The physiological role of mitochondrial calcium revealed by mice lacking the mitochondrial calcium uniporter

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Mitochondrial calcium has been postulated to regulate a wide range of processes from bioenergetics to cell death. Here, we characterize a mouse model that lacks expression of the recently discovered mitochondrial calcium uniporter (MCU). Mitochondria derived from \textit{MCU}\textsuperscript{\textminus/\textminus} mice have no apparent capacity to rapidly uptake calcium. Whereas basal metabolism seems unaffected, the skeletal muscle of \textit{MCU}\textsuperscript{\textminus/\textminus} mice exhibited marked impairment in their ability to perform strenuous work. We further show that mitochondria from \textit{MCU}\textsuperscript{\textminus/\textminus} mice lacked evidence for calcium-induced permeability transition pore (PTP) opening. The lack of PTP opening does not seem to protect \textit{MCU}\textsuperscript{\textminus/\textminus} cells and tissues from cell death, although \textit{MCU}\textsuperscript{\textminus/\textminus} hearts fail to respond to the PTP inhibitor cyclosporin A. Taken together, these results clarify how acute alterations in mitochondrial matrix calcium can regulate mammalian physiology.

Calcium plays a central role in a diverse array of cellular processes including signal transduction, secretion of bioactive molecules, muscle contraction and gene expression. Over fifty years ago, it was demonstrated that fully energized mitochondria could rapidly sequester a large, sudden increase in intracellular calcium\textsuperscript{1,2}. Calcium entry into this organelle requires that the ion traverses both the outer and inner mitochondrial membrane (IMM). Subsequent studies have demonstrated that passage of calcium through the ion-impermeable IMM requires the large membrane potential difference generated by the action of the electron transport chain\textsuperscript{3}. Subsequent physiological and biophysical studies identified that large amounts of calcium could rapidly enter the mitochondrial matrix through this transport mechanism\textsuperscript{4,5}. These observations, along with observations that entry of calcium was not directly coupled to the movement of another ion\textsuperscript{4}, established that mitochondrial calcium uptake occurred through a specific channel, termed the mitochondrial calcium uniporter (MCU), that could bind calcium with nanomolar affinity\textsuperscript{6}. Although it was well known that the entry of calcium could be inhibited by the cell-impermeant compound ruthenium red\textsuperscript{6}, for nearly four decades the identification of this ruthenium red sensitive mitochondrial uniporter remained elusive. That situation changed when two groups recently reported the existence of a transmembrane protein CCDC109A that seemed to fulfil the requirement of the long elusive MCU protein\textsuperscript{9,10}. These groups identified that MCU is a protein with a relative molecular mass of approximately 40,000 (M, 40K) that is widely expressed and localized, as expected, to the IMM (refs 9,10).

Although the molecular identity of MCU was unknown until recently, the role of mitochondrial calcium has been intensively studied over the past four decades. These studies have collectively demonstrated that mitochondrial calcium acutely regulates a range of mitochondrial enzymes involved in either the supply of reducing equivalents\textsuperscript{11} or metabolic substrates\textsuperscript{12}, or electron transport\textsuperscript{13}. Together, these observations supported the notion that MCU-dependent entry of calcium represented a central component of metabolic regulation. Indeed, it had been known that cells and tissues seem capable of exquisitely matching the rate of ATP production with ATP utilization such that even with large fluctuations in power output, levels of metabolic intermediates such as ATP, ADP and \textit{P}, remain

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Figure 1. MCU−/− mice lack MCU expression and evidence for rapid mitochondrial calcium uptake. (a) Besides their size, MCU−/− mice (on the right) lack a discernible phenotype. (b) MCU−/− mice are smaller than WT mice (mean ± s.e.m., \( P < 0.01 \) by analysis of variance; \( n = 14 \) female WT and \( n = 13 \) female MCU−/− mice). (c) MCU mRNA expression by real-time PCR analysis in various tissues of WT, heterozygous and MCU−/− mice (\( n = 3 \) animals per genotype, mean ± s.e.m., \( ^* P < 0.05 \), \( ^{**} P < 0.01 \) by analysis of variance compared with WT expression). (d) MCU protein expression in various tissues using a rabbit polyclonal antibody generated against the C terminus of MCU. Tubulin is used as a loading control. (e) Assessment of mitochondrial calcium levels using the fluorescent mitochondrial calcium sensor Fluo-4FF. Calcium addition over the physiological (micromolar) range results in increasing calcium levels in mitochondria isolated from WT skeletal muscle. This uptake in WT mitochondria is inhibited by Ru360 addition. MCU−/− mitochondria lack any demonstrable uptake. Shown is one experiment that is representative of three similar experiments. Inset: western blot analysis of MCU expression in purified WT and MCU−/− mitochondria with cytochrome C oxidase subunit IV (isoform 1) used as a loading control. Arrows indicate calcium addition. (f) Similar experiment performed using cardiac mitochondria. At higher Ca\(^{2+}\) concentrations, there is a small non-Ru360-inhibitable increase in Fluo-4FF fluorescence in MCU−/− mitochondria observed. (g) Parallel assessment of extramitochondrial calcium measurements demonstrating that only WT cardiac mitochondria seem capable of calcium uptake. Uncropped images of blots are shown in Supplementary Fig. 9.

unchanged\(^{14,15}\). This has been extensively studied in tissues such as the heart or skeletal muscle that see large and acute changes in their energy utilization when, for instance, the organism goes from a resting state to a full speed sprint. Under these conditions, it has been widely believed that the entry of mitochondrial calcium augments mitochondrial ATP production to acutely match the rapid increase in ATP demand\(^{11,16-18}\).

