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# MCUb Induction Protects the Heart from Post-Ischemic Remodeling

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## Abstract

**Rationale:** Mitochondrial $Ca^{2+}$ loading augments oxidative metabolism to match functional demands during times of increased work or injury. However, mitochondrial $Ca^{2+}$ overload also directly causes mitochondrial rupture and cardiomyocyte death during ischemia-reperfusion injury by inducing mitochondrial permeability transition pore opening. The mitochondrial $Ca^{2+}$ uniporter (MCU) mediates mitochondrial $Ca^{2+}$ influx, and its activity is modulated by partner proteins in its molecular complex, including the MCUb subunit.

**Objective:** Here we sought to examine the function of the MCUb subunit of the MCU-complex in regulating mitochondria $Ca^{2+}$ influx dynamics, acute cardiac injury and long-term adaptation after ischemic injury.

**Methods and Results:** Cardiomyocyte-specific MCUb overexpressing transgenic mice and *Mcub* gene-deleted ($Mcub^{-/-}$) mice were generated to dissect the molecular function of this protein in the heart. We observed that MCUb protein is undetectable in the adult mouse heart at baseline, but mRNA and protein are induced after ischemia-reperfusion injury. MCUb

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DISCLOSURES

All authors confirm no conflict of interest.

SUPPLEMENTAL MATERIALS

1) Detailed Online Methods

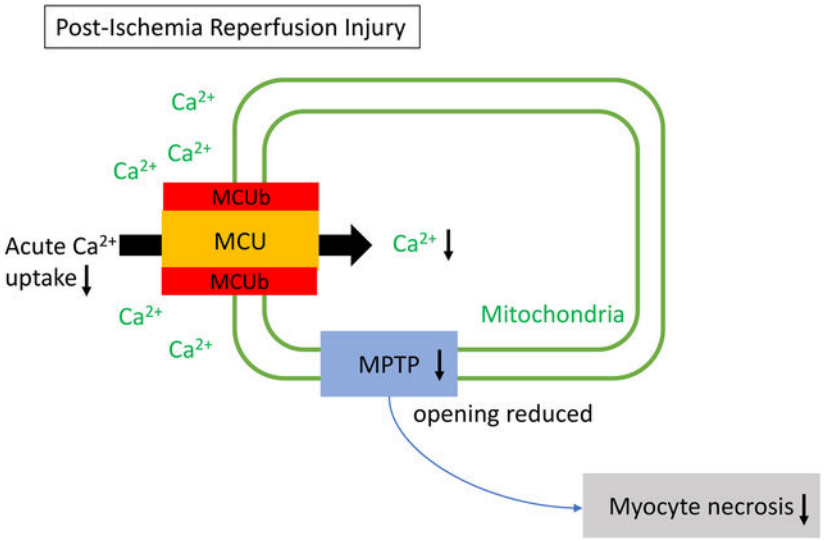
2) Online Figures I-VI with legends

3) References 41–45

overexpressing mice demonstrated inhibited mitochondrial $Ca^{2+}$ uptake in cardiomyocytes and partial protection from ischemia-reperfusion injury by reducing mitochondrial permeability transition pore opening. Antithetically, deletion of the *Mcub* gene exacerbated pathologic cardiac remodeling and infarct expansion after ischemic injury in association with greater mitochondrial $Ca^{2+}$ uptake. Furthermore, hindlimb remote ischemic pre-conditioning induced MCUb expression in the heart, which was associated with decreased mitochondrial $Ca^{2+}$ uptake, collectively suggesting that induction of MCUb protein in the heart is protective. Similarly, mouse embryonic fibroblasts from *Mcub*$^{-/-}$ mice were more sensitive to $Ca^{2+}$ overload.

**Conclusions:** Our studies suggest that *Mcub* is a protective cardiac inducible gene that reduces mitochondrial $Ca^{2+}$ influx and permeability transition pore opening after ischemic injury to reduce ongoing pathological remodeling.

## Graphical Abstract




Our study uniquely demonstrates the physiological relevance of MCUb in the heart. We utilized both MCUb-overexpressing and *Mcub*$^{-/-}$ mice. Importantly, this is the first report of the phenotype of genetic loss of *Mcub* in the heart. We show that MCUb is uniquely induced in the heart within 2–3 days after ischemic injury, where it then decreases mitochondrial $Ca^{2+}$ uptake via the mitochondrial $Ca^{2+}$ uniporter, which reduces the extent of cardiac damage in the proceeding days. We also observed that MCUb expression is induced in the heart by remote ischemia preconditioning of the hindlimb, which similarly reduces mitochondrial Ca2+ influx. Taken together, our data show that MCUb induction is an endogenous protective compensatory measure that reduces mitochondrial $Ca^{2+}$ overload-induced injury and ongoing borderzone expansion through cardiomyocyte death.

**Subject Terms:**

Animal Models of Human Disease; Basic Science Research; Calcium Cycling/Excitation-Contraction Coupling; Heart Failure; Ischemia

**Keywords**

Mitochondrial calcium; mitochondria; $Ca^{2+}$ handling; heart; ischemic injury; reperfusion injury; calcium regulation; infarction

## INTRODUCTION

Ischemic heart disease is one of the leading causes of death worldwide. During ischemia, obstruction of the coronary arteries restricts blood flow causing a shortage of oxygen and nutrients in the affected myocardium, which can directly kill cardiomyocytes.[1, 2] In some settings vascular flow can be reestablished by surgical intervention that often reduces the total level of myocardial wall necrosis, although the reperfusion phase itself can also kill cardiomyocytes.[3] In animal models ischemia-reperfusion (I/R) injury causes cardiomyocyte death by exposing these cells to high levels of free cytosolic $Ca^{2+}$, which then causes mitochondrial permeability transition pore (mPTP) opening.[1, 3] The mPTP is a pore-forming protein complex spanning the mitochondrial inner and outer membranes opens in response to high intracellular $Ca^{2+}$ and reactive oxygen species, where it leads to dissipation of mitochondrial membrane potential, organelle swelling and rupture, ultimately leading to necrotic cell death.[4, 5] Cyclophilin D is an important regulator of mPTP opening and use of cyclophilin inhibitory drugs such as cyclosporine A (CsA) desensitize pore opening, resulting in less cardiomyocyte death after I/R injury.[1, 6] Moreover, reducing mitochondrial $Ca^{2+}$ uptake in the heart during I/R injury can also reduce mPTP opening and subsequent myocyte necrosis.[7]

Transport of $Ca^{2+}$ into the mitochondrial matrix is mediated by the mitochondrial $Ca^{2+}$ uniporter (MCU)-complex, which serves as the major $Ca^{2+}$ influx pathway across the inner membrane, and this activity can be inhibited by Ru360 or ruthenium red.[8, 9] $Ca^{2+}$ efflux is due to the activity of the $Na^{+}/Ca^{2+}/Li^{+}$ exchanger (NCLX), which is also in the mitochondrial inner membrane.[10] The MCU-complex contains four MCU-subunits, produced by either the *Mcu* or *Mcub* gene.[11] If the tetramer is comprised of the *Mcu* gene product (MCU) it forms a pore that readily transduces $Ca^{2+}$; however, the presence of the *Mcub* gene product (MCUb) appears to antagonize $Ca^{2+}$ influx by the MCU-complex.[12] The MCU-complex also contains a number of regulatory components that alter the kinetics of $Ca^{2+}$ uptake or even assembly of the complex in the inner membrane, such as mitochondrial $Ca^{2+}$ uptake 1/2 proteins (MICU1/MICU2) and the essential MCU regulator, mitochondria (EMRE).[13, 14] The minimal functional $Ca^{2+}$ influx channel has recently been reported to consist of MCU and EMRE.[15]

MCUb is encoded by the gene originally annotated as *ccdc109b*, which is now officially known as *Mcub*. MCUb is structurally similar to MCU although the critical “DIME” motif present in MCU that acts as the $Ca^{2+}$ selectivity region of the channel is slightly different in MCUb.[12] As stated above, *in vitro* studies suggest that MCUb becomes part of the tetrameric core of the MCU-complex where it appears to inhibit $Ca^{2+}$ influx.[12] However, deletion of MCU from neurons of the mouse brain did not eliminate all Ca uptake (only 80% inhibited) and the observed residual activity was suggested to arise from MCUb.[16] Cardiac-

specific transgenic (TG) mice overexpressing a dominant negative (dn) MCU mutant were generated, which like MCUb, has alterations in the "DIME" motif.[17] Surprisingly, dnMCU mice were not protected from I/R injury despite near complete inhibition of acute mitochondrial $Ca^{2+}$ uptake.[12] Consistent with this, *Mcu*$^{-/-}$ mice, which also show a complete lack in acute mitochondrial $Ca^{2+}$ uptake, did not show cardioprotection after I/R injury.[18, 19] In contrast, inducible deletion of the *Mcu* gene from the adult mouse heart did protect from I/R injury and reduce mPTP opening.[7, 20] This inconsistency was attributed to an unknown compensatory effect that occurs during development in total somatic *Mcu*$^{-/-}$ mice, but which does not occur when the gene is deleted in the adult heart for the first time.[18, 19] More recently, cardiac-specific MCUb TG mice were generated using a tamoxifen inducible, DNA recombination-dependent overexpression strategy.[21] These inducible MCUb-TG mice showed extreme lethality (12/13) when I/R was performed 1 wk after acute tamoxifen-mediated activation of MCUb expression, although when performed 1 month after tamoxifen treatment this increased lethality was no longer observed and instead mice showed significantly reduced infarct sizes.[21] Collectively, these results highlight the complexity and continuing uncertainty surrounding MCUb function in the heart, and no one has yet investigated mice lacking the *Mcub* gene in the heart.

