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ORIGINAL RESEARCH

MCUb Induction Protects the Heart From Postischemic Remodeling

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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 MCU (mitochondrial Ca^{2+} uniporter) 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 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 pathological cardiac remodeling and infarct expansion after ischemic injury in association with greater mitochondrial Ca^{2+} uptake. Furthermore, hindlimb remote ischemic preconditioning 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: A graphical abstract is available for this article.

Key Words: calcium ■ heart ■ infarction ■ mitochondria ■ reperfusion injury

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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 that 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

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Novelty and Significance

What Is Known?

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

What New Information Does This Article Contribute?

- Mcub expression is induced in the heart after ischemia-reperfusion injury or remote ischemic preconditioning.
- 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.

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 to 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 Ca^{2+} 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.

Nonstandard Abbreviations and Acronyms

α-MHC	α-myosin heavy chain
Co-IP	co-immunoprecipitation
dnMCU	dominant negative MCU mutant
EMRE	essential MCU regulator
I/R	ischemia-reperfusion
MCU	mitochondrial calcium uniporter
MEF	mouse embryo fibroblast
MICU1	mitochondrial Ca^{2+} uptake 1
MICU2	mitochondrial Ca^{2+} uptake 2
mPTP	mitochondrial permeability transition pore
NCLX	$Na^{+}/Ca^{2+}/Li^{+}$ exchanger
RIPC	remote ischemic preconditioning
tTA	tetracycline transactivator
WT	wildtype

cyclophilin inhibitory drugs such as cyclosporine A 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 because of the activity of the $Na^{+}/Ca^{2+}/Li^{+}$ exchanger (NCLX), which is also in the mitochondrial inner membrane.[10] The MCU-complex contains 4 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) seems 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 seems 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.[17] 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 lethality (12/13) when I/R was performed 1 week 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 physiological 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 preconditioning (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 physiological role in the heart where its induction after I/R injury limits ongoing pathological remodeling and infarct expansion.

METHODS

Detailed methods are available in the 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 tTA (tetracycline transactivator) TG mouse line with a cardiac-specific promoter (α-MHC [α-myosin heavy chain]) 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 first and second 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] 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, 0.03 mg/mL, subcutaneous injection) to minimize discomfort. For myocardial infarction injury, mice were challenged with permanent left coronary artery ligation and pain medication was given (buprenorphine, 0.03 mg/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 buffer with 1.8 mmol/L Ca^{2+}. Sarcoplasmic reticulum Ca^{2+} load was evaluated by measuring 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 (Figure 1A and 1B). WT mice were challenged with I/R injury to their hearts (60 minutes of ischemia) and then harvested 1, 3, or 7 days afterward. The left ventricle of the heart was used for analysis 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 with sham controls, and this induction was even stronger 7 days after injury (Figure 1A and 1B). 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 nonspecific or did not