Although the entry of small amounts of calcium may have beneficial effects for metabolic homeostasis, there is a significant amount of data demonstrating that the uptake of large amounts of calcium can induce cell death\(^{19,20}\). The basis for this phenomenon involves opening of the permeability transition pore (PTP). Although the precise molecular make-up of the PTP has remained elusive, evidence suggests that the entry of calcium through a MCU-dependent mechanism is the central mediator of PTP opening\(^{21-23}\). Once opened, the PTP results in depolarization of the IMM leading to collapse of the mitochondrial membrane potential and thus inhibition of electron transport and mitochondrial-dependent ATP production. This has led to the widespread belief that targeting this pathway, including the development of potential inhibitors of MCU, might be a robust strategy...
MCU regulates mitochondrial calcium uptake in permeabilized MEFs. (a) Comparison of cytosolic calcium levels in permeabilized WT and MCU−/− MEFs. Arrows indicate calcium addition. Increasing cytosolic calcium results in a rapid increase in the fluorescent signal in both cell types but the subsequent decline in the fluorescent signal, representing mitochondrial calcium uptake, is observed only in WT cells. Shown is one tracing that is representative of three similar experiments. (b) Ruthenium red (Ru360; 3 µM) inhibits mitochondrial calcium uptake in permeabilized WT MEFs. Shown is one tracing that is representative of three similar experiments. (c) Western blot analysis of MCU MEFs: +/+ –/– –/– –/– representative of three similar experiments. (d) Cytosolic calcium measurements in permeabilized WT MEFs, MCU−/− MEFs, MCU−/− MEFs reconstituted with WT MCU and MCU−/− MEFs reconstituted with MCUMUT. Uncropped images of blots are shown in Supplementary Fig. 9.

Figure 2 MCU regulates mitochondrial calcium uptake in permeabilized MEFs. In vivo. To determine the physiological functions of MCU, we constructed a mouse model using a gene trap strategy in which a retroviral trapping vector was integrated into the first intron of the CCDC109A locus (Supplementary Fig. S1a,b). Although slightly smaller (Fig. 1a,b), organ weight was proportional to body size and overall body composition was indistinguishable between young and old wild type (WT) and MCU−/− mice (Supplementary Fig. S1c,d). Further examination of MCU−/− embryonic and adult tissues by electron microscopy revealed no obvious abnormalities in mitochondrial number or morphology (Supplementary Fig. S2a). Although mice generated using a gene trap strategy sometimes exhibit hypomorphic expression of the targeted gene, there was little evidence of MCU messenger RNA expression (Fig. 1c). Similarly, western blot analysis using antibodies recognizing either amino-terminal or carboxy-terminal regions of the protein failed to detect any MCU protein expression in tissues derived from MCU−/− mice (Fig. 1d and Supplementary Fig. S2b,c).

We next sought to assess whether the absence of MCU expression altered mitochondrial calcium uptake. When purified mitochondria isolated from skeletal muscle of adult WT and MCU−/− mice were loaded with the calcium-sensitive dye Fluo-4FF, WT mitochondria exhibited robust calcium uptake over a range of physiologically to block injury that occurs in a wide array of clinically important disease processes from ischaemia to neurodegeneration.

Taken together, there is a long and extensive literature suggesting that dynamic alterations in mitochondrial calcium play a central role in a wide range of physiological conditions from acute metabolic regulation to determining the threshold for cell death. Nonetheless, most of these studies have been hampered by the inability to directly alter in vivo mitochondrial calcium uptake. Although ruthenium red and some of its derivatives readily block MCU function, these agents cannot reliably enter cells, thus limiting this as an approach to dynamically regulate its derivatives readily block MCU function, these agents cannot reliably enter cells, thus limiting this as an approach to dynamically regulate.

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relevant extramitochondrial calcium concentrations (Fig. 1e). This uptake was inhibited in WT mitochondria by the addition of the potent ruthenium red subcomponent, Ru360. However, MCU−/− skeletal muscle mitochondria, which lacked detectable MCU expression (Fig. 1e, inset), exhibited no appreciable calcium uptake over a range of physiological calcium concentrations. Further experiments performed with heart mitochondria revealed similar findings, although with cumulative additions of micromolar concentrations of calcium, we did note that MCU−/− cardiac mitochondria marginally increased their Fluo-4PF fluorescence (Fig. 1f), but this signal seemed to represent nonspecific leakage of dye out of the mitochondrial matrix as indicated by rapid quenching of the fluorescent signal with EGTA (Supplementary Fig. S3a,b). We performed parallel experiments using Calcium Green-5N to measure extramitochondrial calcium levels (Fig. 1g). In these experiments, calcium addition to WT cardiac mitochondria resulted in a rapid increase in Calcium Green-5N fluorescence followed by a slower decline in the fluorescence intensity of the calcium sensor (Fig. 1g). This slower decrease in fluorescence after calcium addition is consistent with the known buffering capacity of mitochondria to uptake cytosolic calcium. At higher concentrations of calcium (>15 μM), buffering capacity is eventually exceeded and stored mitochondrial calcium is released into the extramitochondrial space. Over the same range of calcium concentration, MCU−/− mitochondria did not demonstrate evidence for either mitochondrial uptake or release.

To further analyse the function of MCU within intact cells, we generated primary mouse embryonic fibroblasts (MEFs) from WT and MCU−/− embryos. Functional assessment revealed that when calcium was added to permeabilized MEFs, there was an initial rapid spike in Calcium Green-5N fluorescence intensity (Fig. 2a), which was followed by a slower decline in the fluorescence intensity of the cytosolic calcium sensor. As described above, this decline in fluorescence corresponds to mitochondrial calcium uptake. In permeabilized WT MEFs, in the presence of ER calcium uptake inhibition, mitochondrial uptake continued until the calcium retention capacity of the mitochondria was exceeded. At this point (for example, the fourth addition of calcium in Fig. 2a), any further increase in extramitochondrial calcium resulted in a large rise in measured fluorescence. This terminal, large fluorescent increase indicates the irreversible release of mitochondrial calcium stores and is commonly used as an index of PTP opening (similar to Fig. 1g). In contrast, calcium addition to MCU−/− MEFs resulted in a staircase profile consistent with the inability of MCU−/− mitochondria to rapidly uptake calcium (Fig. 2a). The profile seen in MCU−/− MEFs was similar to what is observed when WT MEFs were treated with ruthenium red (Fig. 2b). Retroviral-mediated reconstitution of WT MCU expression restored mitochondrial calcium uptake in MCU−/− MEFs (Fig. 2c,d). In contrast, a mutant of MCU where the critical acidic linker region was mutated at amino acids 261 and 264 was unable to reconstitute calcium uptake when expressed in MCU−/− cells.

