fig. 2n), and had similar \(\Delta \Psi_{\text{mito}}\) compared to WT littermate controls (Fig. 2o). Measuring mitochondrial ROS is technically challenging\(^{43}\), so we considered the possibility that our studies were inadequate to detect differences in ROS between WT and mtCaMKII hearts, potentially of a magnitude to contribute to the dilated cardiomyopathy in mtCaMKII mice. In order to more decisively determine if elevated ROS contributed to mtCaMKII cardiomyopathy, we took an orthogonal approach, and interbred mtCaMKII mice with mice transgenically overexpressing a mitochondrial-targeted form of catalase (mCat), an enzyme that decomposes H\(_2\)O\(_2\) into H\(_2\)O and O\(_2\). The mCat mice are resistant to ROS mediated mitochondrial disease\(^{44}\). However, the mtCaMKII x mCat interbred mice were not protected against left ventricular dilation, nor depressed ejection fraction compared to mtCaMKII mice (Supplementary Fig. 3a). Finally, Nnt is mutated in the C57Bl/6\(^{e}\) strain we used\(^{45}\), suggesting that the left ventricular dilation in mtCaMKII cardiomyopathy phenotype may be artificially disconnected from ROS generation\(^{46}\). In order to examine this possibility, we back crossed mtCaMKII mice for five generations into the CD1 background that expresses WT Nnt. The mtCaMKII mice in the C57Bl/6\(^{e}\) and CD1 genetic backgrounds exhibited similar left ventricular dilation (Supplementary Fig. 3b), suggesting lack of increased ROS generation in mtCaMKII hearts was not due to loss of Nnt. Thus, in vitro and in vivo data strongly suggested that mtCaMKII cardiomyopathy was not a consequence of elevated ROS. Given the lack of effect of increased mitochondrial CaMKII activity on myocyte survival, mitochondrial Ca\(^{2+}\), ROS, or \(\Delta \Psi_{\text{mito}}\) activators and measures of the mitochondrial transition pore (mPTP) opening, we did not anticipate mPTP was involved in the dilated cardiomyopathy present in mtCaMKII hearts. However, to directly test for increased mPTP opening in mtCaMKII hearts in vivo we interbred mtCaMKII mice with mice lacking cyclophilin D (Ppif\(^{-/-}\)), an mPTP subunit that is involved in pore opening\(^{47}\). The Ppif\(^{-/-}\) mice are protected from mPTP opening\(^{48}\). Consistent with our data up to this point, the Ppif\(^{-/-}\) x mtCaMKII interbred mice were not protected against dilated cardiomyopathy (Supplementary Fig. 3c). Taken together, these in vitro and in vivo results strongly argued against defective mitochondrial Ca\(^{2+}\) or its consequences as a cause of cardiomyopathy in mtCaMKII mice.

**Impaired cytoplasmic Ca\(^{2+}\) homeostasis in mtCaMKII hearts.** Energy deficiency may contribute to dilated cardiomyopathy\(^{49}\), but little is known about specific upstream molecular signals initiating metabolic insufficiency. We initially found that the ATP concentration was significantly reduced in myocardial mitochondria lysates from mtCaMKII compared to WT littermate control hearts (Fig. 3a). We next used 3\(^1\)P magnetic resonance (MR) spectroscopy to measure myocardial high-energy phosphates in vivo\(^{50,51}\). Cardiac ATP and creatine phosphate concentrations were significantly reduced, by 30–45\%, in mtCaMKII mice as compared to WT controls (Fig. 3b, c). MR imaging confirmed dilated cardiomyopathy (Fig. 3d, Supplementary Movie 1), similar to our echocardiographic findings (Fig. 1g).

Together, these results show a significant reduction in ATP in mtCaMKII hearts, in vitro and in vivo, and are consistent with the hypothesis that impaired myocardial function in mtCaMKII mice is a consequence of energy deficiency.

Cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) controls cardiac contraction and relaxation, and [Ca\(^{2+}\)]\(_{i}\), homeostasis represents a major physiological energy cost. We next measured [Ca\(^{2+}\)]\(_{i}\), in ventricular myocytes isolated from mtCaMKII and WT hearts in response to increasing stimulation frequencies. Contrary to our expectations, these initial studies showed a decrease in resting [Ca\(^{2+}\)]\(_{i}\), in cardiomyocytes isolated from mtCaMKII and WT hearts (Supplementary Fig. 4a) consistent with previous findings in hearts with CaMKII overexpression\(^{52}\). We next considered that increased cytoplasmic CaMKII seen in mtCaMKII cardiomyocytes (Fig. 2a) could mask effects of ATP deficiency on [Ca\(^{2+}\)]\(_{i}\), in isolated, mechanically unloaded cardiomyocytes, potentially because energy requirements were too low to reveal differences. In order to test this idea, we repeated the [Ca\(^{2+}\)]\(_{i}\), measurements in isolated ventricular myocytes dialyzed with AIP, a CaMKII inhibitory peptide. The AIP peptide was confined to the cytoplasmic compartment by the membrane permeation epitope and was dialyzed across the cell membrane using a microelectrode in whole-cell mode patch-clamp configuration. AIP dialysis had no effect on resting diastolic [Ca\(^{2+}\)]\(_{i}\), in WT cardiomyocytes, but resulted in a significant, stimulation frequency dependent elevation in diastolic [Ca\(^{2+}\)]\(_{i}\), (Fig. 3e, f), unchanged peak Ca\(^{2+}\) amplitude (Supplementary Fig. 4b) and slower Ca\(^{2+}\) decay (Supplementary Fig. 4c) in mtCaMKII cardiomyocytes, consistent with the hypothesis that incompletely targeted mtCaMKII preserved cytoplasmic [Ca\(^{2+}\)]\(_{i}\) homeostasis in ventricular myocytes isolated from mtCaMKII hearts. In order to test if the elevated [Ca\(^{2+}\)]\(_{i}\), in mtCaMKII cardiomyocytes was a consequence of deficient ATP, we fortified the micropipette dialysate with ATP (5 mM) to approximate physiological cytoplasmic ATP activity\(^{53}\). ATP dialysis reduced diastolic [Ca\(^{2+}\)]\(_{i}\), in WT cardiomyocytes, but resulted in no effect on diastolic [Ca\(^{2+}\)]\(_{i}\), in WT control cardiomyocytes (Fig. 3e, f). We interpreted the data up to this point as supporting a model where impaired energetics in mtCaMKII hearts caused defective intracellular [Ca\(^{2+}\)]\(_{i}\) homeostasis, a cardinal feature of myocardial dysfunction.

