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MICU3 plays an important role in cardiovascular function

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Mitochondrial calcium regulates bioenergetics but also serves as a trigger for cell death[1]. With a sustained increase in catecholamine or a large increase in cytosolic calcium as occurs with ischemia, mitochondrial calcium can rise to high levels leading to activation of the mitochondrial permeability transition pore, thus initiating cell death[1]. Calcium uptake into mitochondria occurs via the mitochondrial calcium uniporter (MCU), which is regulated by three EF-hand proteins, mitochondrial calcium uptake (MICU) 1, 2, and 3[2]. MICU1/MCU ratios vary in different tissues[3], and alterations in substrate have been shown to regulate MICU1 levels altering MCU mediated calcium uptake[4]. MICU3 has generally been thought to function primarily in neuronal tissue where it is highly expressed, and thus has been largely ignored in other tissues such as the heart. We performed quantitative proteomics using Tandem Mass Tag labelling coupled to liquid chromatography and tandem mass spectrometry to analyze MCU and MCU regulators in cardiac and hepatic mitochondria (Figure 1A). Normalizing MICU levels to MCU in heart and liver mitochondria, we found that the MICU1/MCU ratio was 0.75 in liver and 0.25 in heart which is consistent with previous studies[3]; however, the MICU3/MCU ratio in heart is more than three-fold higher than that found in liver. To confirm that the MICU3 we observed in heart mitochondria is not due to contamination by mitochondria from nerve tissue in heart, we isolated cardiomyocytes and compared the ratio of MICU3 to MCU in cardiomyocytes and heart mitochondria and found similar ratios (Figure 1B). These data suggest that MICU3 might play a role in regulating MCU in heart.

To examine the physiologic function of MICU3, we generated two independent mouse lines with global deletion of MICU3 (*Micu3*$^{-/-}$) utilizing CRISPR-Cas9 methods. By co-microinjecting sgRNAs targeting Exon 1 and Exon 12 (Figure 1C), we created two

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mutant lines, one with a small frameshift deletion in Exon 1 and the other with the entire region between Exon 1 and Exon 12 deleted. The mice were viable, and there were no gross differences in adult *Micu3*$^{-/-}$ mice compared to wild-type (*WT*) littermates. MICU3 protein expression was not present in *Micu3*$^{-/-}$ cardiomyocytes (Figure 1B). Preliminary results showed no significant differences between the two lines, thus subsequent studies were focused on the line with deletion of Exon 1–12. We found no difference in MCU, MICU1 or NCLX protein levels between the *WT* and *Micu3*$^{-/-}$ hearts. Echocardiograms showed no significant baseline differences in heart rate, fractional shortening (FS) or ejection fraction (EF) between *WT* and *Micu3*$^{-/-}$ mice. MICU3 has been shown to facilitate MICU1-mediated progressive channel activation by calcium[5]. As sustained mitochondria calcium overload associated with long term isoproterenol treatment has been suggested to lead to hypertrophy and cardiac dysfunction, we tested the hypothesis that *Micu3*$^{-/-}$ mice would have less dysfunction following isoproterenol-induced hypertrophy. Both sexes of adult (8–17 weeks) *Micu3*$^{-/-}$ and *WT* littermate mice were treated with continuous infusion of isoproterenol (15mg/kg/day) via an osmotic mini-pump for two weeks. Echocardiograms were performed at baseline and after treatment to assess changes in cardiac dimensions and function. Whereas *WT* mice exhibited depressed function (FS and EF) after 2 weeks of treatment with isoproterenol, *Micu3*$^{-/-}$ mice demonstrated normal function. Correspondingly, *WT* mice developed left ventricular (LV) dilation from baseline, whereas LV dimension remained stable in *Micu3*$^{-/-}$ mice (Figure 1D). To determine whether loss of MICU3 protected hearts from isoproterenol-induced mitochondrial calcium overload, after 2 weeks of isoproterenol treatment we assessed levels of phosphorylated pyruvate dehydrogenase (PDH). *Micu3*$^{-/-}$ hearts exhibited elevated phosphorylated PDH, which has been demonstrated to be inversely proportional to mitochondrial calcium level, indicating lower mitochondrial calcium relative to *WT* hearts (Figure 1E).