Here we observed that MCUb is essential in regulating mitochondrial $Ca^{2+}$ dynamics by limiting MCU-complex $Ca^{2+}$ uptake. Transgene-mediated constitutive overexpression of MCUb in the heart lowered mitochondrial $Ca^{2+}$ uptake rates and was protective during acute I/R injury by reducing cell death and mPTP opening frequency. With respect to physiologic significance, MCUb protein is normally not detectable in the adult heart but upon injury this gene product is induced where it serves a protective function. Indeed, *Mcub*$^{-/-}$ mice show greater mitochondrial $Ca^{2+}$ uptake days after I/R injury that is associated with greater ventricular pathology and remodeling compared with wildtype (WT) controls. Moreover, we observed that remote ischemic pre-conditioning (RIPC) also induces MCUb expression in the heart and that this limits mitochondrial $Ca^{2+}$ uptake. Our results are the first to show that the *Mcub* gene plays a necessary physiologic role in the heart where it's induction after I/R injury limits ongoing pathological remodeling and infarct expansion.

## METHODS

Detailed methods are available in the Online Data Supplement. All original data, additional detailed methods and materials used will be provided upon reasonable request to the corresponding author.

### Animals.

All experimental use of mice was approved by the Institutional Animal Care and Use Committee (IACUC). Cardiac-specific MCUb overexpressing TG mice were generated using the cardiac-specific tetracycline (Tet)-off expression system as described previously.[22] A tetracycline transactivator (tTA) TG mouse line with a cardiac-specific promoter (α-myosin heavy chain, α-MHC) was used along with another TG mouse line in which the mouse Mcub cDNA was under the control of the α-MHC-tetracycline operator sequences. *Mcub*-null mice were also generated from previously targeted ES cells using the "knockout-

first allele" strategy that is targeted between the 1st and 2nd exon (*Mcub*$^{tm1a(KOMP)Mbp}$, European Conditional Mouse Mutagenesis program, MGI ID: 1914065). Mouse embryonic fibroblasts (MEFs) were generated and cultured as described previously.[23] Mouse embryonic fibroblasts (MEFs) were generated from *Mcub-loxP* (fl) and *Mcu*$^{fl/fl}$ mice.[7] These mice were also crossed together to generate *Mcu*$^{fl/fl}$*Mcub*$^{fl/fl}$ double-targeted MEFs.

### Surgery.

I/R injury was performed as previously described.[7, 24] Mice were closely monitored after surgery and received necessary pain relief treatment (Buprenorphine, 0.03mg/mL, subcutaneous injection) to minimize discomfort. For myocardial infarction (MI) injury, mice were challenged with permanent left coronary artery ligation and pain medication was given (Buprenorphine, 0.03mg/mL, subcutaneous injection).

### Mitochondrial Isolation and analyses.

Heart or MEF mitochondria were isolated by differential centrifugation as described previously.[7, 23]

**$Ca^{2+}$ and mPTP measurement in cardiomyocytes.—**Adult cardiac ventricular myocytes were isolated from mouse hearts using previously described methods.[25]

**$Ca^{2+}$ sparks and transient measurements.—**Intact ventricular myocytes were loaded with Fluo-4 AM dye (5 μmol/L) for 30 minutes, transients and sparks were recorded as previously described.[25, 26] Calcium transients were obtained by field stimulation at 1 Hz in normal Tyrode's buffer with 1.8 mmol/L $Ca^{2+}$. Sarcoplasmic reticulum (SR) $Ca^{2+}$ load was evaluated by the $Ca^{2+}$ transient amplitude upon rapid application of caffeine (10 mmol/L). Images were acquired with confocal microscopy (Nikon, ×40 objective) using line scan mode with excitation at 488 nm, emission at >505 nm. Images were analyzed using ImageJ software and Sparkmaster.[27]

## RESULTS

### MCUb expression is induced following I/R injury.

To investigate the function of the *Mcub* gene in regulating mitochondrial $Ca^{2+}$ influx with ischemic injury we first carefully examined *Mcub* mRNA and MCUb protein expression, along with the other components of the MCU-complex, following a time-course of I/R injury (Fig. 1A–B). WT mice were challenged with I/R injury to their hearts (60 minutes of ischemia) and then harvested 1-, 3- or 7-days afterwards. The left ventricle of the heart was used for analysis (either whole tissue) to focus on the infarct and border-zone regions. We observed that both *Mcub* mRNA and MCUb protein expression were increased starting 3 days after I/R injury compared to sham controls, and this induction was even stronger 7 days after injury (Fig. 1A–B). Importantly, the specificity of the MCUb custom made antibody was verified in *Mcub*$^{-/-}$ hearts after I/R injury (and null MEFs), as all commercial antibodies that we surveyed failed and were either non-specific or did not detect MCUb. We believe that the increase in MCUb protein observed from purified heart mitochondria after I/R injury is dominantly due to cardiomyocyte expression since the contribution to the total

mitochondrial content from immune cells, fibroblasts and endothelial cell fraction in the heart is negligible. mRNA for other MCU-complex components (*Mcu*, *Micu1*, *Micu2* and *Smdt1* (EMRE encoding gene)) also showed increased expression following I/R injury, but to a lower degree than *Mcub* (Fig.1A). Interestingly, we also observed that MCU and EMRE protein levels were increased in the heart 7 days post I/R injury (Fig 1B). To extend these results permanent myocardial infarction (MI) injury was performed on WT mice, which similarly showed induction of MCUb protein in the heart by 3 and 7 days after injury (Fig. 1C). However, it should be noted that we were unable to detect MCUb protein in the hearts of uninjured adult mice, suggesting that this protein might only have a functional role in the heart days after an ischemic injury event.

### Cardiomyocyte-specific MCUb overexpressing mice.

To model the observed induction of MCUb in the heart after injury and to examine its potential function we developed cardiac-specific MCUb TG mice using the tet-off system.[28] A tetracycline transactivator (tTA) TG mouse line with cardiac-specific expression (α-myosin heavy chain, α-MHC) was crossed with α-MHC tetracycline-operator responsive TG mice containing a MCUb cDNA (MCUb-TG) to generate experimental double TG (MCUb-DTG) mice (Fig.2A). Western blotting from purified mitochondria from the hearts of tTA-TG, single MCUb-TG and the MCUb-DTG mice was performed at 6 wks of age (Fig. 2B). The data show no expression of MCUb protein in tTA-TG hearts but substantial expression in the hearts of MCUb-DTG mice (Fig. 2B). We should also note that the single MCUb-TG line showed minimally detectable MCUb protein, due to slight leak of the single TG construct (Fig. 2B). MCU, MICU1, MICU2 and NCLX protein levels were not changed with MCUb overexpression in the heart, but interestingly, EMRE levels were reduced compared with the 2 controls (Fig. 2B). As recently proposed, EMRE is essential for MCU complex assembly such that MCUb overexpression might displace MCU from the tetramer and its association with EMRE.[29] To examine this concept further we performed co-immunoprecipitation (Co-IP) for EMRE using MCU antibody, which showed that MCUb overexpression inhibited the presence of EMRE within the MCU complex (Fig. 2C). Previous studies showed that deleting *Mcu* in the heart decreased the expression level of NCLX to maintain overall mitochondrial $Ca^{2+}$ flux.[7] However, we did not observe a difference in NCLX protein levels with MCUb is overexpression in the heart (Fig. 2B), although NCLX activity was reduced in adult cardiomyocytes from these MCUb-DTG mice (Online Fig. I), presumably as a compensatory measure to again maintain total mitochondrial $Ca^{2+}$ flux potential.

Purified mitochondria from hearts of MCUb DTG mice showed inhibited $Ca^{2+}$ uptake compared with the two control groups (Fig. 2D). We also examined mitochondrial $Ca^{2+}$ uptake in living but permeabilized cardiomyocytes in culture using a Rhod-2-fluorescence assay, which again showed that MCUb overexpression blocked mitochondrial $Ca^{2+}$ uptake compared to controls, and this block was similar in effect to treatment with the MCU-complex inhibitor Ru360 (Fig. 2E). Collectively these data suggest that MCUb functions as a type of dominant negative effector of the MCU-complex.