**CKmito prevents dilated cardiomyopathy in mtCaMKII mice.** Loss of creatine kinase activity is a common finding in failing human myocardium and in animal models of heart failure\(^{53,54}\). Creatine kinase maintains energetics by rapidly and reversibly transferring a high-energy phosphoryl moiety between ATP and creatine phosphate, a highly diffusible high-energy phosphate molecule important for cellular energy homeostasis. Overexpression of CK-M, the cytosolic myofibrillar isoform of creatine kinase, in several models of heart failure improves overall cardiac function and survival\(^{55,56}\), but the potential role of CKmito, the mitochondrial isoform, was only recently shown to improve myocardial response to injury. We considered that loss of CKmito could be important to the dilated cardiomyopathy in mtCaMKII
mice, as it associates with the ATP transporter on the mitochondrial intermembrane space, and enhances energy transfer from the mitochondria to the cytosol57. Furthermore, CKmito was hyperphosphorylated in mtCaMKII compared to WT mitochondria (KCRS, Supplementary Table 1). We measured expression of myofibrillar, and mitochondrial creatine kinase isoforms in WT and mtCaMKII hearts with antibodies we validated to be specific for each isoform location (Supplementary Fig. 5a), and found a trend ($P = 0.06$) for reduced CKmito in mtCaMKII compared to WT (Fig. 4a, b). In contrast, the expression of CK-M was similar in WT and mtCaMKII hearts (Fig. 4c, d). However, we did not measure a reduction in CKmito 1 week after MI surgery compared to sham-operated WT mice (Supplementary Fig. 5b), suggesting mitochondrial CaMKII overexpression in mtCaMKII hearts is a more potent upstream signal for reducing CKmito than MI under these conditions. In order to test whether ATP loss and reduced CKmito contributed to the dilated phenotype in the mtCaMKII hearts, we attempted to recover myocardial energetics by interbreeding mtCaMKII mice with mice overexpressing CKmito in myocardium. CKmito was significantly overexpressed at a similar level in both the CKmito and mtCaMKII x CKmito interbred mice (Fig. 4e, f). The mtCaMKII x CKmito interbred mice showed improved in vivo ATP and creatine phosphate levels (Fig. 4g), reduced diastolic $[Ca^{2+}]_i$ in isolated myocytes (Fig. 4h), and exhibited significantly reduced left ventricular dilation compared to mtCaMKII littermates (Fig. 4i and Supplementary Movie 1). Importantly, the recovery of myocardial function and energetics in the mtCaMKII x CKmito mice occurred without a reduction in mitochondrial CaMKII overexpression (Fig. 4j, k). In contrast, interbreeding mtCaMKII mice with transgenic mice overexpressing the cytosolic CK-M did not rescue dilated cardiomyopathy (Supplementary Fig. 5c), but rather worsened adverse structural remodeling compared to mtCaMKII hearts. These data show that defective energetics and dilated cardiomyopathy in the mtCaMKII mice with mice overexpressing CKmito in myocardium. CKmito was significantly overexpressed at a similar level in both the CKmito and mtCaMKII x CKmito interbred mice (Fig. 4e, f). The mtCaMKII x CKmito interbred mice showed improved in vivo ATP and creatine phosphate levels (Fig. 4g), reduced diastolic $[Ca^{2+}]_i$ in isolated myocytes (Fig. 4h), and exhibited significantly reduced left ventricular dilation compared to mtCaMKII littermates (Fig. 4i and Supplementary Movie 1). Importantly, the recovery of myocardial function and energetics in the mtCaMKII x CKmito mice occurred without a reduction in mitochondrial CaMKII overexpression (Fig. 4j, k). In contrast, interbreeding mtCaMKII mice with transgenic mice overexpressing the cytosolic CK-M did not rescue dilated cardiomyopathy (Supplementary Fig. 5c), but rather worsened adverse structural remodeling compared to mtCaMKII hearts. These data show that defective energetics and dilated cardiomyopathy in the mtCaMKII mice can be significantly reversed by CKmito replacement. These findings provide evidence that myocardial dilation can be a metabolic consequence of excessive mitochondrial CaMKII

Fig. 3 mtCaMKII hearts are ATP deficient. a ATP content in mitochondria isolated from WT ($n = 9$) and mtCaMKII ($n = 9$) hearts. b Example MRI images and corresponding $^{31}$P MR spectra and c summary data for ATP and creatine phosphate (PCr) quantification from in vivo $^{31}$P MR spectroscopy in WT ($n = 9$) and mtCaMKII ($n = 8$) hearts. d Summary data from MRI measurements of WT ($n = 9$) and mtCaMKII ($n = 8$) hearts; see Supplementary Movie 1 for MR Cines. e Example intracellular $[Ca^{2+}]$ signals recorded from Fura-2 and f summary data for diastolic (resting) $[Ca^{2+}]_i$ from isolated ventricular myocytes stimulated at 1, 3, or 5 Hz (WT $n = 12$; WT + ATP $n = 14$; mtCaMKII $n = 15$; mtCaMKII + ATP $n = 19$) (WT cells were isolated from 2 heart, mtCaMKII cells were isolated from 3 hearts). Data are represented as mean ± SEM, significance was determined using a two-tailed Student’s t test or one-way ANOVA with Tukey’s multiple comparisons test. ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. Source data, including exact $p$ values, are provided as a Source data file.

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activity leading to ATP deficiency, and suggest that chronic ATP deficiency can result in dilated cardiomyopathy that is potentially preventable or reversible.

**mtCaMKII decreases complex I expression and activity.** Loss of electron transport chain (ETC) complex components are thought to drive reduced metabolic capacity in some genetic and acquired forms of heart failure. With this in mind, we next evaluated the ETC to determine if reduced ATP production in mtCaMKII hearts was associated with loss of ETC complex expression and/or function. We interrogated ETC complex expression in heart lysates, and found a significant decrease in protein expression of a complex I component (NDUFB8), and a significant increase in

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**Fig. 4 CKmito replacement rescues ATP deficiency in mtCaMKII hearts.**

- **a** Western blots and **b** summary data for CKmito expression normalized to CoxIV from WT (n = 5) and mtCaMKII (n = 5) hearts.
- **c** Western blot and **d** summary data for CK-M expression normalized to α-actinin from WT (n = 5) and mtCaMKII (n = 5) hearts.
- **e** Western blot and **f** summary data for CKmito expression normalized to VDAC1 from WT (n = 7), CKmito (n = 7), mtCaMKII (n = 7), and mtCaMKII x CKmito (n = 6) hearts.
- **g** In vivo ATP (left) and PCr (right) quantification from 31P MR spectroscopy in WT (n = 9), CKmito (n = 7), mtCaMKII (n = 8), and mtCaMKII x CKmito (n = 7) hearts.
- **h** Summary data for diastolic [Ca²⁺] measurements made with Fura-2-loaded ventricular myocytes stimulated at 1, 3, and 5 Hz (WT n = 12; CKmito n = 17; mtCaMKII n = 15; mtCaMKII x CKmito n = 13 ventricular myocytes isolated from 2 hearts/group).
- **i** Summary data from in vivo MRI measurements in WT (n = 9), CKmito (n = 7), mtCaMKII (n = 8), and mtCaMKII x CKmito (n = 7) hearts.