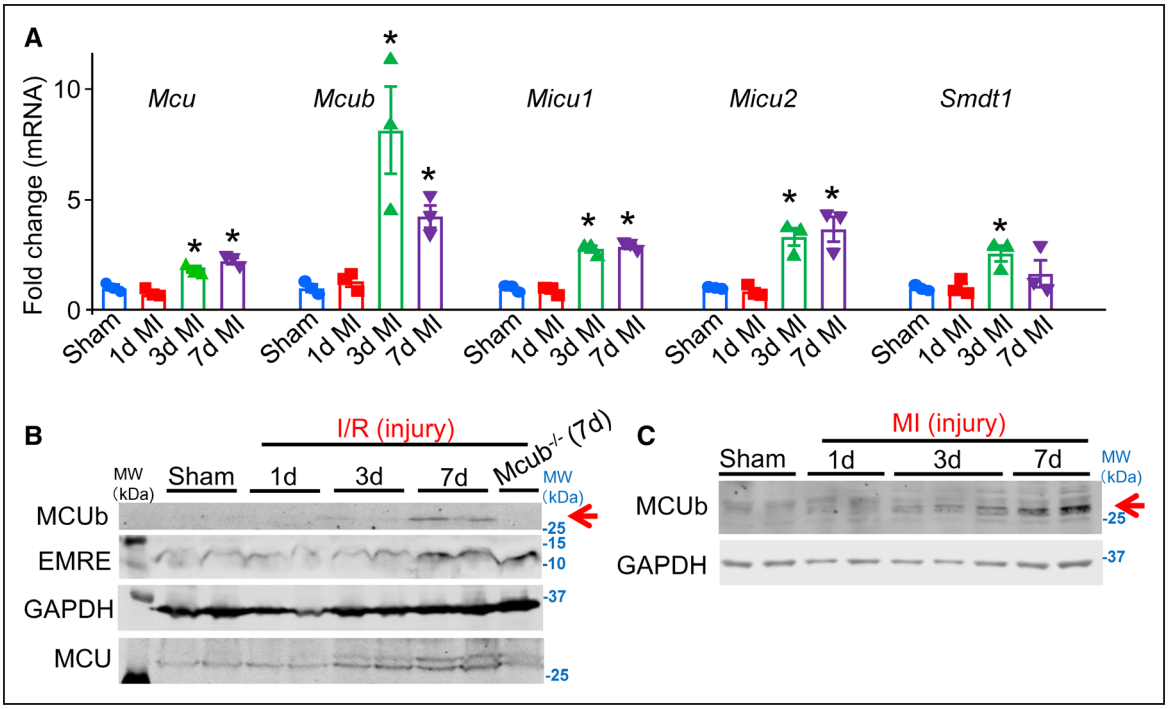

Figure 1. MCUb expression is induced following cardiac ischemic injury.
A, MCU-complex components *Mcu, Mcub, Micu1, Micu2,* and *Smdt1* (EMRE) mRNA levels from cardiac left ventricle tissue and isolated myocytes following ischemia-reperfusion (I/R) injury for 1, 3, or 7 d vs sham. n=3 per group. Data presented as mean±SD of mean (SEM). One-way ANOVA and post hoc Bonferroni test was used for statistical analysis. **P*<0.05 vs sham by post hoc Bonferroni 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 d 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 (Data 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.

detect MCUb. We think that the increase in MCUb protein observed from purified heart mitochondria after I/R injury is dominantly because of cardiomyocyte expression as the contribution to the total mitochondrial content from immune cells, fibroblasts, and endothelial cell fraction in the heart is small. 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* (Figure 1A). Interestingly, we also observed that MCU and EMRE protein levels were increased in the heart 7 days post I/R injury (Figure 1B). To extend these results, permanent myocardial infarction injury was performed on WT mice, which similarly showed induction of MCUb protein in the heart by 3 and 7 days after injury (Figure 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.[22,28] A tTA TG mouse line with cardiac-specific expression (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 (Figure 2A). Western blotting from purified mitochondria from the hearts of tTA-TG, single MCUb-TG, and the MCUb-DTG mice was performed at 6 weeks of age (Figure 2B). The data show no expression of MCUb protein in tTA-TG hearts but substantial expression in the hearts of MCUb-DTG mice (Figure 2B). We should also note that the single MCUb-TG line showed minimally detectable MCUb protein because of the slight leak of the single TG construct (Figure 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 (Figure 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 (Figure 2C). Previous studies