While acute mitochondrial $Ca^{2+}$ uptake was inhibited by MCUb overexpression in both purified mitochondria and intact permeabilized adult cardiomyocytes, there was no effect on oxygen consumption rate (OCR) using a seahorse assay in purified mitochondria from MCUb-DTG hearts, nor was there a reduction in baseline mitochondrial $Ca^{2+}$ content (Fig. 2F–G). We previously showed that mitochondria from conditional *Mcu*-deleted hearts also had no reduction in baseline mitochondrial $Ca^{2+}$ levels nor oxygen consumption.[7] Such results suggest that other $Ca^{2+}$ influx pathways can partially compensate for the loss of MCU-complex activity to allow long-term control of $Ca^{2+}$ levels in the mitochondrial matrix and associated metabolic dynamics.[18] Indeed, overexpression of MCUb in the hearts of MCUb-DTG mice showed no pathological effect as these mice aged, such as no reduction in cardiac fractional shortening measured by echocardiography (Fig. 2H), nor a change in histopathological features, which would be expected if there was a metabolic deficiency (Online Fig. II). MCUb overexpression also did not alter cardiomyocyte intracellular $Ca^{2+}$ handling, such as the amplitude and kinetics of the $Ca^{2+}$ transient, SR $Ca^{2+}$ content, or $Ca^{2+}$ spark frequency (Online Fig. II). In addition, overexpressing MCUb in the heart did not change mitochondrial ultrastructure or show other pathological features by transmission electron microscopy (Online Fig. III). Collectively these findings indicate that MCUb overexpression is not deleterious to cardiac structure-function.

## MCUb overexpression protects from I/R injury.

An area of controversy in the MCU cardiac literature is based on the observation that loss of MCU (*Mcu*$^{-/-}$ mice) in certain genetic backgrounds of mice does not cause lethality, and the viability is presumably due to genetic compensation by other $Ca^{2+}$ influx mechanisms.[19] Consistent with this idea, a study of mitoplasts isolated from cells where MCU expression was knocked-down with siRNA showed that MCU-independent $Ca^{2+}$ currents were increased or induced when MCU levels are inhibited.[30] Moreover, viable germline *Mcu*$^{-/-}$ mice were not protected from I/R injury, yet adult inducible and cardiomyocyte-specific *Mcu* deletion in a pure *C57BL/6* genetic background did result in less I/R injury to the heart.[7, 18] Inducible expression of MCUb in the adult mouse heart also produced protection[21], hence we were uncertain how our mice with constitutive MCUb overexpression in the heart might impact acute injury responsiveness after I/R. Six-wk-old control and MCUb-DTG mice were subjected to 30 minutes of ischemia followed by 24 hours of reperfusion (Fig. 3A). Compared with controls, MCUb-DTG mice showed a significant reduction in ischemic injury with no difference in the area at risk (Fig. 3B–D). Mechanistically, we also observed that overexpression of MCUb decreased mPTP opening frequency to a similar extent as using the mPTP desensitizer CsA in tTA-TG control mitochondria (Fig. 3E). Collectively, these results suggest that chronic MCUb overexpression in the heart is not subject to compensation and that MCUb overexpression conveys acute cardioprotection following I/R injury.

## Deletion of the Mcub gene does not affect the heart at baseline.

To understand the physiological role of MCUb, we generated and analyzed *Mcub*$^{-/-}$ mice in which this genetic locus was targeted with a “knock-out first” cDNA cassette containing β-galactosidase (LacZ) and neomycin (Neo). This allele construction disrupts protein expression without employing the conditional LoxP sites and a Cre-recombinase strategy

that are built-in if needed (Fig. 4A). Since MCUb protein expression is not detectable by western blot in the heart under baseline conditions (Fig. 1B and 2B), we did not measure cardiac MCUb in our *Mcub*$^{-/-}$ groups at baseline. MCU, MICU1, MICU2 and NCLX expression were not altered in hearts of *Mcub*$^{-/-}$ mice compared to controls (Fig. 4B). Interestingly, an increase in EMRE protein expression was observed in *Mcub*$^{-/-}$ mice, which is the antithetic effect observed with MCUb overexpression whereby EMRE was reduced in the heart (Fig. 2B). No alterations were observed in *Mcub*$^{-/-}$ mouse body weights, activity, or gross anatomical features compared to controls.

Mitochondrial $Ca^{2+}$ uptake in isolated heart mitochondria from *Mcub*$^{-/-}$ mice was not altered versus control conditions, nor was baseline mitochondrial $Ca^{2+}$ levels altered as measured by two different assays (Fig. 4C–E). Oxygen consumption rate was also not changed in isolated heart mitochondria from *Mcub*$^{-/-}$ mice compared to controls (Data not shown). Loss of MCUb caused no difference in the Rhod-2-fluorescence assay that measures mitochondrial $Ca^{2+}$ uptake in permeabilized cardiomyocytes (Fig. 4F). Other quantitative aspects of the intracellular $Ca^{2+}$ transient and $Ca^{2+}$ handling kinetics were also unaltered in adult cardiomyocytes from *Mcub*$^{-/-}$ mice (Fig 4G–J). There was also no difference in $Ca^{2+}$ spark frequency, $Na^{+}$-dependent mitochondrial $Ca^{2+}$ efflux rates, or in mPTP activity due to loss of *Mcub*$^{-/-}$ versus control animals (Online Figure IV). Cardiac function measured by echocardiography was also unaffected in *Mcub*$^{-/-}$ mice (Fig. 4K), nor was there discernible pathology in the heart by electron microscopy (Online Fig. III). Thus, loss of MCUb was of no discernable consequence to the heart at baseline.

### MCUb deletion in mouse embryonic fibroblasts.

As presented earlier, adult cardiomyocytes lack detectable MCUb protein expression at baseline, which likely underlies our inability to identify a cardiac phenotype in *Mcub*$^{-/-}$ mice. However, mouse embryonic fibroblasts (MEFs) show MCUb expression at baseline (Online Fig. VA), so they were used as a more physiological model to examine the endogenous function of this gene. To generate *Mcub* targeted MEFs we used mice in which the LacZ-Neo cassette within the *Mcub* "knock-out first" allele was removed with a Flipase deletor mouse line, resulting in the generation of mice containing *LoxP* (*fl*) sites that could be used with Cre-recombinase for subsequent gene deletion. Immortalized MEFs were generated from these homozygous *Mcub*$^{fl/fl}$ mice and infection with an adenovirus expressing Cre recombinase (AdCre) in culture resulted in complete deletion and loss of MCUb protein (Online Fig. VA). We also generated *Mcu*$^{fl/fl}$ MEFs as well as double-targeted *Mcu*$^{fl/fl}$/*Mcub*$^{fl/fl}$-homozygous *loxP* MEFs for current analyses. AdCre infection deleted all MCU protein expression in the appropriate MEF lines (Online Fig. VA). We then assessed mitochondrial $Ca^{2+}$ uptake dynamics from these MEFs. Deletion of *Mcu* (*Mcu*$^{fl/fl}$ +AdCre) completely inhibited all $Ca^{2+}$ uptake versus mitochondria from the same parent MEF cell line without adenovirus infection (Online Fig. VB), while double deletion of *Mcu* and *Mcub* (*Mcu*$^{fl/fl}$/*Mcub*$^{fl/fl}$ +AdCre) produced mitochondrial $Ca^{2+}$ uptake dynamics identical to *Mcu* deletion, showing complete inhibition once again (Online Fig. VC). However, deletion of *Mcub* alone (*Mcub*$^{fl/fl}$ +AdCre) exhibited initial mitochondrial $Ca^{2+}$ uptake dynamics similar to control (first 4 $Ca^{2+}$ pulses) but then a much earlier transition to mPTP and release of all matrix $Ca^{2+}$ was observed (i.e. reduced $Ca^{2+}$ capacity and higher

$Ca^{2+}$ sensitivity of mPTP). This indicates that endogenous MCUb protein in MEFs normally limits $Ca^{2+}$ uptake via the MCU-complex (Online Fig. VD).