To assess mitochondrial calcium uptake in a more physiological context, we isolated adult cardiac myocytes from WT and MCU−/− mice. Consistent with previous results15, in WT myocytes loaded with the mitochondrial-targeted calcium-sensitive dye Rhod-2 in the presence of manganese added to quench any cytosolic signal, isoproterenol addition stimulated a measurable rise in mitochondrial calcium levels (Fig. 3a). This response was absent in cardiac myocytes obtained from MCU−/− mice. Mitochondrial calcium levels were also unchanged in MCU−/− myocytes treated with agents (KCl and caffeine) that have been shown to directly increase cytosolic calcium (Supplementary Fig. S3c,d). Similarly, analysis of MEFs engineered to express a mitochondrial-targeted aequorin construct. Levels of mitochondrial calcium were assessed by aequorin luminescence following histamine stimulation (100 μM) for WT (n = 10) and MCU−/− MEFs (n = 12). Values were normalized to maximal aequorin luminescence observed in permeabilized cells exposed to exogenous calcium. (c) Cytosolic calcium levels in WT (n = 14) or MCU−/− MEFs (n = 17) as measured by Fluo-4 fluorescence following histamine stimulation. All pooled data represent mean ± s.e.m.
We next sought to ascertain the role of MCU in basal metabolism. (Fig. 4a and Supplementary Fig. S4a). This lack of a discernible given glutamate and malate as a substrate (G/M) followed by ADP (state of the oxygen consumption observed in isolated hepatic mitochondria n electron transport inhibitor antimycin A (Fig. 6b). Similarly, the response to ADP and the respiratory control ratio (state 3/state 4) were indistinguishable (Fig. 4b,c). Furthermore, metabolic assessment of total body basal oxygen consumption was not altered (Fig. 5a). In contrast, no calcium-dependent stimulation was observed in the respiration of mitochondria obtained from MCU−/− mice. Although informative, such experiments using isolated mitochondria and high concentrations of calcium are by nature non-physiological. Thus, to better understand the physiological importance of mitochondrial calcium, we concentrated on the role of MCU in skeletal muscle physiology in vivo. One postulated effect of intramitochondrial calcium is the regulation of key mitochondrial enzymes involved in bioenergetics and metabolic flux. In particular, the phosphorylation of pyruvate dehydrogenase (PDH) is thought to be modulated by the calcium-sensitive phosphatase PDP1 (ref. 29). We reasoned that MCU−/− mice should allow a direct test of whether PDH is indeed regulated in vivo by these mechanisms. We first measured resting matrix calcium levels in mitochondria isolated from the skeletal muscle of WT and MCU−/− mice. We noted an approximate 75% reduction in basal matrix calcium levels in the mitochondria of mitochondria, cells derived from MCU−/− mice seem unable to rapidly uptake calcium.

**MCU and metabolism**

We next sought to ascertain the role of MCU in basal metabolism. Surprisingly, when we analysed oxygen consumption of MCU−/− MEFs under basal conditions or following the addition of oligomycin, the uncoupler FCCP or the electron transport inhibitor antimycin A (n = 5). (b) Representative tracings of the oxygen consumption observed in isolated hepatic mitochondria given glutamate and malate as a substrate (G/M) followed by ADP (state

![Figure 4](image-url)
**Figure 5** Altered in vivo skeletal muscle metabolism and PDH activity in MCU⁻/⁻ mice. (a) Mitochondrial oxygen consumption following depolarization induced by the addition of 500 µM calcium with and without the respiratory chain inhibitor antimycin A (15 µM). The average ± s.e.m. of three independent experiments is shown. **P < 0.01 by analysis of variance compared with without calcium. (b) Levels of matrix calcium measured in WT and MCU⁻/⁻ mitochondria derived from skeletal muscle following an overnight 16 h fast (**P < 0.01 by t-test; n = 3 female WT and n = 4 female MCU⁻/⁻ mice). (c) Western blot determination of the levels of phospho-PDH (Ser 293 of the E1 subunit) and total PDH levels in the skeletal muscle of three pairs of WT and MCU⁻/⁻ mice starved for 16 h. (d) Under starved conditions, altered PDH phosphorylation is seen in various muscle types including the extensor digitorum longus (EDL) representing glycolytic/fast twitch fibres, the soleus (SOL) that is predominantly oxidative/slow twitch and the gastrocnemius (GN) that is a mix of fast and slow twitch. (e) Skeletal muscle PDH activity in units of milli optical density (mOD) for WT and MCU⁻/⁻ mice after a 16 h fast (**P < 0.05; n = 3 mice per genotype). (f) Serum lactate levels in WT and MCU⁻/⁻ male mice under fed conditions or after 16 h of starvation (n = 7 WT and n = 6 MCU⁻/⁻; * P < 0.05 by t-test). (g) Metabolomic analysis of skeletal muscle demonstrating levels of various TCA cycle intermediates in mice that were starved overnight (n = 3 male mice per genotype). There was a trend for increased lactate levels in the MCU⁻/⁻ muscle. All pooled data represents mean ± s.e.m. Uncropped images of blots are shown in Supplementary Fig. 9.