As cardiac injury after ischemia-reperfusion is thought to be associated with increased mitochondrial calcium leading to mitochondria-initiated cell death, we tested whether *Micu3*$^{-/-}$ hearts were protected against ischemia-reperfusion injury. *Ex vivo* Langendorff-perfused hearts from *WT* and *Micu3*$^{-/-}$ mice were subjected to 20 minutes of global ischemia followed by 90 minutes of reperfusion. *Micu3*$^{-/-}$ hearts had reduced infarct size normalized to the entire LV and improved contractile function following reperfusion (Figure 1F). Protection in *ex vivo Micu3*$^{-/-}$ hearts strongly supports a role for MICU3 in cardiac mitochondria. Interestingly, germline ablation of MCU or EMRE was not cardioprotective, likely due to adaptations in these germline knockout mice. We speculate that loss of MICU3 does not lead to adaptation and under conditions of prolonged elevated calcium, the loss of MICU3 is protective.

Our results show that loss of MICU3 confers cardioprotection against ischemia-reperfusion injury and isoproterenol-induced cardiac dysfunction. MICU3 levels relative to MCU and MICU1 are higher in heart than in liver, and loss of MICU3 reduces calcium overload during sustained isoproterenol treatment. Taken together these data support the novel finding that MICU3 plays an important role in regulating pathological calcium overload in the heart.

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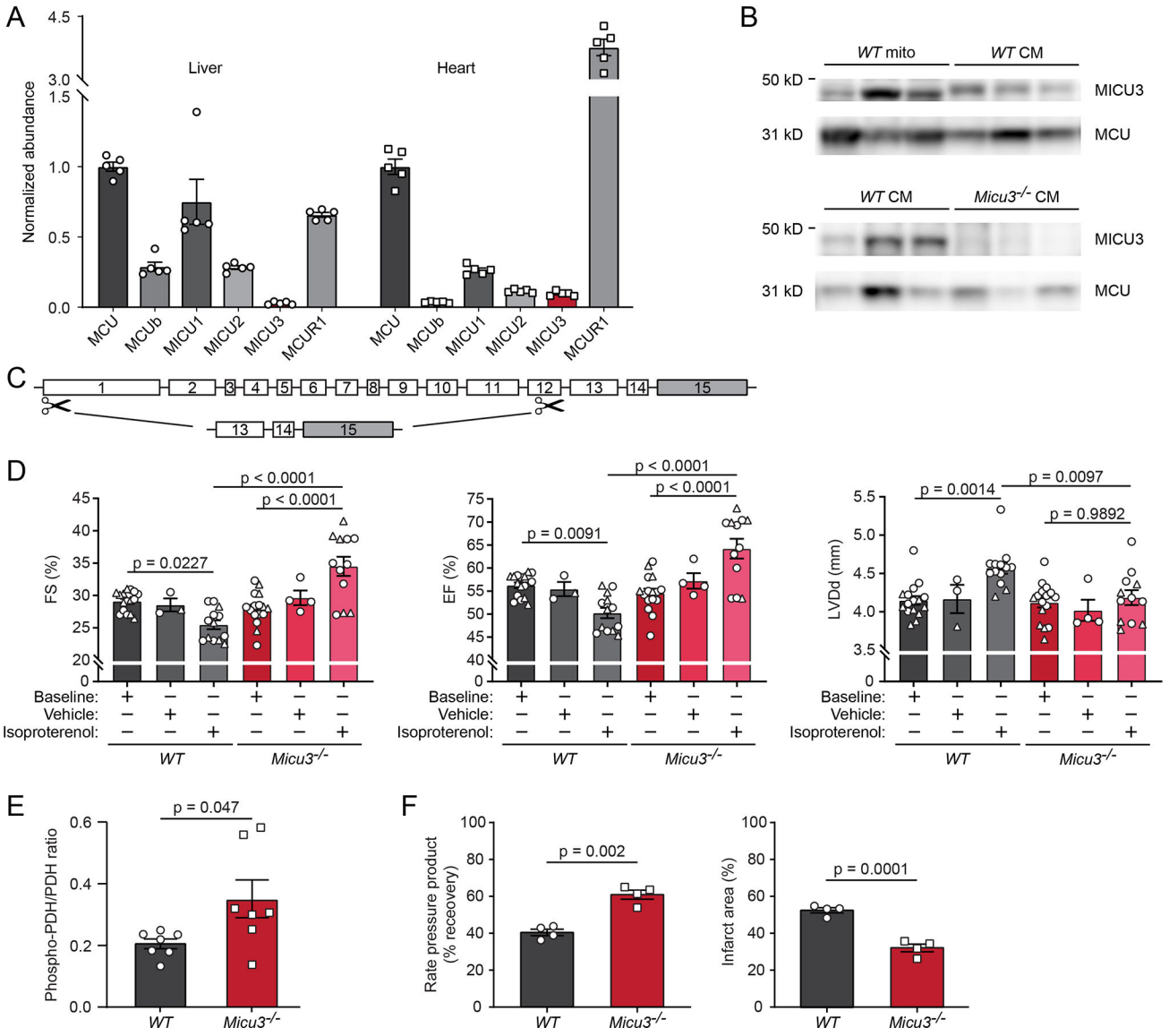