### Endogenous cardiac MCUb induction is cardioprotective.

To examine the physiologic function of MCUb protein induction following I/R injury we used 6-wk-old *Mcub*$^{-/-}$ mice subjected to 60 minutes of cardiac ischemia with a 24-hour reperfusion period, and then assessed injury (Fig. 5A). Importantly, there was no difference in acute injury to the heart as measured by ischemic area (IA) versus the area at risk (AAR) in *Mcub*$^{-/-}$ mice compared with control mice (Fig. 5B). Given that MCUb expression in the heart was observed 3 and 7 days after I/R injury, we next tested whether deleting MCUb alters post-injury infarct remodeling (Fig. 5C). Six-wk-old mice were challenged with 60 minutes of ischemia but were not harvested until 4 wks after surgery and assessed throughout for cardiac function by echocardiography (Fig. 5C). We observed that *Mcub*$^{-/-}$ mice developed significantly expanded left ventricle internal diameters, both end-diastolic and end-systolic, at 2 or 4 wks post injury (Fig. 5D–E, Online Fig. VI). However, *Mcub*$^{-/-}$ mice showed a trend towards a decrease in ventricular fractional shortening by 2 and 4 wks post injury as compared to controls (Fig. 5F). Gravimetric analysis showed a significantly greater increase in heart weight/body weight (HW/BW) and lung weight/body weight (LW/BW) in *Mcub*$^{-/-}$ mice 4 wks post I/R injury compared to control mice, suggesting greater pathology, most likely due to effects at the infarct borderzone (Fig. 5G–H). Indeed, direct measurements of scar area 4 wks after I/R injury showed a significant increase in *Mcub*$^{-/-}$ mice compared with control (Fig. 5I), even though initial infarct size 24 hours after I/R surgery was not different (Fig 5B). Collectively, these data indicate that MCUb induction normally plays a protective role in the heart by limiting $Ca^{2+}$ influx in cardiomyocytes in the viable myocardium and borderzone, likely reducing ongoing cell death and scar expansion.

We also examined the contribution of MCUb to mitochondrial $Ca^{2+}$ uptake post I/R injury in control and the *Mcub*$^{-/-}$ mice (Fig. 5J–K). Western blotting from these same hearts again showed that I/R injury resulted in MCUb protein induction over 7 days (Fig. 5K). Isolated mitochondria from hearts of these WT mice 7-day post I/R injury generated a profile of inhibited mitochondrial $Ca^{2+}$ uptake after injury compared to isolated mitochondria from hearts of sham controls (Fig. 5J). However, mitochondria isolated from hearts of *Mcub*$^{-/-}$ mice 7 days after I/R injury did not show this inhibited profile and instead showed normal $Ca^{2+}$ uptake like that observed in uninjured sham hearts (Fig. 5J). Thus, the induction of MCUb protein expression in the heart in the first wk of I/R injury normally reduces mitochondrial $Ca^{2+}$ influx to have a protective effect on the remaining myocardium. In contrast, *Mcub*$^{-/-}$ mice lack this protective mechanism and show unrestrained $Ca^{2+}$ uptake after I/R injury leading to worsening of cardiac remodeling.

### Remote ischemic pre-Conditioning induces MCUb expression.

We also examined if remote ischemic pre-conditioning (RIPC) induces cardiac MCUb expression prior to I/R injury. RIPC is a noninvasive ischemic procedure in rodents, whereby peripheral blood flow is interrupted to parts of the limb to activate the same molecular players in the heart that underlie protection against I/R injury.[31, 32] The femoral artery of the mouse was occluded for 5-min followed by 5-min release, with a total of 4 cycles per day

for seven consecutive days (Fig. 6A). The left ventricle of the heart was used for consistency with I/R groups in this paper. We found that both mRNA expression and protein levels of MCUb were induced in the heart following the RIPC procedure (Fig. 6B–C). Moreover, mitochondria isolated from the hearts of RIPC WT mice had reduced $Ca^{2+}$ uptake rates compared to mitochondria isolated from sham-treated WT control mice (Fig. 6D). However, mitochondria isolated from pre-conditioned *Mcub*$^{-/-}$ mice showed intermediate $Ca^{2+}$ uptake rates suggesting that MCUb was only part of the reason why RIPC affected cardiac mitochondrial $Ca^{2+}$ uptake dynamics. Collectively, our data demonstrate that RIPC can induce MCUb expression in the heart, suggesting a collective mechanism whereby MCUb protein expression protects from later ischemic insults at the level of the mitochondrion.

## DISCUSSION

The precise biological role of MCUb remains controversial. Some studies have suggested that MCUb likely acts as a dominant negative regulator of MCU by inserting into the quaternary core of the MCU-complex and directly interfering with channel permeation of $Ca^{2+}$.[12] Other studies suggest that MCUb may function as a low conductance mitochondrial $Ca^{2+}$ uptake channel.[16, 33] However, most studies in cells with overexpressed proteins or in planar lipid bilayers with recombinant protein are consistent with mammalian MCUb functioning to inhibit acute $Ca^{2+}$ uptake.[8, 12, 15, 34] For example, Checchetto and Szabo showed that purified MCU protein generates a $Ca^{2+}$ current in a planar lipid bilayer while purified MCUb could not, similar to results of Raffeallo et al.[12, 35] However, the *MCUb* gene from trypanosomes was shown to conduct $Ca^{2+}$ such that its absence caused reduced viability as well as reduced $Ca^{2+}$ influx in isolated systems.[33, 36] Although, it should be noted that the trypanosome MCU-complex is divergent from the mammalian form with additional subunit paralogs of unknown relevance.[33, 36] Our studies support the idea that MCUb acts as a dominant negative regulator of the MCU-complex in mammalian systems, as least for acute mitochondrial $Ca^{2+}$ uptake. In MEFs where MCUb protein is constitutively expressed, deletion of *Mcub* produced increased mitochondrial $Ca^{2+}$ uptake sensitivity suggesting that it functions as a true inhibitor of both acute and slow $Ca^{2+}$ influx.

Given the structural homology between MCUb and MCU it is believed that MCUb inserts into the quaternary core channel of the MCU-complex, and because MCUb has divergence in the “DIME” motif found in MCU, it no longer permits $Ca^{2+}$ conduction through the pore.[12] Alternately, MCUb may inhibit mitochondrial $Ca^{2+}$ uptake by changing the composition of the MCU-complex such that it no longer contains the essential component EMRE, as our data suggest. Structural analysis of the MCU minimal conducting complex showed that EMRE must be present where it binds 1:1 with MCU in generating a proper pore conformation.[15, 29] We observed that MCUb overexpression directly disrupted the interaction between MCU and EMRE by immunoprecipitation from mitochondrial protein extracts (Fig. 2B–C), while *Mcub*$^{-/-}$ hearts appear to show increased EMRE protein levels (Fig. 4B). However, future studies will be required to better define how MCUb inhibits mitochondrial $Ca^{2+}$ influx.

We and others have shown that inhibition of mitochondrial $Ca^{2+}$ influx by *Mcu* deletion in the adult mouse heart renders mitochondria insensitive to acute $Ca^{2+}$ stimulated increases in

mitochondrial energy production.[7, 18] However, in the absence of acute fluxing of mitochondria $Ca^{2+}$ a more dynamic compensation occurs that enhances long-term adaptation producing greater fatty acid utilization.[37, 38] For example, skeletal muscle-specific deletion of *Mcu* in mice inhibited acute mitochondrial $Ca^{2+}$ influx and $Ca^{2+}$-stimulated mitochondrial respiration, resulting in impaired metabolism-contraction coupling and reduced acute exercise performance.[39] However, loss of *Mcu* enhanced muscle performance under conditions of fatigue with a preferential shift towards fatty acid metabolism, resulting in reduced body fat with aging.[39] In the current study we failed to observe an alteration in oxygen consumption in isolated mitochondria from MCUb-DTG or $Mcub^{-/-}$ hearts compared with controls when assayed in pyruvate/malate buffer. By comparison, recently generated adult inducible MCUb TG mice similarly failed to show an effect on basal oxygen consumption in isolated mitochondria, although a significant reduction in reserve and maximal respiratory activity was noted.[21] dnMCU TG mice showed increased respiratory function in a working heart model but not at controlled $Ca^{2+}$ levels in permeabilized myocardial fiber preparations, which the authors interpreted as resulting from greater energy production outside of the mitochondria.[17] Thus, current evidence suggests that inhibition of MCU activity in the heart impacts respiratory function following acute stimulation with $Ca^{2+}$, while baseline mitochondrial respiration is unaffected although during stress greater fatty acid utilization is observed.[37, 38]

A key finding here is that MCUb is an inducible cardioprotective gene in the context of I/R injury. MCUb protein is not detected in the adult mouse heart at baseline, but I/R injury results in a dramatic induction of expression within 3 days. MCUb induction has a delayed effect where it reduces the likelihood of infarct expansion into borderzone cardiomyocytes by reducing mitochondrial $Ca^{2+}$ overload-induced necrosis. This would have a beneficial effect on remodeling after MI injury, supported by the observation that $Mcub^{-/-}$ mice succumb to greater injury in the proceeding days following an ischemic injury event. This is consistent with a mechanism of action referred to as delayed- or post-conditioning[40], which we also observed in mice subjected to RIPC.[31, 32] Indeed, we found that RIPC from the hindlimb also induced MCUb expression in the heart, suggesting that the MCUb gene is sensitive to and likely directly regulated by ischemia-dependent signaling and transcriptional pathways.