WT and mtCaMKII data are the same as in Fig. 3. Student’s t test or one-way ANOVA with Tukey’s multiple comparisons test. ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05. Source data, including exact p values, are provided as a Source data file.
protein expression of a complex II component (SDHB) in mtCaMKII compared to WT hearts (Fig. 5a, b). Because these complexes are made up of many proteins, we quantified assembled complexes and measured complex activity using native gel electrophoresis. We found a significant decrease in assembled complex I (Fig. 5c) and in complex I activity (Fig. 5d) in mtCaMKII mitochondria compared to WT. Based on the apparent decrease in complex I and increase in complex II in mtCaMKII hearts, we measured oxygen consumption rates of isolated mitochondria under conditions selective for complex I (pyruvate/malate) and complex II (succinate/rotenone) metabolism. We observed a decrease in state 3 (ADP activated)
respiration in the presence of complex I substrates (pyruvate/malate) (Fig. 5e), and an increase in state 3 respiration in the presence of complex II substrates (succinate/rotenone) (Fig. 5i) in the mtCaMKII mitochondria compared to WT, consistent with protein expression and activity data. We next repeated the measurement of complex I expression in mtCaMKII mitochondria rescued by CKmito overexpression. We found a partial recovery of expression of a complex I component in mtCaMKII x CKmito compared to mtCaMKII mitochondria (Fig. 5g, h), but no recovery of complex I activity (Fig. 5i). We interpret these data to collectively indicate that the reduction in ATP production in mtCaMKII hearts is a consequence of extensive metabolic remodeling that can be substantially corrected by a modest replacement of CKmito.

Augmented TCA cycle dehydrogenases in mtCaMKII hearts. To more broadly interrogate potential metabolic targets for CaMKII in mitochondria we performed a mass spectrometry-based analysis of phosphorylated peptides enriched from myocardial mitochondria from mtCaMKII and WT littermate hearts (see Methods). We identified 38 proteins exhibiting significantly increased phosphorylation in the mtCaMKII mice compared to the WT controls. Most notably, 22 of these proteins were involved in metabolism and energy production (Supplementary Table 1). The abundance of these targets, together with our finding that mtCaMKII have impaired energetics, added further support to our earlier results showing that CaMKII is involved in regulating mitochondrial metabolism.

We were intrigued by a pattern of increased phosphorylation of pyruvate dehydrogenase, and key tricarboxylic acid (TCA) cycle dehydrogenases in the mtCaMKII hearts (Supplementary Table 1). We next asked if this hyperphosphorylation was associated with a change in activity of these enzymes. The activity of pyruvate dehydrogenase (Fig. 6a), α-ketoglutarate dehydrogenase, fumarase, and malate dehydrogenase (Fig. 6b) were significantly increased in mtCaMKII compared to WT mitochondria, and there was a trend toward increased activity in all TCA cycle enzymes in mtCaMKII compared to WT mitochondria. These findings suggested that mtCaMKII operated, at least in part, to augment metabolism through TCA cycle activity, possibly providing insight into acute metabolic benefits of physiological increases in mitochondrial CaMKII activity. Because the TCA cycle operates to produce NADH, the major electron source for ATP production by oxidative phosphorylation, we measured NADH in ventricular myocytes isolated from mtCaMKII and WT hearts (Fig. 6c). Consistent with augmented TCA cycle activity, resting NADH was increased in mtCaMKII compared to WT cardiomyocytes (Fig. 6d). However, pacing induced NADH increases were only present in cardiomyocytes isolated from WT mice. In contrast, increased pacing frequency caused a reduction in NADH in mtCaMKII cardiomyocytes (Fig. 6d), suggesting that the resting production of NADH was augmented, but lacked capacity to increase NADH production during rapid stimulation. Intriguingly, and in contrast to other models of cardiomyopathy with loss of complex I, we did not detect a significant deficiency of NADH in cardiac lysates (Fig. 6e).

Modeling mtCaMKII and metabolism. We performed analysis of mitochondrial metabolism using a modified computational model simulating the cardiac cycle using pulsatile functions for ATP hydrolysis and cytosolic calcium transients (see Methods) (Fig. 7a). The effect of mtCaMKII was simulated by reduced CKmito activity and complex I respiration, and increased TCA enzyme activities, as observed in experimental measurements (Supplementary Table 2). This mathematical simulation showed decreased ATP and creatine phosphate levels in mtCaMKII, and replacement of CKmito produced a substantial rescue of these parameters (Fig. 7b and Supplementary Fig. 6), similar to our experimental observations. We interpret the consistency between these simulated and experimental outcomes to suggest that the combination of reduced CKmito and complex I activity are sufficient to cause decreased ATP, despite enhanced pyruvate dehydrogenase and TCA cycle activity, and that ATP deficiency can be repaired by CKmito replacement in the presence of excessive mtCaMKII.

Discussion

The association between defective ATP metabolism and heart failure has been recognized for many years and diminished expression of creatine kinase and complex I have been noted in animal models, and in failing human hearts. Despite this, there has been uncertainty whether failed energetics are a cause or a consequence of damaged myocardium. Thus, lack of understanding of upstream signals controlling energetics and cardiomyopathy constitutes an important knowledge gap. Our study provides evidence that mitochondrial CaMKII can orchestrate pathological metabolic remodeling and left ventricular dilation. mtCaMKII hearts are deficient in ATP (Fig. 3c), dilated and manifest contractile dysfunction and deranged Ca2+ homeostasis, but are free of hypertrophy and fibrosis. We acknowledge that the derangement in energetics could be secondary to the severe pathology we observed in the mtCaMKII hearts since we were unable measure ATP in hearts prior to the development of...
these pathological phenotypes due to technical limitations. However, excessive mitochondrial CaMKII activity is almost certainly the inciting trigger due to the nature of mitochondrial-targeted CaMKII overexpression in this model. In contrast to mice with untargeted myocardial CaMKII overexpression, the mtCaMKII do not show a high rate of premature death, despite depressed energetics and cardiac chamber dilation. Restoration of a single enzyme, CKmito, by interbreeding mtCaMKII with CKmito transgenic mice, substantially repaired energetics and cardiac dysfunction, and restored \([\text{Ca}^{2+}]\) homeostasis (Fig. 4g–i). In addition, \([\text{Ca}^{2+}]\) homeostasis can be restored by supplementing ATP directly to the cytosol of myocytes (Fig. 3e, f).