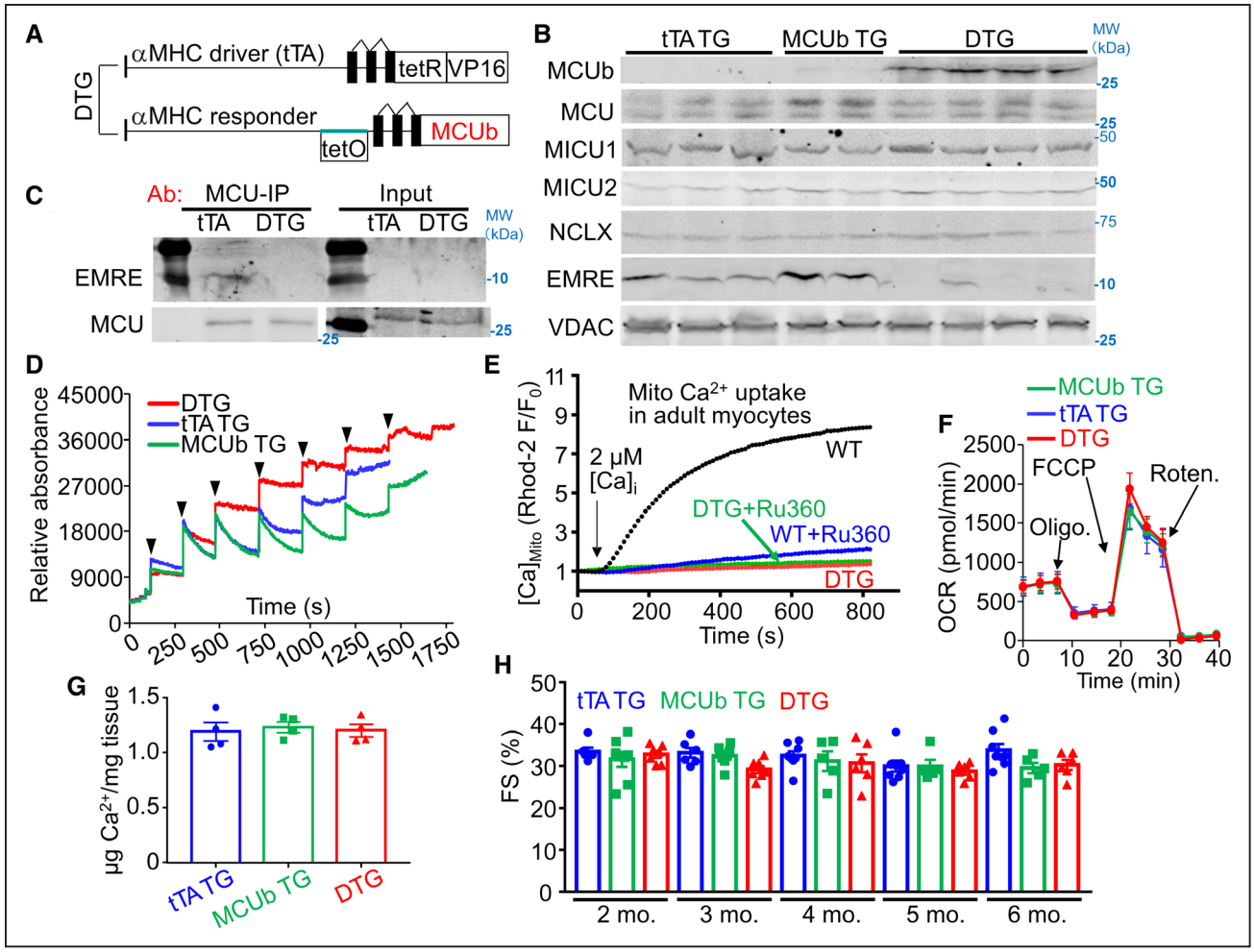

Figure 2. Generation of cardiomyocyte-specific MCUb overexpressing transgenic mice.
A, Schematic of cardiomyocyte-specific MCUb overexpressing mice using a double transgenic (DTG) tet-off system based on the α-MHC (α-myosin heavy chain) promoter. The driver line expresses the tTA (tetracycline transactivator cDNA), 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 protein extracts 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 protein extracts from the indicated 2 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 mo of age. Calcium Green-5 N was used as the Ca^{2+} indicator. The arrows indicate addition of 20 μmol/L $CaCl_2$ 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 mo of age. n=3 per group. Arrows show the position of the 3 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 mo 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.

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 overexpression in the heart (Figure 2B), although NCLX activity was reduced in adult cardiomyocytes from these MCUb-DTG mice (Online Figure 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 2 control groups (Figure 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 with controls, and this block was similar in effect to treatment with the MCU-complex inhibitor Ru360 (Figure 2E). Collectively, these data suggest that MCUb functions as a type of dominant negative effector of the MCU-complex.

Although 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 using a seahorse assay in purified mitochondria from MCUb-DTG hearts, nor was there a reduction in baseline mitochondrial Ca^{2+} content (Figure 2F and 2G). 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 (Figure 2H) nor a change in histopathologic features, which would be expected if there was a metabolic deficiency (Online Figure II). MCUb overexpression also did not alter cardiomyocyte intracellular Ca^{2+} handling, such as the amplitude and kinetics of the Ca^{2+} transient, sarcoplasmic reticulum Ca^{2+} content, or Ca^{2+} spark frequency (Online Figure II). In addition, overexpressing MCUb in the heart did not change mitochondrial ultrastructure or show other pathological features by transmission electron microscopy (Online Figure 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 because of 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-week-old control and MCUb-DTG mice were subjected to 30 minutes of ischemia followed by 24 hours of reperfusion (Figure 3A). Compared with controls, MCUb-DTG mice showed a significant reduction in ischemic injury with no difference in the area at risk (Figure 3B through 3D). Mechanistically, we also observed that overexpression of MCUb decreased mPTP opening frequency to a similar extent as using the mPTP desensitizer cyclosporine A in tTA-TG control mitochondria (Figure 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.