In conclusion, our study showed that MCUb is induced in the heart in response to ischemic stress, either by an I/R injury itself or by RIPC. This induction in MCUb in the heart is protective by limiting $Ca^{2+}$ influx and $Ca^{2+}$ overload in surviving cardiomyocytes at the level of the MCU-complex. We observed that while *Mcub* deletion did not affect initial I/R infarct size, it did limit infarct expansion and pathological remodeling compared with controls. These data suggest that therapeutically targeting the MCU-complex days after an infarction event could still provide benefit if an appropriate permeable and non-toxic MCU-complex inhibitory drug was identified.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Nonstandard Abbreviations and Acronyms:

| | |
|---|---|
| **α-MHC** | α-myosin heavy chain |
| **AAR** | area at risk |
| **$[Ca^{2+}]_{mito}$** | mitochondrial calcium concentration |
| **BW** | body weight |
| **Co-IP** | co-immunoprecipitation |
| **CsA** | cyclosporine A |
| **dnMCU** | dominant negative MCU mutant |
| **DTG** | MCUb overexpressing mice with 2 transgenes (MCUb + tTA) |
| **EMRE** | essential MCU regulator, mitochondrial (Smdt1 gene product) |
| **FCCP** | carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone |
| **FS** | fractional shortening |
| **HW** | heart weight |
| **IA** | ischemic area |
| **I/R** | Ischemia-reperfusion |
| **LacZ** | β-galactosidase |
| **LW** | lung weight |
| **MCU-complex** | mitochondrial calcium uniporter complex |
| **MCU** | mitochondrial calcium uniporter pore-forming subunit |
| ***Mcu*$^{-/-}$** | null mice lacking gene encoding MCU protein |
| **MCUb** | mitochondrial calcium uniporter b subunit |
| ***Mcub*$^{-/-}$** | null mice lacking gene encoding MCUb protein |
| **MCUb TG** | α-MHC tetracycline-operator mouse with MCUb cDNA |
| **MEF** | mouse embryo fibroblast |

| | |
|---|---|
| **MICU1** | mitochondrial calcium uptake 1 |
| **MICU2** | mitochondrial calcium uptake 2 |
| **MI** | myocardial infarction |
| **mPTP** | mitochondrial permeability transition pore |
| **Neo** | neomycin |
| **NCLX** | $Na^+/Ca^{2+}/Li^+$ exchanger |
| **OCR** | oxygen consumption rate |
| **OXPHOS** | mitochondrial oxidative phosphorylation complex |
| **RIPC** | remote ischemic pre-conditioning |
| **SR** | sarcoplasmic reticulum |
| **tTA TG** | tetracycline transactivator cardiac-specific transgenic mouse |
| **VDAC** | Voltage-dependent anion channel |
| **wk** | week |
| **WT** | wildtype |
| **$\Delta\Psi_m$** | mitochondrial membrane potential |

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## NOVELTY AND SIGNIFICANCE

**What Is Known?**

- Ischemia-reperfusion (I/R) injury causes cardiomyocyte death by exposing these cells to high levels of free cytosolic $Ca^{2+}$, which then causes mitochondrial permeability transition pore (mPTP) opening.
- Mcub is an inhibitory subunit of the mitochondrial $Ca^{2+}$ uniporter (MCU) that reduces mitochondrial $Ca^{2+}$ influx.
- Acute overexpression of Mcub protects the heart from I/R injury.

**What New Information Does This Article Contribute?**

- Mcub expression is induced in the heart after ischemia-reperfusion injury or remote ischemic pre-conditioning.
- Genetic deletion of Mcub in the heart exacerbated damage after ischemia-reperfusion injury.
- Mcub induction in the heart is a protective mechanism that reduces mitochondrial $Ca^{2+}$ overload and borerzone cardiomyocyte necrosis.

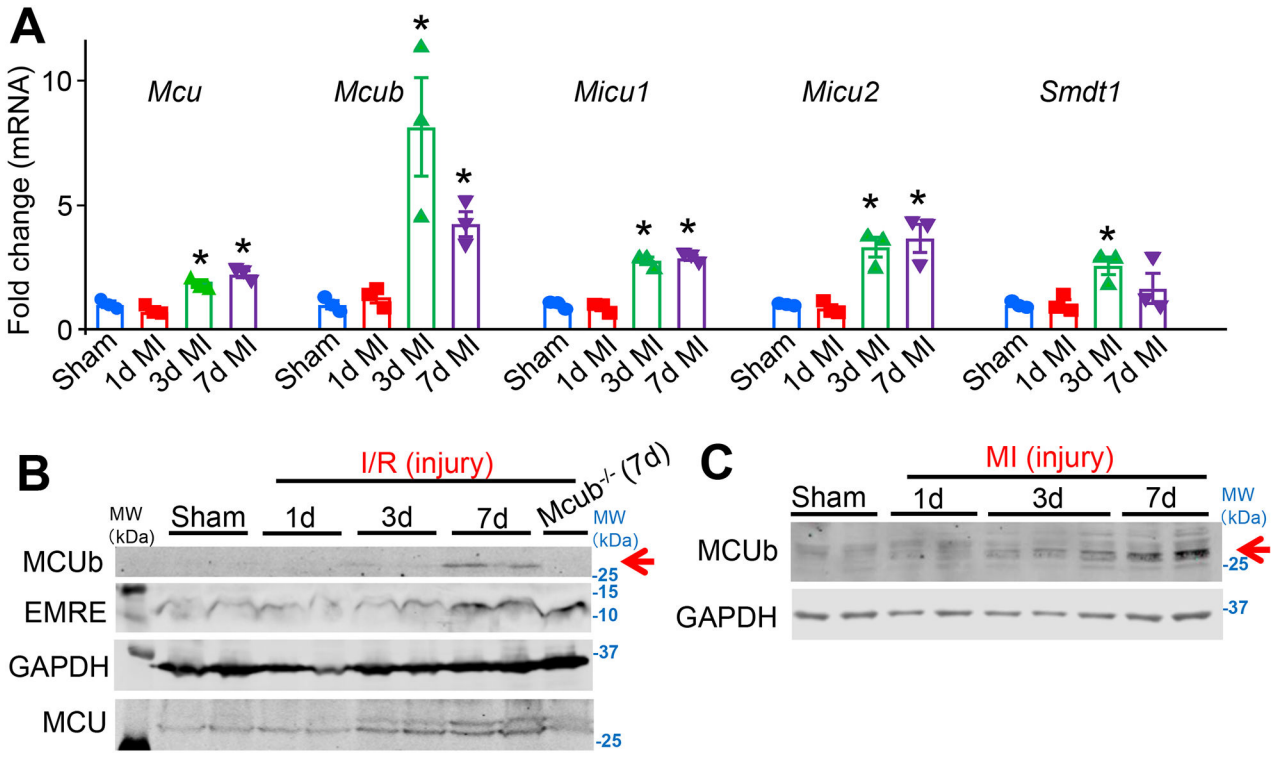


**Figure 1. MCUb expression is induced following cardiac ischemic injury.**

A) MCU-complex components *Mcu, Mcub, Micu1, Micu2* and *Smdt1* (EMRE) mRNA levels from cardiac left ventricle tissue and isolated myocytes following ischemia reperfusion (I/R) injury for 1-, 3- or 7-days versus sham. n=3 per group. Data presented as mean ± standard deviation of mean (SEM). One-way ANOVA and post hoc Bonferroni test was used for statistical analysis. *p<0.05 versus sham by post hoc Bonferroni's multiple comparison test. B) Western blot of MCUb, MCU and EMRE from left ventricle of the WT mouse heart following a time-course of I/R injury as shown in days. GAPDH was used as the protein loading control. *Mcub*$^{-/-}$ heart tissues 7 days after I/R injury is used as an antibody negative control. Arrow shows position of MCUb protein. The molecular weight marker shown is based on the uncropped western blots in which standards can (Online Supplement, and applicable to the remaining western blots in the paper). C) Western blot of MCUb from the heart following a time-course of myocardial infarction (MI) injury as shown. GAPDH was used as the protein loading control.