These findings are corroborated by our computational modeling where replacing CKmito activity improved ATP levels in the
Fig. 6 mtCaMKII activates TCA cycle dehydrogenases and augments resting NADH. a Pyruvate dehydrogenase (PDH) activity in isolated mitochondria from WT (n = 7) and mtCaMKII (n = 9) hearts. b TCA cycle enzyme activities in permeabilized mitochondria from WT (n = 7) and mtCaMKII (n = 9) hearts, CS: citrate synthase, IDH: isocitrate dehydrogenase, α-KGDH: α-ketoglutarate dehydrogenase, SL: succinate-CoA ligase, SDH: succinate dehydrogenase, MDH: malate dehydrogenase. c Representative NADH imaging of isolated ventricular myocytes before pacing (calibration bar = 50 μm). d Quantification of NADH autofluorescence in isolated ventricular myocytes before and after pacing from WT (n = 29 myocytes from two hearts) and mtCaMKII (n = 52 myocytes from three hearts) hearts. Maximum (100%) NADH was measured in the presence of FCCP. Cells from the same genotype were paired in comparisons of before and after pacing. e Quantification of NAD+ and NADH in hearts from WT (n = 4) and mtCaMKII (n = 4) mice. f Western blot and summary data for acetylated proteins normalized to VDAC1 in isolated mitochondria from WT (n = 4) and mtCaMKII (n = 4) hearts. Data are represented as mean ± SEM, significance was determined using a two-tailed Student’s t test. **P < 0.01, *P < 0.05. Source data, including exact p values, are provided as a Source data file.

Fig. 7 Computational modeling of energetics. a Schematic image of key parameters used in a three compartment model of CaMKII, creatine kinase (CK), and mitochondrial energetics. b Computer simulation results were compared in WT, CKmito, mtCaMKII, and mtCaMKII x CKmito interbred conditions. The ratios of PCr/ATP and ATP/ADP were calculated at the end of 5 min cardiac cycle (simulated using pulsatile functions to oscillate ATP hydrolysis and cytosolic calcium).

mtCaMKII model (Fig. 7 and Supplementary Fig. 6). These data suggest that the lack of adequate energy can directly lead to a stable pattern of ventricular dilation and contractile dysfunction, and restoring myocardial energy supply significantly improves cardiac function, despite persistence of excessive mitochondrial CaMKII activity.

Our computational modeling confirmed that ATP deficiency in mtCaMKII hearts is also partly a consequence of loss of complex I activity, and persists despite hyperactivation of the TCA cycle, and increased NADH. Our phosphoproteomic studies identified a site for PDH phosphorylation that is different from the well-known inhibitory sites regulated by mitochondrial calcium71. We observed enhanced PDH activity in the mtCaMKII hearts, but further investigation will be required to know if this phosphorylation site contributes to PDH activation under conditions of excessive CaMKII activity. Diminished complex I content is noted in ischemic and genetic forms of cardiomyopathy68,70. Our computational modeling also identified NDUFB11, a complex I subunit, as a key site for PDH activation under conditions of excessive complex I activity. Loss of mitochondrial CaMKII can selectively drive distinct hypertrophic signaling pathways. Our current findings suggest mitochondrial CaMKII can contribute to pathological injury by remodeling metabolism.

Our results support a view that organelle resident and subcellular specific actions of CaMKII can selectively drive distinct cardiomyopathy phenotypes. Nuclear CaMKII overexpression causes mild myocardial hypertrophy12, and non-targeted CaMKII overexpression leads to massive hypertrophy, myocardial death, fibrosis, chamber dilation, heart failure, deranged Ca2+ homeostasis, and early sudden death13,35,36. Here, we show that targeted mitochondrial CaMKII overexpression causes a surprisingly pure dilated cardiomyopathy linked to adverse metabolic remodeling, and ATP deficiency. Unexpectedly, chronic actions of excessive
mtCaMKII do not cause increased myocardial or sudden death. We have not yet identified a mechanism for CaMKII translocation to mitochondria, but note, similar to the case of CaMKII, that multiple mitochondrial proteins lack canonical TOM/TIM guide sequences. We assume mitochondrial CaMKII is activated by Ca\(^{2+}\)/calmodulin, ROS and O-GlcNAcylation, upstream CaMKII activating signals that are present in mitochondria.

The sequence of compartment specific increases in CaMKII activity in heart following injury are unknown, but our findings suggest that cellular stress pathways, activated by MI, likely contribute to increased mitochondrial CaMKII activity. The potential for CaMKII to operate in various cellular compartments suggests that CaMKII functions as a highly versatile agent, promoting hypertrophy and cardiac chamber dilation, depending on its subcellular location.

Failing human myocardium shows elevated diastolic \([\text{Ca}^{2+}]_c\), and increased cytosolic CaMKII activity, as evidenced by ryanodine receptor hyperphosphorylation and sarcoplasmic reticulum Ca\(^{2+}\) leak. Acute CaMKII inhibition is sufficient to reduce Ca\(^{2+}\) leak, and elevate diastolic \([\text{Ca}^{2+}]_c\), and may prove mechanistic function in failing human myocardial striate. Our studies extend the connections between CaMKII and cellular \([\text{Ca}^{2+}]_c\) homeostasis by demonstrating that loss of cytoplasmic \([\text{Ca}^{2+}]_c\), homeostasis and left ventricular dilation can arise from defective myocardial energetics, independent of increased cytoplasmic CaMKII activity because cardiac chamber dilation persisted in mtCaMKII mice interbred with AC3-I transgenic mice with extramitochondrial CaMKII inhibition (Fig. 2c). Myocardium is highly dependent on a moment to moment balance of ATP production and utilization related, in part, to the high-energy demands of continuous and physiologically changing work. Achieving physiological \([\text{Ca}^{2+}]_c\), homeostasis is a major energetic task for myocardium and elevation of diastolic cytoplasmic \([\text{Ca}^{2+}]_c\) is a common finding in animal heart failure models and in failing human myocardium, conditions where energy supply is insufficient to satisfy demand. The mtCaMKII hearts show a severe loss of ATP and reduction in CKmito and complex I, consistent with a scenario where energy stores are inadequate to support normal function. ATP flux is purposed to specific subcellular compartments, and mitochondrial ATP is primarily responsible for fueling SERCA2a activity that is required to stabilize diastolic cytoplasmic \([\text{Ca}^{2+}]_c\) after each heartbeat. Myofilalement activation and cardiac contraction follow a regenerative release of \(\text{Ca}^{2+}\) to the cytoplasm from the intracellular sarcoplasmic reticulum store, while relaxation and diastole are a consequence of sequestration of cytoplasmic \(\text{Ca}^{2+}\) into the sarcoplasmic reticulum by SERCA2a. We found that decreased ATP in mtCaMKII hearts was associated with elevation of diastolic cytoplasmic \([\text{Ca}^{2+}]_c\), and that elevated \([\text{Ca}^{2+}]_c\) could be repaired in vitro by exogenous ATP and in vivo by replacement of CKmito. We interpret these findings to provide strong support for the hypothesis that mtCaMKII dilated cardiomyopathy arises from ATP deficiency that is exacerbated by CKmito deficiency. We speculate that sustained hyperactivation of mitochondrial CaMKII could contribute to metabolic defects and elevated diastolic \([\text{Ca}^{2+}]_c\) in human heart failure. In addition, others have shown that increases in myocardial ADP and intracellular \([\text{Ca}^{2+}]_c\) can contribute to diastolic dysfunction. While we did not directly quantify diastolic function, this may also be contributing to the phenotype of the mtCaMKII mice.