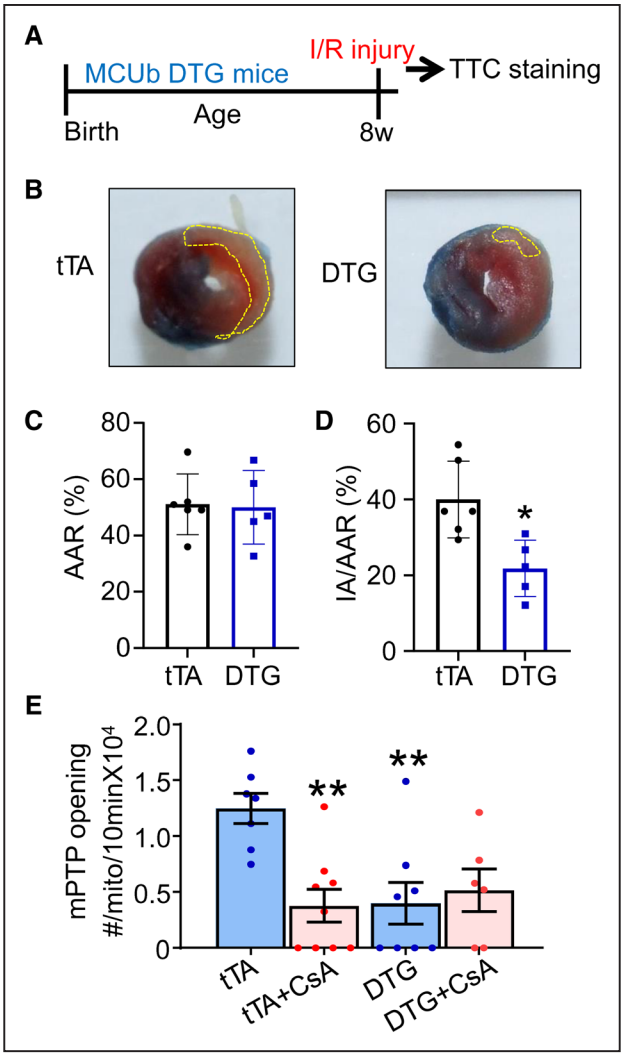

Figure 3. MCUb overexpression generates protection from cardiac ischemia-reperfusion (I/R) injury.
A, Temporal strategy of I/R injury in mice for (**B**), (**C**), and (**D**). Mice at 8 wks of age were challenged with 30 min of ischemia followed by 24 h of reperfusion. **B**, Hearts were sacrificed for 2,3,5-triphenyltetrazolium chloride (TTC) staining. The yellow dotted area shows ischemic region. **C** and **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 (tetracycline transactivator) TG group, n=5 in DTG group. Data presented as mean±SEM. Student *t*-test was used for statistical analysis. **P*<0.05 vs tTA (tetracycline transactivator). **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 multiple comparison test was performed. A significant interaction was found between treatment and genotype (*P*<0.05). ***P*<0.01 vs tTA. WT indicates wild type.

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 (Figure 4A). As MCUb protein expression is not detectable by western blot in the heart under baseline conditions (Figure 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 with controls (Figure 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 (Figure 2B). No alterations were observed in *Mcub*$^{-/-}$ mouse body weights, activity, or gross anatomic features compared with 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 2 different assays (Figure 4C through 4E). Oxygen consumption rate was also not changed in isolated heart mitochondria from *Mcub*$^{-/-}$ mice compared with 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 (Figure 4F). Other quantitative aspects of the intracellular Ca^{2+} transient and Ca^{2+} handling kinetics were also unaltered in adult cardiomyocytes from *Mcub*$^{-/-}$ mice (Figure 4G through 4J). 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 (Figure 4K), nor was there discernible pathology in the heart by electron microscopy (Online Figure 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 Figure 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 Figure VA). We also generated *Mcu*$^{fl/fl}$ MEFs as well as double-targeted *Mcu*$^{fl/fl}$/*Mcub*$^{fl/fl}$-homozygous *loxP* MEFs for analyses. AdCre infection deleted all MCU protein expression in the appropriate MEF lines (Online Figure 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 Figure VB), although 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 Figure 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 a much earlier transition to mPTP and release of all matrix Ca^{2+} was observed (ie, 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 Figure VD).