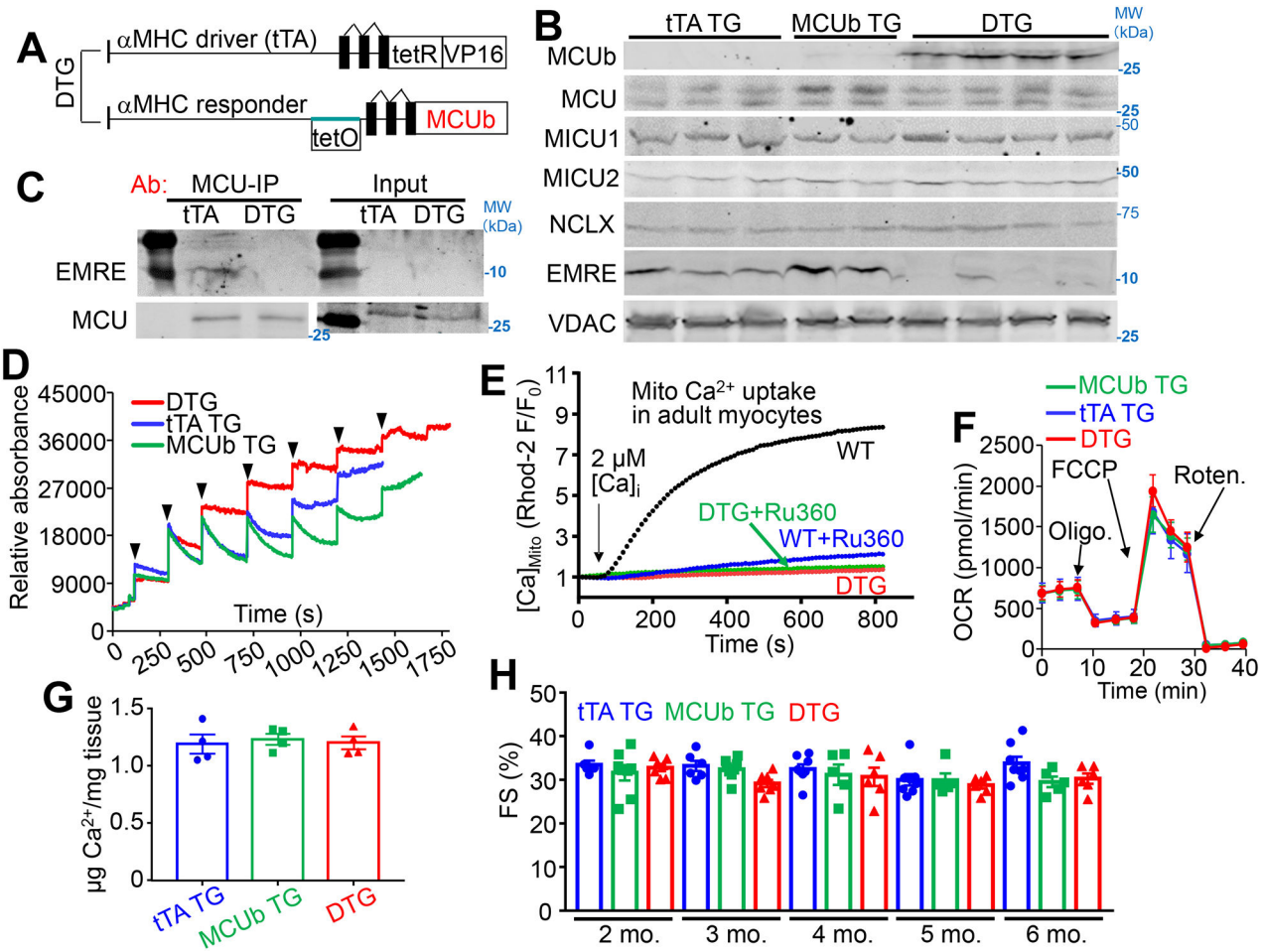


**Figure 2. Generation of cardiomyocyte-specific MCUb overexpressing transgenic mice.**

A) Schematic of cardiomyocyte-specific MCUb overexpressing mice using a double transgenic (DTG) tet-off system based on the α-myosin heavy chain (α-MHC) promoter. The driver line expresses the tetracycline transactivator cDNA (tTA), and the responder line contains the tet-operator and the MCUb cDNA. B) Western blot of MCUb, MCU, MICU1, MICU2, NCLX and EMRE in cardiac mitochondria from the indicated groups of mice. VDAC was used as a processing and loading control. C) Co-immunoprecipitation of EMRE with an MCU antibody from isolated heart mitochondria from the indicated two groups of mice. Western blotting for MCU from both immunoprecipitated samples as well as input samples is shown as controls. D) Mitochondrial $Ca^{2+}$ uptake in isolated heart mitochondria from the indicated groups at 3 months of age. Calcium Green-5N was used as the $Ca^{2+}$ indicator. The arrows indicate addition of 20 μM $CaCl_2$ to the solution. E) Mitochondrial $Ca^{2+}$ uptake in permeabilized adult cardiomyocytes from hearts of the indicated groups, with or without Ru360 under 2 μmol/L $CaCl_2$ perfusion. F) Oxygen consumption rate (OCR) measurement in cardiac mitochondria from the indicated mouse groups of mice at 3 months of age. n=3 per group. Arrows show the position of the three different drugs given in temporal sequence. G) Quantification of baseline mitochondrial $Ca^{2+}$ levels in isolated cardiac mitochondria from the indicated groups of mice at 6 months of age. n=4 per group. Data presented as mean ± SEM. One-way ANOVA was used for statistical analysis. H)

Echocardiographic measurement of fractional shortening (FS, %) from indicated groups at the indicated ages in months. n=7 in tTA TG group, n=5 in MCUb TG group, and n=6 in DTG group. Data presented as mean ± SEM. Two-way ANOVA was used for statistical analysis, with no significant interactions or effects of variables.

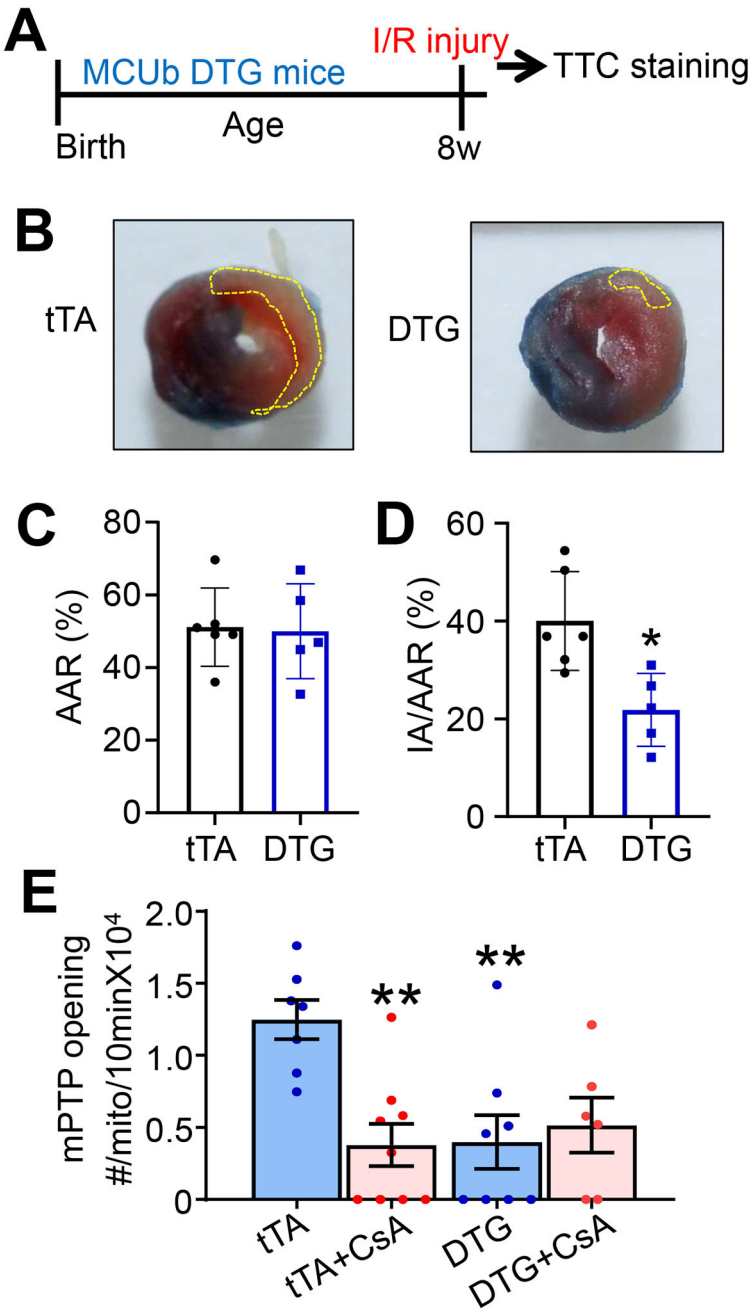


**Figure 3. MCUb overexpression generates protection from cardiac I/R injury.**

A) Temporal strategy of I/R injury in mice for panels “B”, “C” and “D”. Mice at 8 wks of age were challenged with 30 minutes of ischemia followed by 24 hours of reperfusion. B) Hearts were sacrificed for 2,3,5-triphenyltetrazolium chloride (TTC) staining. The yellow dotted area shows ischemic region. C,D) Average area at risk (AAR) and ischemic area (IA)/AAR of hearts from mice subjected to I/R injury from the indicated mouse groups. n=6 in tTA TG group, n=5 in DTG group. Data presented as mean ± SEM. Student’s t-test was used for statistical analysis. *p<0.05 vs tTA. E) Mitochondrial permeability transition pore (mPTP) opening frequency measurements in permeabilized cardiomyocytes. mPTP inhibitor

cyclosporine A (CsA) was used as a control. n=7 in tTA TG group, n=9 in tTA group with CsA treatment, n=8 in DTG group, n=6 in DTG group with CsA treatment. Data presented as mean ± SEM. Two-way ANOVA with a post-hoc Bonferroni's multiple comparison test was performed. A significant interaction was found between treatment and genotype ($p<0.05$). **$p<0.01$ vs tTA.