The core components of adverse cardiac remodeling are myocardial hypertrophy and dilation. However, the biological connections between hypertrophy and dilation are uncertain. One paradigm is that myocardial hypertrophy is a compensatory reaction to pathological stress, but sustained and excessive hypertrophy can progress to chamber dilation over time. Although the interrelationship between hypertrophy and dilation is not completely understood, dilation may be favored by myocyte death interposed upon hypertrophied myocardium that eventually impairs myocardial ability to sustain elevated ventricular wall stress. However, the rate of progression to left ventricular dilation in patients with left ventricular hypertrophy detectable by sonographic imaging is low, suggesting that the processes of hypertrophy and dilation are not tightly coupled, nor necessarily driven by the same biological mechanisms. Our findings regarding mtCaMKII cardiomyopathy indicate that myocardial dilation can arise as a sustained response to myocardial energy deprivation in the absence of myocardial hypertrophy, or death. The cardiac dilation in mtCaMKII hearts is potentially reversible, because it does not involve increases in myocardial death, or fibrosis, and can be rescued by replacement of CKmito. Furthermore, \([\text{Ca}^{2+}]_c\), homeostasis can be repaired in ventricular myocytes isolated from mtCaMKII hearts by addition of ATP. Thus, it is possible that CKmito replacement, or mitochondrial CaMKII inhibition could prevent or reverse some acquired forms of dilated cardiomyopathy, a life-threatening disease phenotype that has been largely considered irreversible.

Many studies have provided evidence affirming a role of excessive CaMKII in myocardial disease. However, relatively less is known about the physiological roles of CaMKII in heart. Surprisingly, CaMKII activity appears dispensable for basal cardiac function, although complete ablation of CaMKIIy and CaMKII\(\delta\) in myocardium causes mild hypertrophy due to loss of a tonic negative regulatory action on calcineurin signaling. We interpret the available evidence to suggest that CaMKII promotes fight or flight responses that increase myocardial performance during extreme physiological stress. Many of these responses are linked to CaMKII catalyzed phosphorylation of Ca\(^{2+}\) homeostatic proteins that enhance the excitation–contraction coupling mechanism. For example, CaMKII may augment the treppe effect in ventricular myocardium, and enhance the dynamic range of heart rate acceleration in cardiac pacemaker cells by catalyzing ryanodine receptor phosphorylation. Our findings showing enhanced TCA cycle activity indicate that mitochondrial CaMKII can increase delivery of NADH to the electron transport chain. While we acknowledge that chronic overexpression of CaMKII creates an artificial condition, and although we currently lack tools to tightly control mitochondrial CaMKII expression or activity on a moment to moment timescale, we speculate that acute, physiological activation of mitochondrial CaMKII could be advantageous by increasing ATP flux from oxidative phosphorylation by enhanced NADH production through the TCA cycle. However, mtCaMKII mice, burdened by chronic, pathological elevation in mitochondrial CaMKII, exhibit loss of CKmito and complex I that prevent this metabolic benefit, and instead contribute to metabolic insufficiency, elevated diastolic cytoplasmic \([\text{Ca}^{2+}]_c\), and severe dilated cardiomyopathy.

**Methods**

**Animal models.** All the experiments were carried out in accordance with the guidelines and approval of Institutional Animal Care and Use Committees at the University of Iowa and Johns Hopkins University (PHS Animal Welfare Assurance, A3021-01 (Univ. Iowa), A3272-01 (JHU)). Mice were housed in a facility with 14 h light/10 h dark cycle at 23 ± 1 °C and 30%–70% humidity. Body temperature is maintained at 37 °C using a rectal thermometer and infrared heating lamp. A left thoracotomy and pericardiotomy is performed, and the left main coronary artery is completely ligated. When a toe pinch confirms proper depth of anesthesia, mice are given a single dose of succinylcholine (2 mg/kg). Body temperature is maintained at 37 °C using a rectal thermometer and infrared heating lamp. A left thoracotomy and pericardiotomy is performed, and the left main coronary artery is completely ligated.

**Myocardial infarction.** Mice are anesthetized with 1.5–2% isoflurane and given a pre-emptive dose of buprenorphine (0.03–0.07 mg/kg). Mice are then intubated and ventilated (100% O\(_2\), 200 μl TV, 120 bpm) using a Harvard Apparatus MiniVent. When a toe pinch confirms proper depth of anesthesia, mice are given a single dose of succinylcholine (2 mg/kg). Body temperature is maintained at 37 °C using a rectal thermometer and infrared heating lamp. A left thoracotomy and pericardiotomy is performed, and the left main coronary artery is completely ligated.
occluded by tying a knot with 7–0 prolene. After verification that MI has occurred (blanching of the tissue distal to the suture), the ribs and skin are closed with 5–0 silk. Once the chest is closed, the mouse remains under isoflurane until succinylcholine is completely metabolized. Isoflurane is turned off, the mouse is allowed to regain respiration on its own, and then extubated. When performing a shart surgery, mice undergo the same procedure, but the ligation of the artery is performed. The resulting TetCKmito mice were bred with aMHC-Tet-off mice to generate compound hetozygous mice as the CKmito transgenic mice. LV EF was calculated from the difference in end-diastolic and end-systolic cavity volumes divided by the end-diastolic volume. For studies.

Echocardiography. Transthoracic echocardiography was performed in unsedated mice using a Vevo 2100 (VisualSonics Inc) system, equipped with a 30–40 MHz linear array transducer. The left ventricle end-diastolic and end-systolic ventricular volumes (EDV, ESV), and the percent ejection fraction (EF) were estimated using the Simpson’s method from the apical two chamber view of the heart. Measurement of EDV, ESV were manually traced according the American Society of Echocardiography. The EF was automatically calculated by the system building software (Vevo Lab 3.1.1) using the following formula: 

\[
EF\% = \frac{EDV - ESV}{100}
\]

The M-mode echocardiogram was acquired from the parasternal short axis view of the left ventricle (LV) at the mid-papillary muscles level and at sweep speed of 200 mm/s. From this view left ventricle mass (LV mass) is calculated from interventricular septal thickness at end of diastole (IVSD), LV chamber diameter at end of diastole (LVEDD), posterior wall thickness at end of diastole (PWTED) measurements using the following formula where 1.055 is the specific gravity of the myocardium:

\[
LV\text{ Mass (mg)} = 1.055 \times (LVSD + LVEDD + PWTED)^3 - (LVEDD)^3
\]

The echocardiographer was blinded to the genetic identity of the mice for all studies.