Endogenous Cardiac MCUb Induction Is Cardioprotective

To examine the physiological function of MCUb protein induction following I/R injury, we used 6-week-old *Mcub*$^{-/-}$ mice subjected to 60 minutes of cardiac ischemia with a 24-hour reperfusion period and then assessed injury (Figure 5A). Importantly, there was no difference in acute injury to the heart as measured by quantifying ischemic area versus the area at risk in *Mcub*$^{-/-}$ mice compared with control mice (Figure 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 postinjury infarct remodeling (Figure 5C). Six-week-old mice were challenged with 60 minutes of ischemia but were not harvested until 4 weeks after surgery and assessed throughout for cardiac function by echocardiography (Figure 5C). We observed that *Mcub*$^{-/-}$ mice developed significantly expanded left ventricle internal diameters, both end-diastolic and end-systolic, at 2 or 4 weeks post-injury (Figure 5D and 5E; Online Figure VI). However, *Mcub*$^{-/-}$ mice showed a trend toward a decrease in ventricular fractional shortening by 2 and 4 weeks post-injury as compared with controls (Figure 5F). Gravimetric analysis showed a significantly greater increase in heart weight/body weight and lung weight/body weight in *Mcub*$^{-/-}$ mice 4 weeks post-I/R injury

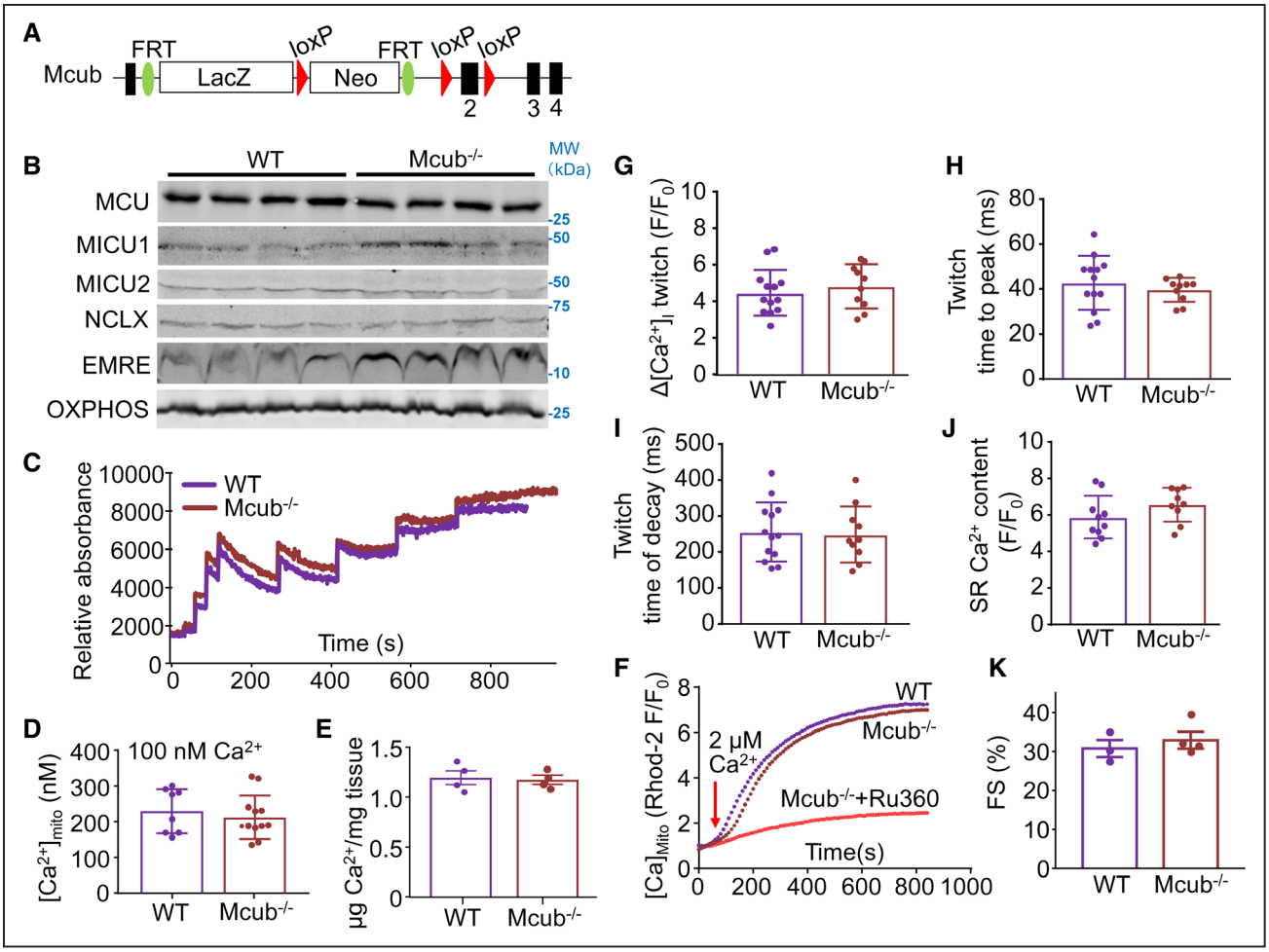

Figure 4. Deletion of *Mcub* does not affect mitochondrial Ca^{2+} uptake or cardiac function at baseline.