Figure 1.

1A: Proteomic analysis of MCU regulators in hepatic and cardiac mitochondria (n=5). **1B**: Western blot showing similar levels of MICU3 normalized to MCU in *WT* mitochondria and *WT* adult cardiomyocytes, and Western blot from adult cardiomyocytes isolated from *WT* and *Micu3*$^{-/-}$ mice demonstrating no MICU3 expression (MCU was the loading control) in *Micu3*$^{-/-}$ cardiomyocytes. Each lane is a separate cardiomyocyte or mitochondrial isolation from an individual mouse. **1C**: CRISPR-mediated targeted deletion of exon 1–12 of the *Micu3* gene. Exons 1 through 14 encompass the coding sequence, while exon 15 is non-coding (shown in gray). The scissors indicate the targeting sites for the CRISPR short guide RNAs (PAM sequences underlined), which were CGGCTCCCGGGATGTCGGGCCGG (exon 1) andCGTGCTGTATACGTAGCTACTGG (exon 12). **1D**: Fractional shortening (FS), ejection fraction (EF), and left ventricular end diastolic dimension (LVDd) at baseline and after two weeks of isoproterenol treatment in *Micu3*$^{-/-}$ mice (n = 16) vs. *WT* (n

= 16) mice. Statistical differences are shown. No sex differences were observed (circles and triangles represent males and females, respectively). Consistent with the data showing increased LV dilation in WT compared to *Micu3*$^{-/-}$ we found no difference in heart weight to body weight (data not shown). **1E**: Quantitated densitometry ratio of phosphor-PDH to total PDH from hearts of *Micu3*$^{-/-}$ (n = 7) and *WT* (n=7) exposed to 2 weeks of isoproterenol infusion. Calcium was measured with phospho-PDH/total PDH ratio because we were interested in *in vivo* mitochondrial levels following isoproterenol treatment, and there are currently no methods to measure mitochondrial calcium *in vivo*. **1F:** Quantitated rate pressure product and infarct size assessment by TTC staining post-reperfusion between *Micu3*$^{-/-}$ (n = 4) and *WT* (n = 4) hearts subjected to I/R injury using the Langendorff *ex vivo* perfusion system. Rate pressure product (the product of heart rate and left ventricular developed pressure) was measured with a fluid-filled balloon. There were no differences in pre-ischemic function (heart rate or left ventricular developed pressure) between *WT* and *Micu3*$^{-/-}$ hearts. Following ischemia and reperfusion *Micu3*$^{-/-}$ hearts had improved recovery of rate pressure product and reduced infarct size. Error bars represent standard error of the mean (SEM).