In vivo murine cardiac MRI/MRS. Anesthesia was induced under 2% isofluorane and maintained under 1% isofluorane and the in vivo studies carried out on a Bruker Biospec MRS/MRI spectrometer equipped with a 4.7 T/70 cm Oxford magnet and a 12 cm (i.d) actively shielded BGA-12TM gradient set, as previously described. For MRI for ventricular size and function, complete set of high temporal and spatial resolution multi-slice cine images were acquired and analyzed as previously reported. LV EF was calculated from the difference in end-diastolic and end-systolic cavity volumes divided by the end-diastolic volume. For 31P MRS, after shamming, a one-dimensional 31P chemical shift imaging (1D-CSI) sequence, using adiabatic excitation pulses to obtain uniform field across the field of view, was acquired as previously described. The PCr and ATP peaks in 31P MR localized spectra were quantified by integrating peak areas. Voxel shifting, a process that shifts the reason to the slice boundaries fractionally, permits the redefinition of the slice boundaries after data collection to align them with the LV border and minimize contamination from the chest wall, using high-resolution 1H images as a guide. Concentrations of PCr and ATP as well as the ratio of PCr/ATP were measured as previously described. The MRI studies were performed without knowledge of the genetic identities of the mice.

Western blot. Heart lysates were prepared in RIPA buffer containing protease and phosphatase inhibitors. Heart lysate or cytoplasm and mitochondria fractions were loaded into 4–12% Bis-Tris NuPAGE gels (ThermoFisher) or 4–15% Mini Protein gels (Bio-Rad). Proteins were transferred to nitrocellulose membrane using Turbo-blotter (Bio-Rad). Primary antibodies (CamKII (Abcam), 1:1000; phospho-CaMKII (Thermo Scientific), 1:1000; VDAC1 (Abcam), 1:2000; FLAG (Rockland), 1:2000; GAPDH (Cell Signaling), 1:10,000; CKmito (Sigma), 1:6000; CoxIV (Cell Signaling), 1:1000; CK-M (Sigma), 1:10,000; α-actinin (Sigma), 1:1000; OxPhos (Abcam), 1:500; Ac13s (Cell Signaling), 1:1000; HA (Sigma), 1:5000; SERCA2a (Babbeda), 1:5000; PDH (Abcam), 1:1000) were incubated with the membrane overnight at 4 °C. Secondary antibodies were incubated with the membrane at room temperature for 1 h. Blots were imaged using an Odyssey Fc Imager (Llicer). Bands were quantified using Image Studio Software (Licor), coomassie-stained membranes were quantified using Image J software.

Mitochondrial isolation. Mitochondria were isolated using homogenization method on ice as previously described. In brief, the heart was minced and homogenized in ice-cold isolation buffer (75 mM sucrose, 225 mM mannitol, 1 mM EGTA, and 0.2% fatty acid free BSA, pH 7.4) using a Potter-Elvehjem glass homogenizer. The homogenates were centrifuged for 10 min at 500 × g. The resulting supernatants were centrifuged for 10 min at 10,000 × g. The mitochondrial pellets were washed twice with isolation buffer and centrifuged at 7700 × g for 6 min each. Mitochondrial pellets were suspended in a small amount of isolation buffer to 5 mg/ml. The protein concentration was determined using the BCA Assay kit (ThermoFisher Scientific).

Mitochondrial ATP measurements. Mitochondrial ATP content was measured using ATP Bioluminescence Assay Kit (Roche Life Science). Isolated mitochondria (15 µg) were suspended in KCl-based buffer at 37 °C. The ATP production was calculated from a standard curve for a series of ATP concentrations.

Mitochondrial Ca2+ content. Total mitochondrial Ca2+ content was measured in isolated mitochondria with the Calcium Assay Kit (Cayman Chemical). Total Ca2+ was normalized to protein content.

Mitochondrial ROS production. Amplex UltraRed, a H2O2 sensitive fluorescent probe, was used to monitor ROS production in isolated mitochondria. Hydrogen peroxide was used to establish the standard curve as previously described. Briefly, 15 µg of isolated mouse heart mitochondria was suspended in 0.2 ml KCl-based buffer solution (137 mM KCl, 0.25 mM EGTA, 2 mM MgCl2, 2 mM KH2PO4, 20 mM HEPES, 5 mM NaCl, pH 7.2) with 4 U/ml horseradish peroxidase, 40 U/ml superoxide dismutase, 10 µM Amplex UltraRed in a 96-well plate, followed by sequential additions of substrate 5 mM pyruvate/malate, complex I inhibitor 0.5 µM rotenone, and complex III inhibitor 1 µM antimycin A. Amplex UltraRed fluorescent signal was read by a microplate reader (BioTek) at 553 nm excitation and 595 nm emission.

Mitochondrial membrane potential. Mitochondrial membrane potential (ΔΨm) was monitored by the ratiometric dye tetramethylrhodamine methyl ester (TMRM) (546/573-nm excitation and 590-nm emission). Isolated mitochondria (25 µg) were suspended in 0.2 ml KCl-based buffer solution (137 mM KCl, 0.25 mM EGTA, 2 mM MgCl2, 2 mM KH2PO4, 20 mM HEPES, 5 mM NaCl, pH 7.2) in a 96-well plate and read by using a BioTek microplate reader. Glutamate/malate (5 mM/5 mM) was used as substrates to energize mitochondria (state 2) followed by addition of 1 mM ADP (state 3).

Blue native gel and complex I activity. Mitochondrial isolates from heart tissue lysates were prepared according to the NativePAGE Sample prep kit (ThermoFisher) (8 g/g: digitoxin/protein). The mitochondrial resuspensions were run on 3–12% Bis-Tris Native Page gels (ThermoFisher). Gels were run using the NativePAGE Running Buffer kit (ThermoFisher). Gels were either stained using the Coomassie R-250 Staining kit (ThermoFisher) or used for in-gel activity. For this, the gel was equilibrated in 5 mM Tris HCl, pH 7.4 for 10 min and then developed in complex I activity solution (5 mM Tris pH 7.4, 2.5 mg/ml NBT, 0.1 mg/ml NADH). The reaction was terminated by soaking the gel in 10% acetic acid. Gels were imaged using an Epson Perfection V800 Photo scanner. For the microplate plate assay, Complex I activity was measured using the Complex I Enzyme Activity Microplate Assay Kit (Abcam ab109721) as specified by the manufacturer’s instructions. Briefly, 100 µg mitochondrial pellets were resuspended in 25 µL of 50 mM Tris/HCl buffer (pH 7.4). Complex I activity was determined by following change in 450 nm absorbance per minute (mOD/min) for 30 min following the addition of Incubation Solution (Abcam) to a total of 1 mL. Each sample was loaded in triplicate (200 µL/well) and incubated for 3 h at room temperature.