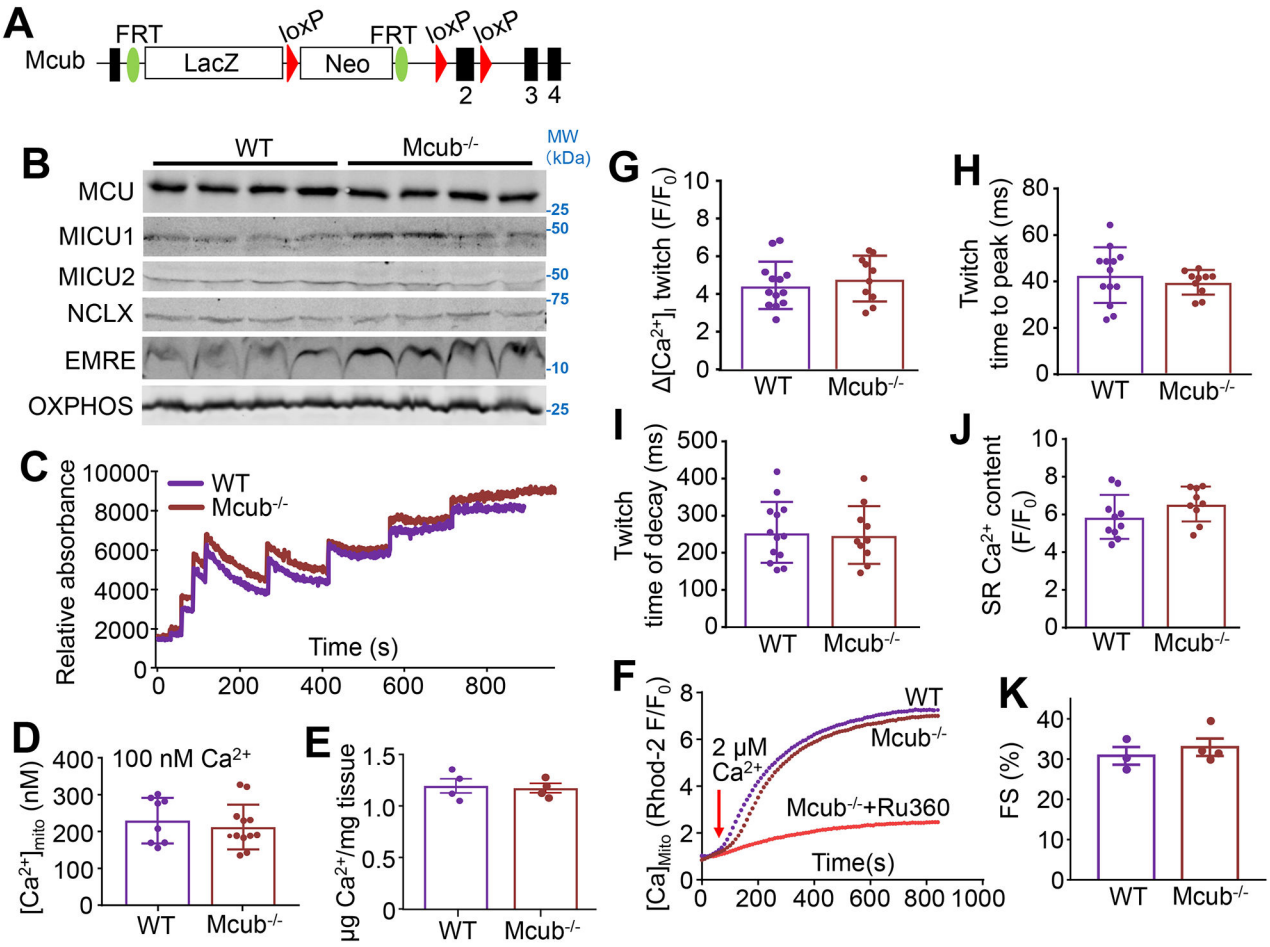


**Figure 4. Deletion of *Mcub* does not affect mitochondrial $Ca^{2+}$ uptake or cardiac function at baseline.**

A) Strategy for generating *Mcub*-null mice with a "knock-out first" allele strategy in which a combined β-galactosidase/neomycin cDNA cassette was inserted between exon 1 and exon 2 of the *Mcub* gene. B) Western blot of MCU, MICU1, MICU2, NCLX and EMRE in cardiac mitochondria from the indicated groups of mice. OXPHOS antibody was used as a control. C) Mitochondrial $Ca^{2+}$ uptake assay in isolated heart mitochondria from the indicated groups of mice at 4 months of age. D) Quantification of baseline mitochondrial $Ca^{2+}$ content ($[Ca^{2+}]_{mito}$) in permeabilized adult cardiomyocytes from the indicated groups. n=8 in WT group, n=12 in *Mcub*$^{-/-}$ group. Data presented as mean ± SEM. Student's t-test was used for statistical analysis. E) Quantification of baseline $[Ca^{2+}]_{mito}$ levels in isolated cardiac mitochondria from the indicated groups of mice at 4 months of age. n=4 per group. Data presented as mean ± SEM. Student's t-test was used for statistical analysis. F) Measurement of mitochondrial $Ca^{2+}$ uptake in permeabilized adult cardiomyocytes under 2 μmol/L $CaCl_2$. Ru360 was used as a control. G-I) Measurements $Ca^{2+}$ transient amplitude, time to peak and twitch decay (tau) from adult cardiomyocytes from hearts of the indicated groups of mice. n=13 in WT group, n=10 in *Mcub*$^{-/-}$ group. Data presented as mean ± SEM. Student's t-test was used for statistical analysis. J) Measurements of sarcoplasmic reticulum (SR) $Ca^{2+}$ content from adult cardiomyocytes from hearts of the indicated groups of mice. n=10 in WT

group, n=9 in *Mcub*$^{-/-}$ group. Data presented as mean ± SEM. Student's t-test was used for statistical analysis. K) Echocardiographic measurement of fractional shortening (FS, %) from the indicated groups at 4 months of age. n=3 in WT group, n=4 in *Mcub*$^{-/-}$ group. Data presented as mean ± SEM. Student's t-test was used for statistical analysis.