A, Strategy for generating *Mcub*-null mice with a "knock-out first" allele strategy in which a combined β-galactosidase/neomycin cDNA cassette was inserted between exon 1 and exon 2 of the *Mcub* gene (exons 2-4 are labeled). **B**, Western blot of MCU (mitochondrial Ca^{2+} uniporter), MICU1, MICU2, NCLX, and essential MCU regulator (EMRE) in cardiac mitochondria from the indicated groups of mice. OXPHOS antibody was used as a control. (This antibody recognizes 5 different mitochondrial Oxphos complexes, although only the 25 kDa band is shown.) **C**, Mitochondrial Ca^{2+} uptake assay in isolated heart mitochondria from the indicated groups of mice at 4 mo 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 *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 mo of age. n=4 per group. Data presented as mean±SEM. Student *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 *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 *t*-test was used for statistical analysis. **K**, Echocardiographic measurement of fractional shortening (FS, %) from the indicated groups at 4 mo of age. n=3 in WT group, n=4 in *Mcub*$^{-/-}$ group. Data presented as mean±SEM. Student *t*-test was used for statistical analysis.

compared with control mice, suggesting greater pathology, most likely because of the effects at the infarct borderzone (Figure 5G and 5H). Indeed, direct measurements of scar area 4 weeks after I/R injury showed a significant increase in *Mcub*$^{-/-}$ mice compared with control (Figure 5I), although initial infarct size 24 hours after I/R surgery was not different (Figure 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 (Figure 5J and 5K). Western blotting from these same hearts again showed that I/R injury resulted in MCUb protein induction over 7 days (Figure 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 with isolated