MCU⁻/⁻ mice (Fig. 5b). Using a phoso-specific PDH E1 subunit antibody, we next analysed the level of PDH phosphorylation in three pairs of WT and MCU⁻/⁻ mice. Consistent with the role for the calcium-sensitive phosphatase PDP1 regulating PDH phosphorylation, lower levels of matrix calcium in the MCU⁻/⁻ mice led to markedly increased levels of PDH phosphorylation in these animals (Fig. 5c). We noted these changes across a variety of muscle types including skeletal muscle groups that were either fast twitch, slow twitch or mixed fibres (Fig. 5d). These phosphorylation differences were most evident under starved conditions and largely disappeared when the animals were re-fed (Supplementary Figs 5a–c). Similarly, direct addition of calcium to WT mitochondria led to a rapid reduction in PDH E1 phosphorylation, a response not evident in isolated MCU⁻/⁻ mitochondria (Supplementary Fig. 5d). Previous results have indicated that this phosphorylation change on the E1 subunit is known to negatively regulate PDH activity.³⁰ Consistent with these past results, PDH activity was significantly reduced in the skeletal muscle of starved MCU⁻/⁻ mice, whereas citrate synthase activity was not altered (Fig. 5e and Supplementary Fig. S5e). Finally, a decrease in PDH activity often correlates with a rise in serum lactate levels. We noted that MCU⁻/⁻ mice indeed had a significantly elevated serum lactate level (Fig. 5f). Direct measurement of muscle lactate levels demonstrated a similar trend, whereas other tricarboxylate acid (TCA) metabolites were not altered (Fig. 5g).

**MCU regulates skeletal muscle work**

The entry of calcium into the mitochondria during various physiological stresses is thought to increase mitochondrial energy production.¹¹ We next designed a number of functional tests that
required a rapid increase in skeletal muscle work load. When we assessed skeletal muscle peak performance by placing mice on a rapid, steep treadmill, we observed a statistically significant impairment in the exercise capacity of MCU−/− mice (Fig. 6a). Similarly, when we assessed forearm grip strength, a measure of predominantly isometric muscle contraction, we also noted a small, but significant decrease in the strength of MCU−/− mice (Fig. 6b). This defect was more pronounced when we measured the ability of the mice to perform a pull up manoeuvre that requires concentric muscle contraction and hence increased power output (Fig. 6c). These observed defects in the ability of MCU−/− animals to generate maximal power output were not due to any apparent alterations in skeletal muscle fibre composition (Fig. 6d and Supplementary Fig. S6).

The role of MCU in cell death

The opening of the PTP causes a rapid inflow of solutes inducing mitochondrial swelling and can be readily monitored by a number of approaches including a rapid fall in absorbance. Consistent with previous results, the addition of extramitochondrial calcium to WT cardiac mitochondria induced PTP opening (Fig. 7a). For WT mitochondria, this opening was inhibited by addition of either cyclosporin A (CsA) or Ru360. In contrast, MCU−/− mitochondria isolated from either liver or heart exhibited no evidence of PTP opening even after exposure to high levels of calcium for prolonged periods of time (Fig. 7a,b and Supplementary Fig. S7a). As such, these observations are consistent with our previous observations in permeabilized cells (see Fig. 2a) and demonstrate that MCU is required for calcium-induced PTP opening.

We next sought to understand the physiological importance of these observations. We took MCU−/− or WT MEFs and exposed them to a range of potential inducers of cell death. These agents included hydrogen peroxide as a prototypical example of oxidative stress; the ER stress inducing agent tunicamycin; the chemotherapeutic and DNA damaging agent doxorubicin; C2-ceramide, an agent that can activate both apoptotic and necrotic pathways; and thapsigargin, which triggers cell death by interfering with ER calcium uptake. We monitored cell death using the combination of annexin V and staining. Although all of these agents were effective at inducing cell death, we noted no difference in the kinetics or magnitude of cell death in MEFs with or without MCU expression (Fig. 7c). We also noted that the release of cytochrome C from the mitochondria into the cytosol was not affected by MCU expression, nor did we observe a significant difference in cytosolic calcium levels during cell death (Fig. 7d and Supplementary Fig. S7b,c). For both tunicamycin and doxorubicin we also measured the degree of caspase-3 activity. Again, we noted indistinguishable levels of caspase-3 activation in WT and MCU−/− cells (Fig. 7e).

Finally, to assess whether similar effects were observed in tissues and organs, we subjected hearts of WT and MCU−/− mice to ischaemia–reperfusion injury. When the degree of injury was assessed by functional measurements such as the post-ischaemic recovery in the rate pressure product or by pathological measurements such as direct assessment of the infarct area, our analysis revealed that MCU−/− mice exhibited no evidence for protection from ischaemia–reperfusion-mediated injury (Fig. 8a,b). Similarly, the level of apoptosis and the magnitude of ischaemic contracture was equivalent in WT and MCU−/− hearts subjected to ischaemia–reperfusion injury (Supplementary Fig. S8a,b). Interestingly, whereas, as expected, the PTP inhibitor CsA provided significant protection to WT hearts, this agent had no demonstrable effect on MCU−/− hearts (Fig. 8a,b).