Mitochondrial respiration/oxygen consumption. Mitochondrial oxygen consumption rate was assayed using a high-throughput automated 96-well extra- cellular flux analyzer (XF96; Seahorse Bioscience). Freshly isolated mitochondria (15–20 µg) in suspension in isolation buffer (137 mM KCl, 2 mM KH2PO4, 0.5 mM EGTA, 2.5 mM MgCl2, and 20 mM HEPES at pH 7.2, and 0.2% fatty acid free BSA), transferred into a 96-well XF96 plate, and
centrifuged at 3000 x g for 20 min at 4 °C to attach to the plate. The respiration was evaluated with substrates of complex I (5 mM pyruvate/malate), complex II (5 mM succinate), following addition of 1 mM ADP. Oxygen consumption was measured with one cycle of 0.5 min mix, 3 min measurement and another 0.5 min mix in each step after port injection.

**TCA cycle enzyme activities.** Pyruvate dehydrogenase (PDH) activity was measured with the Pyruvate Dehydrogenase Enzyme Activity Microplate Assay Kit (Abcam) using 20 µg of isolated mitochondria for each assay. Citrate synthase (CS) activity was measured using a MitoCheck Citrate Synthase Activity Assay Kit (Cayman Chemicals). The rest of TCA cycle enzymes activities were measured using colorimetric assay based kinetic assays as described. Briefly, the isolated mitochondria were freeze/thawed three times to lyse the mitochondria. Mitochondrial lystate (15 µg) was then suspended in phosphate based solution (50 mM KH₂PO₄, 1 mg/ml BSA, pH 7.2 in the first assay, 10 mM KH₂PO₄ in the second assay). The first assay used dichlorophenol Indophenol (DCPIP) reduction (λ_reduced = 600 nm) to measure succinyl-CoA ligase (SL), succinate dehydrogenase (SDH), lumbarase (FM), and malate dehydrogenase (MDH) by sequential addition of reagents 140 µM DCPIP, 100 µM Duroquinone, 0.8 mM phenazine methosulfate (PMS) and imaged with a 510-nm long-pass filter. Data were collected and analyzed using NIS Elements 4.0 software (Nikon).

**Transmission electron microscopy and mitochondrial scoring.** Hearts were fixed in 2.5% gluteraldehyde in 0.1 M Na cacodylate buffer overnight at 4 °C, washed 3 × 20 min in 0.1 M Na cacodylate buffer, pH 7.2, fixed in 4% OsO₄, washed in 0.1 M Na cacodylate buffer then dH₂O, 2.5% uranyl acetate, ETOH series to dehydrate then EIOH and Spurr’s with final solution 100% Spurr’s. Hearts were embedded in Spurr’s at 60 °C for 24–48 h. Ultramicrotomy sections were cut at 90 nm and samples collected on 200 mesh for formvar grids for staining with uranyl and lead. Stained sections were examined with a Phillips/FEI BioTwin CM120 Transmission Electron Microscope and digital images were collected with a Gatan Oris high-resolution cooled digital camera (2Kx2K).

**Quantitative PCR.** Total DNA was prepared using DNeasy Blood and Tissue Kit (Qagen). Validated primers for Mitochondrial (CCATCTCCACCTGTCGTCC, ACCGCTCAGGTAGTACTCATCACGCAAG, and nuclear GGAATCGTCCGACGAGATTCAATCTGCGA) genes were synthesized and used for qPCR on the CFX Connect™ Real-Time PCR Detection System (Bio-Rad) with CFX Manager 3.1 software (Bio-Rad). Mitochondrial to nuclear DNA ratios were quantified using the ΔΔCt method.

**TUNEL measurements.** Cryosections (10 µm) of ventricular tissue were fixed in 4% paraformaldehyde and stained with In-situ Cell Death Detection Kit (Roche). Images were acquired on a laser-scanning confocal microscope (LSM 510, Carl Zeiss), TUNEL positive and total nuclei were counted from five images per sample, and the averages reported.

**Fibrosis.** Mouse hearts were formalin fixed and paraffinized. Heart sections were cut along the coronal plane at a 60° for 30 min and deparaffinized through xylene, 100% ethanol, 95% ethanol and then water. The slides were then stained using a Masson’s Trichrome Aniline Blue Stain Kit (Newcomer Supply, 9179B) and imaged at ×20 with an Aperio Scanscope CS. The images were then analyzed with Aperio Imagescope, using a lower intensity threshold of 150 and a hue value of 0.66. Six left ventricular free wall images were analyzed per sample. The percentage percentages of the six images were then averaged and the average positivity values were reported.

**Liquid chromatography and mass spectrometry.** Frozen extracted mitochondria were lysed by sonication in 300 µl of lysis buffer (8 M urea, 50 mM ammonium bicarbonate, 4 µl protease inhibitors (Complete mini EDTA-free mixture [Roche Applied Science], 10 µl protease inhibitor mixture [PhosSTOP, Roche Applied Science]). After centrifugation (20,000 × g for 10 min at 4 °C), the protein concentration of the supernatant was measured using the Bradford assay (Bio-Rad) and ~150 µg of proteins for each condition were subjected to digestion. Protein reduction and alkylation were performed using a final concentration of 2 mM dithiothreitol and 4 mM iodoacetamide, respectively. Proteins were first digested for 4 h at 37 °C with Lys-C (enzyme/substrate ratio 1:100). The second digestion was performed overnight at 37 °C with trypsin (enzyme/substrate ratio 1:100) in 2 M Urea. The resulting peptides were chemically labeled using stable isotope dimethyl labeling as described previously. After protein digestion the mix of the six images were then averaged and the average positivity values were reported.