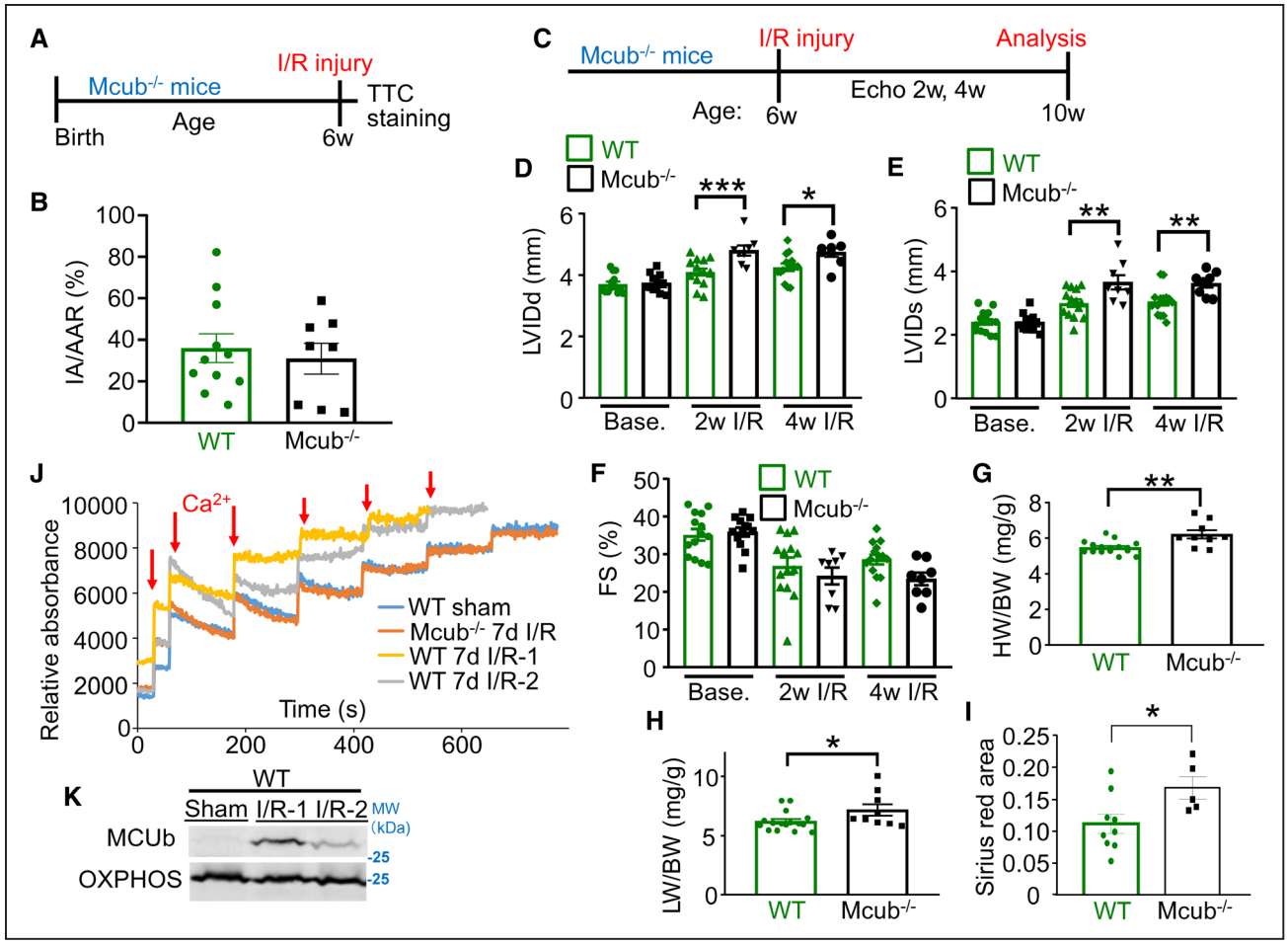

Figure 5. Cardiac MCUb protein induction protects the heart from damage post ischemia-reperfusion (I/R) injury. **A**, Temporal schematic of the I/R injury model in mice for (**B**). Mice at 6 wks of age were challenged with 60 min of ischemia followed by 24 h of reperfusion. Hearts were sacrificed for TTC staining. **B**, Both ischemic area (IA) and average area at risk (AAR) were analyzed and averaged for the indicated groups following I/R injury. n=11 in wild-type (WT) group, n=8 in *Mcub*$^{-/-}$ group. Data presented as mean±SEM. Student *t*-test was used for statistical analysis. **C**, Temporal schematic of the strategy for assessing infarct expansion following I/R injury, for (**D**)–(**H**). Mice at 6 wks of age were challenged with 60 min 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. 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 multiple comparison test was performed. For (**D**) and (**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 2-way ANOVA showed no significant interaction. Markings denote the following: *$P<0.05$, **$P<0.01$, ***$P<0.001$. **G** and **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 *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 wk post-I/R injury in the indicated groups. n=14 in WT group, n=9 in *Mcub*$^{-/-}$ group. Data presented as mean±SEM. Student *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 d post-injury. **K**, Western blot of MCUb in the same isolated hearts used in (**J**). OXPHOS antibody was used as the protein loading control. FS indicates fractional shortening; LVIDd, end-diastolic left ventricle internal diameter; and LVIDs, end-systolic left ventricle internal diameter.