DISCUSSION

In summary, we provide an in vivo description of the physiological effects seen in the absence of MCU expression. Remarkably, although mitochondria and cells from MCU−/− mice lack evidence for high-capacity calcium uptake, these animals are grossly normal. Although this result is unexpected, it should be noted that a previous study found no significant organ pathology following continuous short interfering RNA (siRNA)-mediated knockdown of MCU in the liver. Furthermore, few discernible phenotypes were observed in mice genetically deficient for myoglobin, creatine kinase or creatine, molecules that were all originally thought to be absolutely essential for cardiac and skeletal muscle bioenergetics. Indeed, in the context of the whole animal, the absence of MCU produces relatively minor alterations in basal energetics. However, we should note that we found a significant reduction, but not a complete absence, of mitochondrial matrix calcium in mice lacking MCU. For instance, in our analysis, matrix calcium seemed to be reduced to about 25% of WT levels in the skeletal muscle mitochondria of starved MCU−/− mice. This non-zero value suggests that alternative mechanisms must exist for calcium entry, although on the basis of our cellular and isolated mitochondrial studies; the high capacity, rapid entry mode that occurs through the uniporter is clearly absent. It is conceivable that these slower and presumably low capacitance mechanisms might allow for some physiological adaptation over time.
We also observed that although MCU−/− mitochondria lacked evidence for calcium-induced PTP opening, the absence of MCU expression did not result in a demonstrable alteration in the magnitude of in vitro or in vivo cell death. Previous results with transient manipulation had suggested that altering MCU expression modulated the sensitivity to cell death in many but not all cellular systems. Interestingly, we observed that although the absence of MCU did not alter the magnitude of cell death, MCU−/− hearts were insensitive to CsA treatment. One interpretation is that in the absence of MCU, additional CsA-independent and calcium-independent cell death pathways emerge and predominate. Interestingly, yeast mitochondria lack an MCU equivalent but still seem to undergo PTP opening, although this event seems to be insensitive to both calcium and CsA.

Finally, although there is unequivocal evidence for the in vitro regulation of mitochondrial dehydrogenase function by calcium, the observation that MCU−/− mice have limited defects in basal metabolism suggests that the in vivo effects of altering matrix calcium may be most important under certain stress conditions. Our results demonstrate that in tissues such as skeletal muscle, which exhibit a large dynamic energetic range, mitochondrial calcium regulates the intrinsic metabolism of the tissue as well as the peak performance. The observation that the defect in maximal skeletal muscle power output of MCU−/− mice is qualitatively similar to what was observed in mice deficient in creatine kinase suggests that this property of skeletal muscle, that is, the ability to rapidly boost and maintain peak power output, is for obvious reasons, under intense evolutionary pressure.

**Figure 7** MCU expression is necessary for calcium-induced PTP opening but not required for cell death. (a) Only WT mitochondria undergo PTP opening after calcium addition (500 μM) as evidenced by a rapid drop in absorbance. Shown is one experiment using heart mitochondria that is representative of three similar experiments. (b) Average change in absorbance following calcium addition from three independent experiments using isolated cardiac mitochondria in the presence or absence of CsA (0.2 μM) and Ru360 (3.0 μM). **P < 0.01 by analysis of variance.** (c) Cell viability as measured by annexin V/propidium iodide staining in WT and MCU−/− MEFs following a wide array of challenges including hydrogen peroxide exposure (1 mM), tunicamycin (2 μg ml−1), doxorubicin (2 μM), C2-ceramide (100 μM) and thapsigargin (1 μM). The time course and magnitude of cell death was not altered by the absence of MCU expression (P value is not significant; n = 3 per genotype). (d) Cytosolic cytochrome C levels in WT or MCU−/− MEFs following the addition of hydrogen peroxide. Tubulin is shown as a loading control. (e) Caspase-3 activity was measured under basal conditions or 24 h after treatment with tunicamycin or doxorubicin (P value is not significant; n = 3). All pooled data represent mean ± s.e.m. Uncropped images of blots are shown in Supplementary Fig. 9.
**Figure 8** Role of MCU in ischaemia-reperfusion injury. (a) Assessment of the rate pressure product (RPP; heart rate times systolic blood pressure) after ischaemia-reperfusion injury in the hearts of female WT or MCU<sup>−/−</sup> mice with and without CsA (0.2µM) for five minutes before ischaemia. (b) Infarct size in WT and MCU<sup>−/−</sup> mice following 20 min of global ischaemia and 90 min of reperfusion. *P < 0.05, **P < 0.01 by analysis of variance (NS, not significant); all pooled data represent mean ± s.e.m.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

X.P., J.L. and T.N. designed, performed and analysed the experiments and aided in writing the manuscript; C.L. helped construct the mouse model, J.S., Y.T., M.M.F., A. Wiederkehr for the original mito-aequorin adenovirus. This work was supported by NHLBI Electron Microscopy Core Facility for assistance with electron microscopy, C. Petucci of the Metabolomics Core Facility Sanford-Burnham Medical Research Institute for aiding in the metabolomic profiling, C. Combs and the NHLBI Microscopy Core for help with the Rhod-2 fluorescent measurements and A. Wiederkehr for the original mito-aequorin adenovirus. This work was supported by NIH Intramural funds.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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METHODS

Generation of MCU knockout mouse and MEF cells. Two independent gene-trapped embryonic stem cell clones with confirmed integration within the CCDC109A (MCU) locus were obtained from the Texas A&M Institute of Genomic Medicine (College Station). These two embryonic stem cell clones were microinjected into blastocysts collected from albinio C57BL/6 mice (JAX Stock No. 000058), but only one embryonic stem cell clone (ID#: IST116698) gave rise to chimaeas signifying germline transmission. As shown in Supplementary Fig. S1, the retrovirus-based gene-trapping vector, Omnimab Vector 76, was integrated into Intron 1 of the MCU (also known as Cak109a) gene. The mutant mouse line was maintained on a mixed genetic background of C57BL/6 and CD1 (Charles River Laboratories). Physiological assessment was performed on 3–5-month-old mice. Body composition (muscle, fat and free fluid) was measured in non-anesthetized mice using an EchomRI QMR machine. All mouse embryonic fibroblasts (MEFs) were primary cultures generated from 11.5 to 13.5 day mouse embryos using standard methods. All animal studies were done in accordance and approval of the NHLBI Animal Care and Use Committee.