**Mitochondrial Ca²⁺ uptake.** Mitochondrial Ca²⁺ uptake was measured as previously described. Briefly, single isolated isolated ventricular myocytes were loaded with 4 µM Fura-2 acetoxymethyl (AM) for 30 min and then perfused with Tyrode’s solution for 30 min to de-esterify the Fura-2 AM. After placement on a recording chamber, the cells were bathed in buffer solution composed of (mM): 137 NaCl, 10 Hepes, 10 glucose, 1.8 CaCl₂, 0.5 MgCl₂, 2 mM CaCl₂, 2 mM MgCl₂, 2 mM α-ketoglutarate, 100 mM thiamine pyrophosphate (TPP) and 0.1% Triton X100 by sequential additions of 0.5 mM CoASH, 0.8 mM NADP, 2 mM MgCl₂, 2 mM α-ketoglutarate, 100 mM thiamine pyrophosphate (TPP) and 0.1% Triton X100 at 37 °C. Myocytes were dispersed by gentle agitation, collected in serial solutions for 30 min to de-esterify the Fura-2. The resulting Ca²⁺-loaded ventricular myocytes as previously described were then perfused with a nominally Ca²⁺ free solution. Final perfusion was with collagenase-containing low Ca²⁺ Tyrode solution for 30 min to de-esterify the Fura-2. The resulting Ca²⁺-loaded ventricular myocytes were then bathed in nominally 10 mM malate. The second spectrofluorometric assay measures α-ketoglutarate dehydrogenase (KGDH), aconitate (AC) and isocitrate dehydrogenase (IDH) by monitoring NADH/NADPH reduction in the presence of 0.1% Triton X100 (100 µg/mL EDTA, 2 mM CaCl₂, 2 mM MgCl₂, 2 mM α-ketoglutarate, 100 mM thiamine pyrophosphate (TPP) and 0.1% Triton X100 by sequential additions of 0.5 mM CoASH, 0.8 mM NADP⁺, 5 mM MgCl₂, 0.5 mM citric-acid, and 15 mM isocitrate. Absorbance measurements were made using a BioTek Microplate reader (SpectraMax MX-96 well plate reader).

**Immunofluorescence imaging.** Isolated ventricular myocytes were fixed in 100% ethanol prior to incubation with antibodies Flag (Rockland), CoxIV (Cell Signal) and then washed with 1% BSA and then incubated with antibodies anti-FM (1:100) and second antibody (1:200). The resulting antibody stainings were visualized with a Zeiss LSM 510 with Zen software. NADH was excited at 485-nm excitation, 535-nm centered fluorescence was monitored and reconstituted in 80% ACN, 6% triethylamine. The resulting antibodies were visualized with a Zeiss LSM 510 with Zen software. NADH was excited at 485-nm excitation, 535-nm centered fluorescence was monitored and reconstituted in 80% ACN, 6% triethylamine. The resulting antibodies were visualized with a Zeiss LSM 510 with Zen software. NADH was excited at 485-nm excitation, 535-nm centered fluorescence was monitored and reconstituted in 80% ACN, 6% triethylamine. The resulting antibodies were visualized with a Zeiss LSM 510 with Zen software.
was achieved with an in-house pulled and gold coated fused silica capillary (360-μm outer diameter, 20-μm inner diameter, 10-μm tip inner diameter) and an applied voltage of 1.7 kV. Full-scan MS spectra (from m/z 350 to 1350) were acquired in the Orbitrap with a resolution of 30,000. Up to ten most intense ions above the threshold of 500 counts were selected for fragmentation. For the fragmentation a decision tree method was used as described previously. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD004631.

**LC-MS data analysis.** For the raw data files recorded by the mass spectrometer, peak lists were generated using Proteome Discoverer (version 1.3, Thermo Scientific, Bremen, Germany) using a standardized workflow. Peak list was searched against a Swiss-Prot database (version 23.02, taxid=9606) containing 13,402 protein entries) supplemented with frequently observed contaminants, using Mascot (version 2.3.02 Matrix Science, London, UK). The database search was performed by using the following parameters: a mass tolerance of 50 ppm for the precursor masses and ±0.6 Da for CID/ETD fragment ions. Enzyme specificity was set to Trypsin with two missed cleavages allowed. Carbamidomethylation of cysteines was set as fixed modification, oxidation of methionine, dimethyl labeling (L, D) of lysine residues and N termini, and phosphorylation (S, T, Y) were used as variable modifications. Percolator was used to filter the PSMs for <1% false discovery-rate. Phosphorylation sites were localized by applying phosphoRS (pRS) (v2.0). Double dimethyl labeling was used as quantification method, with a mass precision of 2 ppm for consecutive precursor mass scans. A retention time tolerance of 0.5 min was used to account for the potential retention time shifts due to deuterium. To further filter for high-quality data, we used the following parameters: high-confidence peptide-spectrum matches, minimal Mascot score of 20, minimal peptide length of 6 only, and unique and the search rank 1 peptide. The phosphopeptides that showed an on/off situation in the mtCaMKII or WT were manually quantified by giving them an arbitrary value of 100 or 0.01 for extreme up- or downregulation, which corresponds to the maximum allowed fold change in the used Proteome Discoverer settings.

**Modeling and simulation.** A three compartment computational model was constructed to include mitochondrial matrix, intermembrane space (IMS), and cytosolic compartments. The compartments were separated by inner mitochondrial membrane (IMM), and outer mitochondrial membrane (OMM). The computational model consists of 48 nonlinear ordinary differential equations. The mitochondrial processes include oxidative phosphorylation, TCA cycles and transmitters across the mitochondrial inner membrane. The mitochondrial creatine kinase in the intermembrane space (IMS) and in the cytosol were modeled as random sequential bi-bi enzyme reactions and creatine, creatine phosphate, ATP and ADP were diffused between IMS and cytosol. The equations were solved by ODE solver ode15s in MATLAB 2018B (The MathWorks, Natick, MA). The computational model of mitochondrial and cellular CaMKII and creatine kinase system was constructed to simulate overexpressed mitochondrial CaMKII condition and mitochondrial creatine kinase (CK). The mitochondrial CaMKII overexpressing (mtCaMKII) condition was simulated by decreasing complex I activity to 45% of control, decreasing mitochondrial CK to 80% of control, and increasing complex II (130%) and TCA cycle enzyme activities (isocitrate dehydrogenase (IDH) 120%, α-ketoglutarate dehydrogenase (αKGDH) 120%, fumarate hydratase (FH) 120%), malate dehydrogenase (MDH) 20% more) according to the experimental measurements of these enzyme activities. Mitochondrial CK overexpression was simulated by increasing mitochondrial CK concentration to 120% of control. ATP hydrolysis and cytosolic calcium transient during cardiac systole was simulated using a pulsatile function. A detailed description of the mathematical equations used is available in the Supplementary Methods (see Model Validation).

**Statistical analysis.** Statistical analyses were performed using Graph Pad Prism 7 software. Sample size and information about statistical tests are reported in the figure legends. Data are presented as mean ± SEM. Pairwise comparisons were performed using a two-tailed t test. For experiments with more than two groups, data were analyzed by one-way ANOVA followed by Tukey’s post hoc multiple comparisons test.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

The authors declare that the data supporting the findings of this study are available within the paper and its supplementary information files. Source data are available with this paper. The mass spectrometry proteomics data are available via the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD004631. The data that support the findings of this study and unique materials are available from the corresponding authors upon reasonable request. Source data are provided with this paper.

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