mitochondria from hearts of sham controls (Figure 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 (Figure 5J). Thus, the induction of MCUb protein expression in the heart in the first week 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 Preconditioning Induces MCUb Expression

We also examined if RIPC induces cardiac MCUb expression before 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 minutes followed by 5-min release, with a total of 4 cycles per day for seven consecutive days (Figure 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 (Figure 6B and 6C). Moreover, mitochondria isolated from the hearts of RIPC WT mice had reduced Ca^{2+} uptake rates compared with mitochondria isolated from sham-treated WT control mice (Figure 6D). However, mitochondria isolated from preconditioned *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 Szabò[35] showed that purified MCU protein generates a Ca^{2+} current in a planar lipid bilayer while purified MCUb could not, similar to results of Raffaello et al.[12] 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

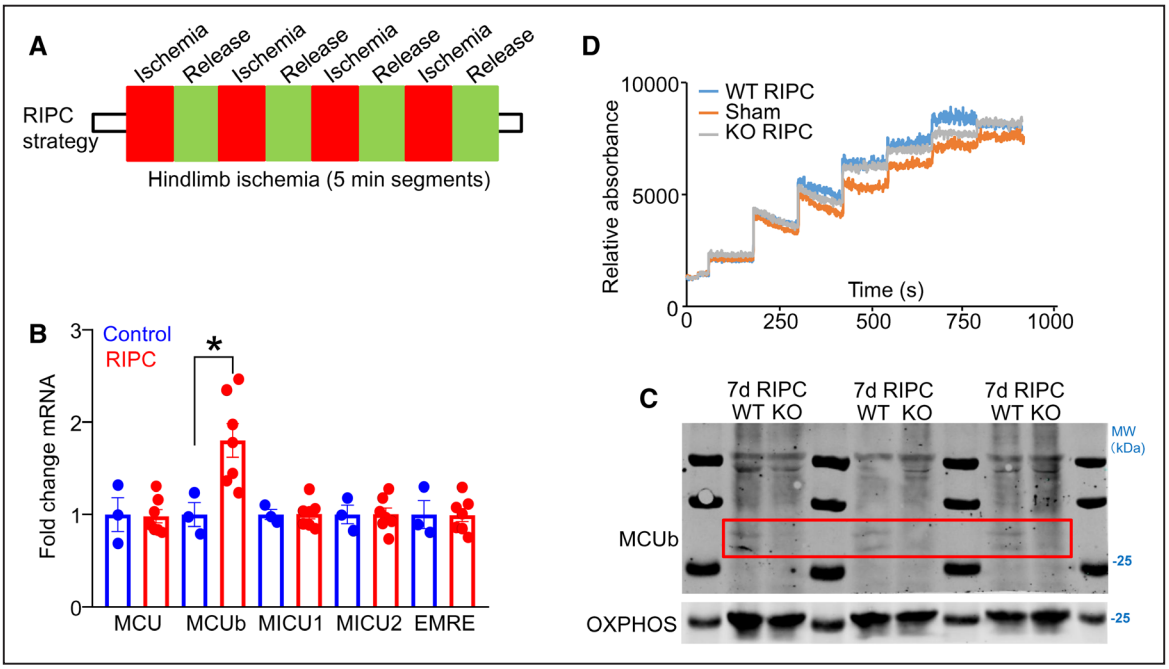

Figure 6. MCUb is induced in the heart by remote ischemic preconditioning from the hindlimb.
A, Temporal strategy of the remote ischemic preconditioning (RIPC) in mice for (**B**), (**C**), and (**D**). Mice at 9–12 wks of age were challenged with hindlimb femoral artery occlusion for 5 min followed by 5 min 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-d RIPC protocol, vs sham. n=3 in sham group, n=7 in RIPC group. Data presented as mean±SEM. Student *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-d RIPC protocol, and then hearts were collected and mitochondria were purified for analyses.

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 (Figure 2B and 2C), although *Mcub*$^{-/-}$ hearts seem to show increased EMRE protein levels (Figure 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 toward 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+}, although 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 myocardial infarction 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 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 although *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 nontoxic MCU-complex inhibitory drug was identified.

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Disclosures

None.

Supplemental Materials

Detailed Online Methods
Online Figures I–VI with legends
Reference[41]

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