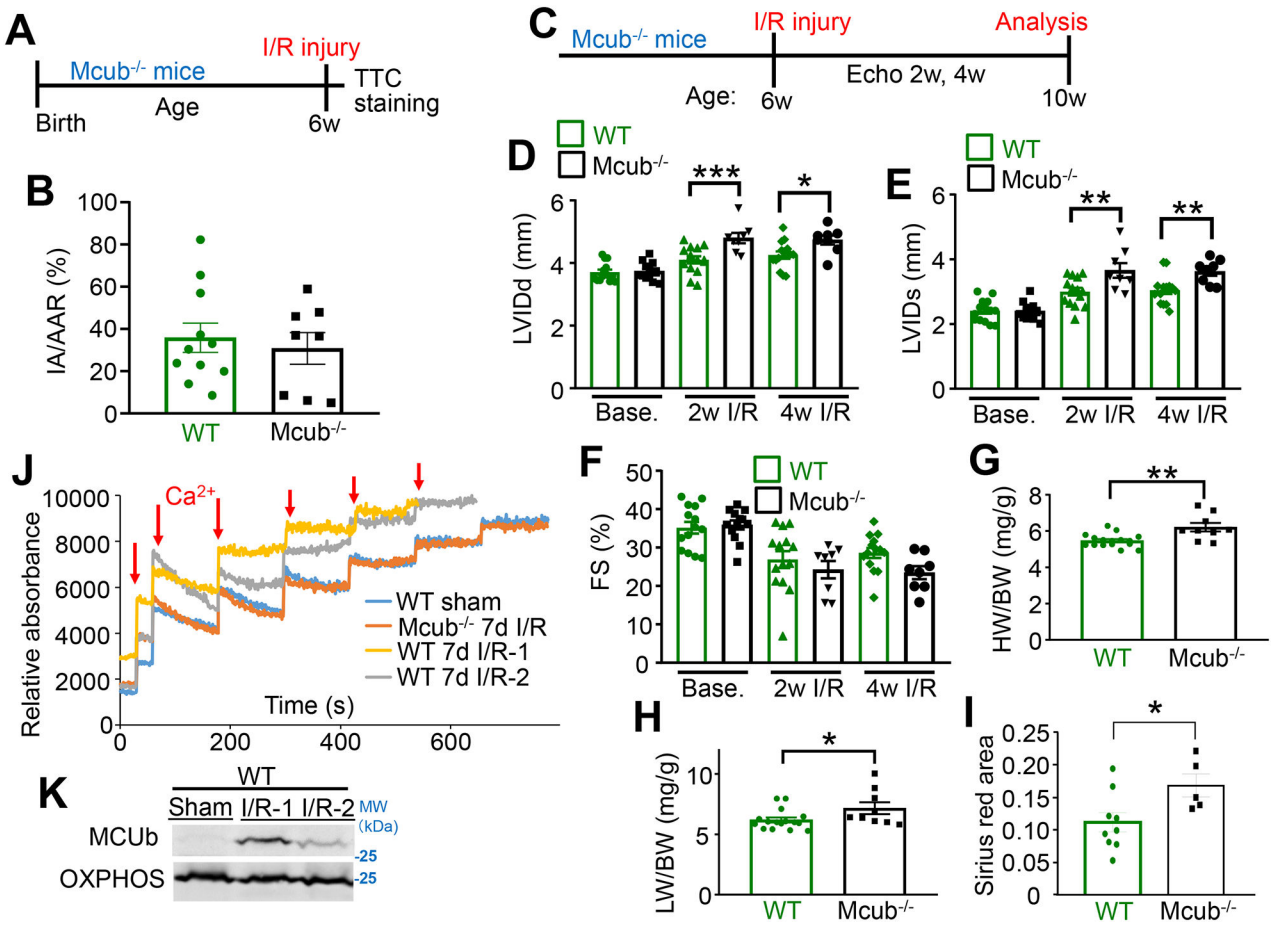


**Figure 5. Cardiac MCUb protein induction protects the heart from damage post I/R injury.**

A) Temporal schematic of the I/R injury model in mice for panel "B". Mice at 6 wks of age were challenged with 60 minutes of ischemia followed by 24 hours of reperfusion. Hearts were sacrificed for TTC staining. B) Both IA and AAR were analyzed and averaged for the indicated groups following I/R injury. n=11 in WT group, n=8 in *Mcub*$^{-/-}$ group. Data presented as mean ± SEM. Student's t-test was used for statistical analysis. C) Temporal schematic of the strategy for assessing infarct expansion following I/R injury, for panels "D-H". Mice at 6 wks of age were challenged with 60 minutes of ischemic injury. Echocardiographic measurements were performed before injury, then 2 and 4 wks of reperfusion. Mice were sacrificed at the 4 wk time point for additional analyses. D-F) Echocardiographic parameters in the indicated groups of mice at the indicated timepoints. LVIDd, end-diastolic left ventricle internal diameter; LVIDs, end-systolic left ventricle internal diameter; FS, fractional shortening. n=14 in WT group, n=12 in *Mcub*$^{-/-}$ group at baseline; n=14 in WT group, n=8 in *Mcub*$^{-/-}$ group post injury. Data presented as mean ± SEM. Two-way ANOVA with a post hoc Bonferroni's multiple comparison test was performed. For "D&E", there was a significant interaction ($p<0.05$) between time and genotype variables. For "F", there was only a significant time effect ($p<0.05$) as the two-way ANOVA showed no significant interaction. Markings denote the following: *$p<0.05$, **$p<0.01$, ***$p<0.001$. G–H) Heart weight/body weight (HW/BW) ratio and lung weight/body weight (LW/BW) ratio in the indicated groups of mice 4 wks post I/R injury. n=14 in

WT group, n=9 in *Mcub*$^{-/-}$ group. Data presented as mean ± SEM. Student's t-test was used for statistical analysis. *p<0.05, **p<0.01. I) Quantification of Sirius Red staining of histological sections from hearts of mice 4 wks post I/R injury in the indicated groups. n=14 in WT group, n=9 in *Mcub*$^{-/-}$ group. Data presented as mean ± SEM. Student's t-test was used for statistical analysis. *p<0.05. J) Mitochondrial $Ca^{2+}$ uptake in isolated mitochondria from the left ventricle of hearts in the indicated groups of mice. Mice were challenged with sham or I/R injury, and then collected 7 days post injury. K) Western blot of MCUb in the same isolated hearts used in "J". OXPHOS antibody was used as the protein loading control.

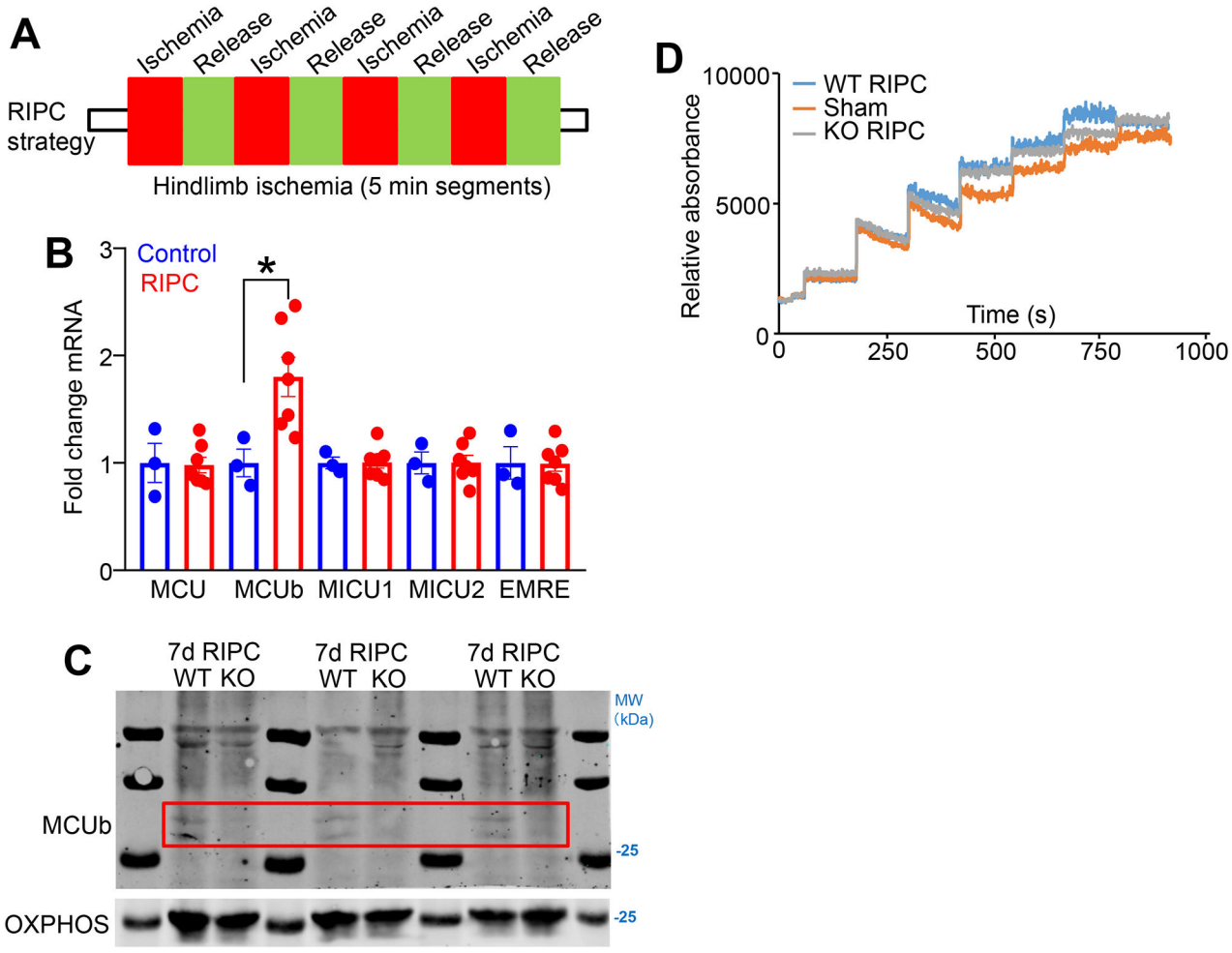


**Figure 6. MCUb is induced in the heart by post remote ischemic pre-conditioning from the hindlimb.**

A) Temporal strategy of the remote ischemic pre-conditioning (RIPC) in mice for panels "B", "C", and "D". Mice at 9–12 wks of age were challenged with hindlimb femoral artery occlusion for 5 minutes followed by 5 minutes of reperfusion, which was repeated with 4 cycles per day for 7 consecutive days. B) mRNA for MCU-complex components *Mcu, Mcub, Micu1, Micu2*, and *Smdt1* (EMRE) from cardiac left ventricle tissue following the 7-day RIPC protocol, versus sham. n=3 in sham group, n=7 in RIPC group. Data presented as mean ± SEM. Student's t-test was used for statistical analysis. *p<0.05. C) Western blot of MCUb in the same isolated hearts used in "D". OXPHOS antibody was used as the protein loading control. The red box shows the area where MCUb protein is induced in the WT heart samples subject to RIPC but not in the $Mcub^{-/-}$ mice (KO). D) Mitochondrial $Ca^{2+}$ uptake assay in isolated mitochondria from the left ventricle of hearts in the indicated groups of mice. Mice were challenged with sham or the 7-day RIPC protocol, and then hearts were collected and mitochondria were purified for analyses.