Mitochondria isolation and metabolic studies. Mitochondria were isolated by standard differential centrifugation procedures. For cardiac mitochondria, hearts were first minced in mitochondrial isotonic buffer (225 mM mannitol, 75 mM sucrose, 5 mM MOPS, 0.5 mM EGTA and 2 mM taurine (pH 7.25)), and subsequently homogenized using an Ultra-Turrax homogenizer (IKA Labortecknik). To help digest the contractile protein apparatus, trypsin (0.001 g per 0.1 g wet tissue) was added to the homogenate for 5 min on ice. This digestion was stopped by addition of 0.2% BSA. The homogenate was initially centrifuged at 900g and the resulting supernatant was spun at 11,000g to pellet the mitochondria. The final mitochondrial pellet was resuspended in mitochondrial isotonic buffer + 0.2% BSA.

For isolation of hepatic mitochondria, liver tissue was washed 4X with PBS and then minced in mitochondrial isotonic buffer containing 0.2% BSA. Skeletal muscle mitochondria, the overlying fat and connective tissue was removed, and the skeletal muscle was subsequently minced in mitochondrial isotonic buffer, and then incubated for 2 min in 10 ml of protease buffer (10 ml isotonic buffer and 1 mg nasear [Sigma, 10.6 U mg⁻¹]). The digestion as above was stopped by addition of 0.2% BSA. The homogenate was centrifuged and resuspended as above.

Oxygen consumption of isolated mitochondria was measured in a chamber connected to a Clark-type O₂ electrode (Inotech) and O₂ monitor (Model 5300, YSI Inc) at 25 °C. Mitochondria were incubated in respiration buffer (120 mM KCl, 5 mM MOPS, 0.1 mM EGTA, 5 mM KH₂PO₄ and 0.2% BSA). After addition of 10 mM glutamate/2 mM malate, state 3 (maximal) respiration was measured by addition of 0.5 mM ADP, or where indicated, 0.5 mM CaCl₂. The respiratory control ratio was determined by the state 3/state 4 respiration rates. Measurement of intact muscle respiration was performed using the Seahorse XF24 analyzer. Respiration was measured under the basal condition, in the presence of the mitochondrial electron transport inhibitors oligomycin (5 μM) and in the presence of the mitochondrial uncoupler FCCP (10 μM) to assess maximal oxidative capacity. Respiration was routinely measured using 25 mM glucose as the extracellular substrate, although further experiments were performed using pyruvate (2 mM) or for cells maintained in the presence of galactose (25 mM).

Total body oxygen consumption and CO₂ production were measured by an open circuit indirect calorimeter (CLAMS, Columbus Instruments). Mice had ad libitum access to food and water and were singly housed one day before the experiment in sealed plastic metabolic cages that were connected to O₂ and CO₂ sensors. After the one day acclimation period, energy expenditure and respiratory exchange ratio were measured for three consecutive days at 30-min intervals.

Calcium retention capacity assay. Calcium uptake in MEFs was assessed using the calcium retention capacity assay as previously described. In brief, WT and MCU−/− MEFs were collected using trypsin and then washed in PBS. The calcium retention capacity assay was performed in a buffer containing 120 mM KCl, 10 mM Tris-HCl at pH 7.4, 5 mM MOPS, 5 mM Na₂HPO₄, 10 mM glutamate, 2 mM malate, 0.002% digitonin, and 0.5 mM thapsigargin to inhibit ER calcium uptake. Cytoplasmic calcium was measured using the fluorescent Ca²⁺ indicator Calcium Green-SN (1 μM; Molecular Probes). Experiments were performed in the presence and absence of 3 μM ruthenium red.

Intramitochondrial calcium assays. Direct mitochondrial calcium uptake was measured essentially as previously described. In brief, isolated mitochondria were loaded with 20 μM Fluor-4 FF AM for 30 min at room temperature. After loading, mitochondria were washed 3X with mitochondrial isotonic buffer (225 mM mannitol, 75 mM sucrose, 5 mM MOPS, 0.5 mM EGTA and 2 mM taurine (pH 7.25)). Assays were performed in a buffer solution composed of 137 mM KCl, 20 mM EGTA, 20 mM HEPES, 5 mM glutamate/malate, 2 mM KH₂PO₄, at pH 7.15, 5 mM NaCl, 10 μM GPP37157, 2.5 μM thapsigargin and, where indicated, 3 μM Ru360. Free Ca²⁺ changes of 0.011, 0.2, 0.5, 5.7 and 15.3 μM were added and Fluor-4-FF fluorescence determined. In some experiments, parallel determination of extramitochondrial calcium was performed using Calcium Green-5N as described above.

Measurement of mitochondrial [Ca²⁺] in intact cells. Adult cardiomycocytes were isolated from hearts of WT and MCU−/− mice as previously described. Myocytes were then attached to Cell-Tak (BD Biosciences)-coated glass-bottom culture dishes. After attachment, myocytes were loaded with 5 μM Rhod-2 AM along with 200 μM MnCl₂ to allow selective quenching of the cytosolic fluorescence. After 15 min preincubation of the myocytes in saline with the adenovirus, a series of images were captured every 2–3 s by confocal microscopy using a Zeiss LSM 510 microscope (Carl Zeiss) using 561 nm laser excitation. For measurement of mitochondrial calcium in intact MEFs, we infected primary WT and MCU−/− MEFs with an adenovirus that expressed a mitochondrial-targeted aequorin construct. The targeted aequorin was amplified by PCR using a previously described adenovirus (Ad-RIP-mitoaequorin) a gift from A. Wiedeker, Univ. of Geneva, Switzerland) as template and subcloned into the new adenoviral vector: pShuttle–IRE6–hGFP-2. Aequorin activity was assessed using 5 μM coelenterazine for 2 h in Krebs–Ringer modified buffer (125 mM NaCl, 5 mM KCl, 1 mM Na₂PO₄, 1 mM MgSO₄, 5.5 mM glucose and 20 mM HEPES, at pH 7.4) supplemented with 1 mM CaCl₂ at 37 °C. Light emission after 100 mM histamine stimulation was measured using a luminoimeter (FLUOstar OPTIMA). At the end of the experiment, 100 μM diltigion and 10 mM CaCl₂ (final concentrations) were added to measure the total aequorin luminescence so as to allow normalization of the data. To measure cytosolic calcium levels in intact MEF cells, cells were loaded with 5 μM Fluor-4-AM for 30 min at room temperature. After 100 μM histamine stimulation, a series of images were captured every 2 s by confocal microscopy using a Zeiss LSM 510 microscope (Carl Zeiss, Germany).

Western blot analysis, MCE expression, autophagy determinations and electron microscopy analysis. For western blot analysis, cells were collected in RIPA buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 1% (vol/vol) Nonidet P-40; 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail (Roche)). For tissue analysis, snap-frozen tissues were homogenized and lysed in RIPA buffer plus phosphatase inhibitor (Sigma). Following transfer to nitrocellulose membranes were immunoblotted with the indicated primary antibodies including: PDH (sc-377092, Santa Cruz Biotechnology, 1:5000); phospho-PDH Ser293 (#AB2024, Millipore, 1:5000); tubulin (#T7816, Sigma, 1:10,000); Cox subunit IV (#ab16,056, Abcam, 1:5,000); GAPDH (#ab8245, Abcam, 1:10,000). For detection of MCE protein expression, we generated a rabbit polyclonal antibody using the C-terminal peptide (CILEKYNQLDKDAQAEEDLRK) conjugated to KLH as an immunogen, or where indicated, a commercial antibody (HPA016480, Sigma, 1:250) that recognized the N terminus of the protein. To reconstitute MCE expression, full-length MCE human complementary DNA was cloned into the pBabe-puro retroviral vector. MCE-containing constructs containing the combined D261Q and E264Q mutation were created using Stratagene QuikChange mutagenesis. To determine autophagic flux we analysed primary MEF cells in culture or tissues from WT and MCU−/− mice using antibodies against p62 (GP62-C, Progen, 1:5,000) or LC3 (FL-8918, Sigma, 1:10,000) as previously described. To determine the effects of calcium on PDH phosphorylation in vitro, skeletal muscle mitochondria were washed with respiration buffer without EGTA (120 mM KCl, 5 mM MOPS, 5 mM KH₂PO₄, and 0.2% BSA) and treated with 300 μM CaCl₂ for 1 min at room temperature. The mitochondria were then lysed and subjected to western

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Mitochondrial swelling assay. Ca\(^{2+}\)-induced swelling of isolated mitochondria was measured spectrophotometrically as a decrease in absorbance at 540 nm. Isolated liver or heart mitochondria (100 μg) were resuspended in swelling buffer (120 mM KCl, 10 mM Tris-HCl, 5 mM MES, 5 mM Na\textsubscript{4}P\textsubscript{2}O\textsubscript{7}, 10 mM glutamate, 2 mM malate and 0.1 mM EGTA) in a total volume of 200 μL. Pore opening was induced and detected by the addition of 500 μM of total CaCl\(_2\) while monitoring absorbance at 540 nm. Where indicated, cyclosporin A (0.2 μM) or Ru360 (3.0 μM) was added.

PDH, citrate synthase and lactate determination. PDH activity was measured using the pyruvate dehydrogenase enzyme activity microplate assay kit (Abcam). Serum lactate levels under the fed or starved condition (overnight, 16 h) were measured using the lactatePro analyser (Arkray). Citrate synthase activity was measured in skeletal muscle mitochondria using a commercial kit (ScienCell). One unit of citrate synthase activity is defined as the amount of enzyme required to convert 1.0 μM of total CaCl\(_2\) while monitoring absorbance at 540 nm. Where indicated, cyclosporin A (0.2 μM) or Ru360 (3.0 μM) was added.
into cross-sections and the area of infarct as a percentage of the whole heart was quantified as previously described.39

**Statistical analysis.** All paired data are represented as mean ± s.e.m. Statistical significance of differences was evaluated by unpaired Student’s t-test with Welch’s correction or when multiple comparisons were employed, a Bonferroni-corrected analysis of variance. No statistical method was used to predetermine sample size. No samples were excluded. Unless specified, the investigators were not blinded to allocation during the experiments and outcome assessment.

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**Supplementary Figure 1** Disruption of the **MCU** gene by gene-trapping.  

**a)** Graphical representation of the mouse **MCU** genomic locus and the trapping vector insertion site. The Omnibank Gene Trap Vector 76, which contains a **b-geo** (fusion of β-Gal gene and neomycin-resistance gene) selection marker flanked by a splice acceptor (SA), a transcription termination sequence (pA), as well as the retroviral LTR sequences was inserted within Intron 1 of the mouse **MCU** gene.  

**b)** The genomic DNA sequence surrounding the gene trap insertion site identified in the IST11669F8 mouse ES clone. The insertion site is denoted with a red asterisk.  

**c)** Individual organ weight at necropsy normalized to total body weight of WT (**MCU**+/+) and **MCU**-/- mice (n=4 per genotype).  

**d)** Body composition as determined by quantitative magnetic resonance in young mice (3-4 months of age, n=7 per genotype) and older mice (10-12 months, n=5 per genotype). All pooled data represents mean +/- S.E.M.
Supplementary Figure 2 Mitochondrial morphology and MCU expression in MCU−/− mice. a) Electron micrographic images of both fetal (E12.5) and adult liver and heart. WT and MCU−/− tissues show similar mitochondrial morphology and abundance. Scale bars = 500 nm. b) Analysis of MCU expression in tissues from WT and MCU−/− mice. Protein lysates were identical to those shown in Figure 1d, however a different antibody against MCU (Sigma) recognizing the N-terminal region of MCU was used. Tubulin, identical to Figure 1d, is again used as a loading control. c) Protein expression in MEFs obtained from wild type or MCU−/− embryos. GAPDH is used as a loading control.
Supplementary Figure 3 Mitochondrial calcium uptake using isolated mitochondria and intact cells. a) Cardiac mitochondria were loaded with the calcium indicator Fluo-4FF. Calcium addition over the physiological (micromolar) range results in increasing calcium levels in mitochondria isolated from WT hearts. This uptake in WT mitochondria is blocked by Ru360 addition. MCU−/− mitochondria lack any demonstrable uptake at low calcium concentrations. At higher Ca2+ concentrations, there is a small, non Ru360-inhibitable, increase in Fluo-4FF fluorescence in MCU−/− mitochondria observed. Addition of EGTA (20 mM) returns fluorescence back to baseline, consistent with dye leakage as the basis of this non Ru360-inhibitable increase in Fluo-4FF fluorescence. b) A similar experiment where the MCU−/− mitochondria were briefly pelleted at the end of the experiment. All fluorescence seen with the MCU−/− mitochondria appears to reside in the supernatant, suggesting again that the fluorescence observed following the addition of higher concentrations of calcium is most consistent with dye leakage out of the mitochondria. c) Cardiac myocytes were isolated from WT or in MCU−/− hearts and adult myocytes were loaded with Rhod-2 to measure mitochondrial calcium levels. Shown is a representative experiment following addition of KCl (50 mM) with average fluorescence calculated from WT myocytes (n=9 cells) or MCU−/− myocytes (n=11 cells). d) Similar analysis using caffeine (20 mM) as a stimulus to increase cytosolic calcium with subsequent imaging of WT myocytes (n=16 cells) and MCU−/− myocytes (n=8 cells). All pooled data represents mean +/- S.E.M.
Supplementary Figure 4  Basal oxygen consumption and autophagic flux in MCU⁺/⁻ cells and tissues.  

**a**) Relative oxygen consumption measured using a Seahorse X-24 analyzer analyzing WT and MCU⁺/⁻ MEFs under basal conditions with glucose as the substrate (25 mM), or a similar analysis using pyruvate (2 mM) or in the presence of galactose (25 mM). Results are the average +/- S.E.M. of three independent experiments each performed in quadruplicate.

**b**) Autophagic flux as determined by levels of p62 and the ratio of LC3I/LC3II. MEF cells were shifted to a low nutrient media (Hanks buffered salt solution; t=0) and subsequently assessed for markers of autophagic flux. Consistent with increased autophagic flux, starvation led to decreased levels of p62 and an increased LC3II/LC3I ratio. However, this response was similar between WT and in MCU⁺/⁻ MEFs. A representative example from three similar experiments is shown.

**c**) Similar assessment in vivo in both liver and heart tissues under randomly fed conditions. Again, no consistent differences were observed between genotypes. GAPDH is shown as a loading control.
Supplementary Figure 5 Regulation of skeletal muscle PDH phosphorylation by MCU. 

a) Measurement of matrix calcium levels under fed conditions reveals that the differences between WT and in MCU−/− mitochondria are less pronounced than under fasting conditions (n=3 mice per genotype).

b) Mice were fasted overnight, re-fed and then harvested four hours later. Phosphorylation of serine 293 of skeletal muscle PDH E1-α subunit was determined.

c) Densitometric quantification of the intensity of phosphorylated to total PDH was determined. n=3; p=NS.

d) Isolated skeletal muscle mitochondria were analyzed in the absence or presence of extramitochondrial calcium (500 µM for 1 minute). Calcium addition led to a brisk reduction in PDH phosphorylation in WT skeletal muscle mitochondria but this effect was not observed in MCU−/− mitochondria. PDH phosphorylation was determined by a phospho-specific antibody.

e) Analysis of citrate synthase activity in WT and in MCU−/− skeletal muscle mitochondria. n=3 mice per genotype, p=NS by t-test, all pooled data represents mean +/- S.E.M.
Supplementary Figure 6: Fiber type profile of the gastrocnemius WT and MCU⁻/⁻ mice. Immunohistochemical analysis of soleus (SOL) and extensor digitorum longus (EDL) muscle using antibodies for slow and fast twitch muscle. No differences were observed between genotypes. One representative experiment from two similar experiments is shown. Scale bar equals 100 µm.
Supplementary Figure 7 MCU expression and cell death. 

a) Calcium-induced PTP opening in hepatic mitochondria isolated from WT or MCU−/− mice. One representative experiment from four similar experiments is shown. Experiments were performed in the presence or absence of cyclosporin A (CsA; 0.2 µM) and the arrow indicates the addition of calcium (500 µM).

b) Cytosolic extracts were prepared from WT and MCU−/− MEFs at the indicated time points after the addition of doxorubicin (2 µM). Cytochrome C release was similar in magnitude and kinetics between WT and MCU−/− cells. Tubulin is shown as a loading control.

c) Measurement of cytosolic calcium using Fluo-4AM fluorescence in primary MEF cells following the addition of 2 mM hydrogen peroxide. Cells were analyzed at the indicated times by flow cytometry. While calcium levels rose after hydrogen peroxide, no discernable differences were observed between genotypes.
**Supplementary Figure 8** Role of MCU in ischaemia/reperfusion injury. 

a) Regions of infarcted myocardium were assessed for TUNEL positive cells (n=3 mice per genotype). Analysis was from three mice per genotype using at least three random sections per mouse. 

b) Magnitude of ischemic contracture, a measure of cytosolic calcium levels, was assessed in WT and MCU<sup>−/−</sup> hearts. There does not appear to be an appreciable difference between WT (n=7) and MCU<sup>−/−</sup> (n=6) mice suggesting that under ischemic conditions, MCU<sup>−/−</sup> hearts do not develop significantly higher levels of cytosolic calcium. All pooled data represents mean +/- S.E.M., statistical significance was evaluated by t-test.